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TOXICOLOGICAL REVIEW

OF

TRICHLOROETHYLENE

APPENDIX E

(CAS No. 79-01-6)

In Support of Summary Information on the Integrated Risk Information System (IRIS)

September 2011

E. ANALYSIS OF LIVER AND CO-EXPOSURE ISSUES FOR THE TCE TOXICOLOGICAL REVIEW

The purpose of this Appendix is to provide scientific support and rationale for the hazard and dose-response sections of the *Toxicological Review of Trichloroethylene (TCE)* regarding liver effects and those of co-exposures. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of TCE. Please refer to the *Toxicological Review of Trichloroethylene (TCE)* for characterization of EPA's overall confidence in the quantitative and qualitative aspects of hazard and dose-response for TCE-induced liver effects. Matters considered in this appendix include knowledge gaps, uncertainties, quality of data, and scientific controversies. This characterization is presented in an effort to make apparent the scientific issues regarding the data and mode-of-action considerations for experimental animal data for liver effects in the TCE assessment.

E.1. BASIC PHYSIOLOGY AND FUNCTION OF THE LIVER—A STORY OF HETEROGENEITY

The liver is a complex organ whose normal function and heterogeneity are key to understanding and putting into context perturbations by TCE, cancer biology, and variations in response observed, and anticipated for susceptible lifestages and background conditions.

E.1.1. Heterogeneity of Hepatocytes and Zonal Differences in Function and Ploidy

Malarkey et al. (2005) state that: (1) the liver transcriptome (i.e., genes expressed as measured by mRNA) is believed only second to the brain in its complexity and includes about 25–40% of the approximately 50,000 mammalian genes; (2) during disease states, the transcriptome can double or triple and its increased complexity is due not only to differential gene expression (up- and downregulation of genes) but also to the mRNA contributions from the heterogeneous cell populations in the liver; and (3) when one considers that over a dozen cell types comprise the liver in varying proportions, particularly in disease states, knowledge about the cell types and cell-specific gene expression profiles help unravel the complex genomic and proteomic data sets. Gradients of gene and protein activity varying from the periportal region to the centrilobular region also exist for sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and the matrix in the space of Disse. Malarkey et al. (2005) also estimate that hepatocytes constitute 60%, sinusoidal endothelial cells 20%, Kupffer cells 15%, and stellate cells 5% of liver cells. Therefore, in experimental paradigms where liver homogenates are used for the determination of "changes in liver," gene expression, or other parameters, the individual changes from cells residing in differing zones and by differing cell type is lost. Malarkey et al. (2005) define the need to better characterize the histological cellular components of the tissues from

which mRNA and protein is extracted and referred to "phenotypic anchoring" and cite acetaminophen as a "model hepatotoxicant under study to assess the strengths and weaknesses of genomics and proteomics technologies" as well as "a good example for understanding and utilizing phenotypic anchoring to better understand genomics data." After acetaminophen exposure "there is an unexplained and striking inter and intralobular variability in acute hepatic necrosis with some regions having massive necrosis and adjacent areas within the same lobe or other lobes showing no injury at all." Malarkey et al. (2005) go on to cite similar lobular variability in response for "copper distribution, iron and phosphorous, chemical and spontaneous carcinogenesis, cirrhosis and regeneration" and suggest that although uncertain "factors such as portal streamlining of blood to the liver, redistribution of blood to core of the liver secondary to nerve stimulation, and exposures during fetal development and possibly lobular gradients are important." Hepatic interlobe differences exist for initiating agents in terms of DNA alkylation and cell replication. In the rat, diethylnitrosamine (DEN) alkylation has been reported to occur preferentially in the left and right median lobes, while cell replication was higher in the right median and right anterior lobes (Richardson et al., 1986). Richardson et al. (1986) reported that exposure to DEN induced a 100% incidence of HCC in the left, caudate, left median, and right median lobes of the liver by 20 weeks vs. only 30% in the right anterior and right posterior hepatic lobes. There was a reported interlobe difference in adduct formation, cell proliferation, liver lobe weight gain, number and size of γ-glutamyltranspeptidase (GGT)+ foci, and carbon 14 labeling from a single dose of DEN. Richardson et al. (1986) suggest that many growth-selection studies utilizing the liver to evaluate the carcinogenic potential of a chemical often focus on only one or two of the hepatic lobes, which is especially true for partial hepatectomy, and that for DEN and possibly other chemicals, this procedure removes the lobes most likely to get tumors. Thus, the "distribution of toxic insult may not be correctly assessed with random sampling of the liver tissue for microarray gene expression analysis" (Malarkey et al., 2005) and certainly any such distributional differences are lost in studies of whole-liver homogenates.

The liver is normally quiescent with few hepatocytes undergoing mitosis and, as described below, normally occurring in the periportal areas of the liver. Mitosis is observed only in approximately 1 in every 20,000 hepatocytes in adult liver (Columbano and Ledda-Columbano, 2003). The studies of Schwartz-Arad et al. (1989), Zajicek et al. (1991), Zajicek and Schwartz-Arad (1990), and Zajicek et al. (1989) have specifically examined the birth, death, and relationship to zone of hepatocytes as the "hepatic streaming theory." They report that hepatocytes and littoral cells continuously stream from the portal tract toward the terminal hepatic vein and that the hepatocyte differentiates as it goes with biological age closely related to cell differentiation. In other words, the acinus may be represented by a tube with two orifices, one for cell inflow situated at the portal tract rim and the other for cell outflow, at the terminal hepatic vein with hepatocytes streaming through the tube in an orderly fashion. In normal liver, cell proliferation is suggested as the only driving force of this flow with each mitosis associated

with displacement of the cells by one cell location and the greater the cell production, the faster the flow and vice versa (Zajicek et al., 1991). Thus, the microscopic section of the liver "displays an instantaneous image of a tissue in flux" (Schwartz-Arad et al., 1989). Schwartz-Arad et al. (1989) further suggest that:

> throughout its life the hepatocyte traverses three acinus zones; in each it is engaged in different metabolic activity. When young it performs among other functions gluconeogenesis, which is found in zone 1 hepatocytes (i.e. periportal), and when old it turns into a zone 3 cell (i.e., pericentral), with a pronounced glycolitic make up. The three zones thus represent differentiation stages of the hepatocyte, and since they differ by their distance from the origin, e.g. zone 2 (i.e., midzonal) is more distant than zone 1, again, hepatocyte differentiation is proportional to its distance.

Chen et al. (1995) report that:

> Hepatocytes are a heterogeneous population that are composed of cells expressing different patterns of genes. For example, gamma-glutamyl transpeptidase and genes related to gluconeogenesis are expressed preferential in periportal hepatocytes, whereas enzymes related to glycolysis are more abundant in the centrilobular area. Glutamine synthetase is expressed in a small number of hepatocytes surrounding the central veins. Most cytochrome p450 enzymes are expressed or induced preferentially in centrilobular hepatocytes relative to periportal hepatocytes.

Along with changes in metabolic function, Vielhauer et al. (2001) reported that there is evidence of zonal differences in carcinogen DNA effects and, also, chemical-specific differences for DNA repair enzyme and that enhanced DNA repair is a general feature of many carcinogenic states including the enzymes that repair alkylating agents but also oxidative repair. As part of this process of differentiation and as livers age, the hepatocyte changes and increases its ploidy with polyploid cells predominant in zone 2 of the acinus (Schwartz-Arad et al., 1989). The reported decrease in DNA absorbance in zone 3 may be due to: (1) a decline in chromatin affinity to the dye; (2) cell death; and (3) DNA exit from intact cells and Zajicek and Schwartz-Arad (1990) suggest that the fewer metabolic demands in Zone 3, under normal conditions, causes the cell to "deamplify" its genes and for DNA excess to leak out cells adjacent to the terminal hepatic vein or to be eliminated by apoptosis reflecting cell death. Thus, the three acinus zones represent differentiation states of one and the same hepatocyte, which increase ploidy as functional demands change. Zajicek and Schwartz-Arad (1990) also report that nuclear size is generally proportional to DNA content and that as DNA accumulates, the nucleus enlarges. This has import for histopathological descriptions of hepatocellular hypertrophy and attendant nuclear changes after toxic insult as well.

The gene amplification associated with polyploidy is manifested by DNA accumulation that involves the entire genome (Zajicek and Schwartz-Arad, 1990). Polyploidization is always attended by the intensification of the transcription and translation and in rat liver the amino acid label and activity of many enzymes increases proportionately to their ploidy. "Individual chromosomes of a tetraploid genome of a hepatocyte reduplicate in the same sequence as in a diploid one. In this case the properties of the chromosomes evidently remain unchanged and polyploidy only means doubling the indexes of the diploid genome" (Brodsky and Uryvaeva, 1977). Polyploidy will be manifested in the liver by either increases in the number of chromosomes per nucleus in an individual cell or by the appearance of two nuclei in a single cell. Most cell polyploidization occurs in youth with mitotic polyploidization occurring predominantly from 2 to 3 weeks postnatally and increases with age in mice (Brodsky and Uryvaeva, 1977). Hepatocytes progress through a modified or polyploidizing cell cycle, which contains gaps and S-phases, but proceeds without cytokinesis. The result is the formation of the first polyploidy cell, which is binucleated with diploid nuclei and has increased cell ploidy but not cell number. The subsequent proliferation of binucleated hepatocytes occurs with a fusion of mitotic nuclei during metaphase that gives rise to mononucleated cells with higher levels of ploidy. Thus, during normal liver ontogenesis, a polyploidizing cell cycle without cytokinesis alternates with a mitotic cycle of binucleated cells and results in progressive and irreversible increases in either cell or nuclear ploidy (Brodsky and Uryvaeva, 1977).

Polyploidization of the liver occurs during maturation in rodents, and therefore, experimental paradigms that treat or examine rodent liver during that period should take into consideration the normally changing baseline of polyploidy in the liver. The development of polyploidy has been correlated in rodents to correspond with maturation. Brodsky and Uryvaeva (Brodsky and Uryvaeva, 1977) report that it is cells with diploid nuclei that proliferate in young mice, but that among the newly formed cells, the percentage of those with tetraploid nuclei is high. By 1 month, most mice (CBA/C57BL mice) already have a polyploid parenchyma, but binucleated cells with diploid nuclei predominate. In adult mice, the ploidy class with the highest percentage of hepatocytes was the 4n X 2 class. The intensive proliferation of diploid hepatocytes occurs only in baby mice during the first 2 weeks of life and then toward 1 month, the diploid cells cease to maintain themselves and transform into polyploid cells. In aged animals, the parenchyma retains only 0.02% of the diploid cells of the newborn animal. While the weight of the liver increases almost 30 times within 2 years, the number of cells increase much less than the weight or mean ploidy. Hence, the postnatal growth of the liver parenchyma is due to cell polyploidization (Brodsky and Uryvaeva, 1977). In male Wistar rats, fetal hepatocytes (22 days gestation) were reported to be 85.3% diploid (2n) and 7.4% polyploid (4n + 8n) cells with 7.3% of cells in S-phase (S1 and S2). By 1 month of age (25-day-old suckling rats) there were 92.9% diploid and 2.5% polyploid; at 2 months, 47.5% diploid and 50.9%

polyploid; at 6 months, 29.1% diploid and 69.6% polyploid; and by 8 months, 11.1% diploid and 87.3% polyploidy (Sanz et al., 1996). However, mouse and rat differ in their polyploidization.

> In the mouse, which has a higher degree of polyploidy than the rats, the scheme of polyploidization differs in that each cell class, including mononucleate cells, forms from the preceding one without being supplemented by self-maintenance. Each cell class is regarded as the cell clone and it is implied that the cells of each class have the same mitotic history and originate from diploid initiator cells with similar properties. In this model 1 reproduction would give a 2n × 2 cell, the second reproduction a 4n cell, and third reproduction a 4n X 2 cell all coming from an originator diploid cell (Brodsky and Uryvaeva, 1977).

The cell polyploidy is most extensive in mouse liver, but also common for rat and humans livers. The livers of young and aged mice differ considerably in the ploidy of the parenchymal cells, but still perform fundamentally the same functions. In some mammals, such as the mouse, rats, dog and human, the liver is formed of polyploid hepatocytes. In others, for example, guinea pig and cats, the same functions are performed by diploid cells (Brodsky and Uryvaeva, 1977). One obvious consequence of polyploidization is enlargement of the cells. The volume of the nucleus and cytoplasm usually increases proportionately to the increase in the number of chromosome sets with polyploidy reducing the surface/volume ratio. The labeling of tritium doubles with the doubling of the number of chromosomes in the hepatocyte nucleus (Brodsky and Uryvaeva, 1977). Kudryavtsev et al. (1993) have reported that the average levels of cell and nuclear ploidy are relatively lower in humans than in rodent, but the pattern of hepatocyte polyploidization is similar, and at maturity and especially during aging, the rate of hepatocyte polyploidization increases with elderly individuals having binucleated and polyploid hepatocytes constituting about one-half of liver parenchyma. Gramantieri et al. (1996) report that in adult human liver, a certain degree of polyploidization is physiological; the polyploidy compartment (average 33% of the total hepatocytes) includes both mononucleated (28%) and binucleated (72%) cells and the average percentage of binucleated cells in the total hepatocyte population is 24% (Melchiorri et al., 1994).

Historically, aging in human liver has been characterized by fewer and larger hepatocytes, increased nuclear polyploidy, and a higher index of binucleate hepatocytes (Popper, 1986), but Schmucker (2005) notes that data concerning the effect of aging on hepatocyte volume in rodent and humans are in conflict with some showing increases in volume to be unchanged and to increase by 25% by age 60 by others in humans. The irreversibility of hepatocyte polyploidy has been used in efforts to identify the origin of tumor progenitor cells (diploid vs. polyploidy) (see Section E.3.1.8, below). The associations with polyploidy and disease have been an active area of study in cancer mode-of-action studies (see Sections E.3.1.4 and E.3.3.1, below).

Not only are polyploid cells most abundant in zone 2 of the liver acinus and increase in number with age, but polyploid cells have been reported to be more abundant following a number of toxic insults and exposure to chemical carcinogens. Wanson et al. (1980) reported that one of the earliest lesions obtained in the liver after *N*-nitrosomorpholine treatment development of hypertrophic parenchymal cells presenting a high degree of ploidy. Gupta (2000) reports hepatic polyploidy is often encountered in the presence of liver disease and that for animals and people, polyploidy is observed during advancement of liver injury due to cirrhosis or other chronic liver disease (often described as large-cell dysplasia referring to nuclear and cytoplasmic enlargement, nuclear pleomorphisms, and multinucleation and probably representing increased prevalence of polyploidy cells) and in old animals with toxic liver injury and impaired recovery. Gorla et al. (2001) report that weaning and commencement of feeding, compensatory liver hypertrophy following partial hepatectomy, toxin and drug-induced liver disease, and administration of specific growth factors and hormones may induce hepatic polyploidy. They go on to state that "although liver growth control has long been studied, whether the replication potential of polyploidy hepatocytes is altered remains unresolved, in part, owing to difficulties in distinguishing between cellular DNA synthesis and generation of daughter cells." Following carbon tetrachloride intoxication, the liver ploidy rises and more cells become binucleate (Zajicek et al., 1989). Minamishima et al. (2002) report that in 8–12-week-old female mice before partial hepatectomy, there were 78.6% 2C, 19.1% 4C, and 2.3% 8C cells but 7 days after, there were 42.0% 2C, 49.1% 4C, and 9.0% 8C. Zajicek et al. (1991) describe how hepatocyte streaming is affected after the rapid hepatocyte DNA synthesis that occurs after the mitogenic stimulus of a partial hepatectomy. These data are of relevance to findings of increased DNA synthesis and liver weight gain following toxic insults and disease states. Zajicek et al. (1991) suggest that following a mitogenic stimulus, not all DNA synthesizing cells do divide but accumulate newly formed DNA and turn polyploid (i.e., during the first 3 days after partial hepatectomy in rats 50% of synthesized DNA was accumulated) and that since the acinus increased 15% and cell density declined 10%, overall cell mass increased 5%. However, cell influx rose 1,300%. "In order to accommodate all these cells, the 'acinus-tube' ought to swell 13-fold, while in reality it increased only 5%" and that on day 3 "the liver remnant did not even double in its size." Zajicek et al. (1991) conclude that apparently "cells were eliminated very rapidly, and may have even been sloughed off, since the number of apoptotic bodies was very low" and therefore, "partial hepatectomy triggers two processes: an acute process lasting about a week marked by massive and rapid cell turnover during which most newly formed hepatocytes are eliminated, probably sloughed off into the sinusoids; and a second more protracted process which served for liver mass restoration mainly by forming new acini." Thus, a mitogenic stimulus may induce increased ploidy and increased cell number as a result of increased DNA synthesis, and many of the rapidly expanding number of cells resulting from

such stimulation are purged, and therefore, do not participate in subsequent disease states of the liver.

Zajicek et al. (1989) note that the accumulation of DNA rather than proliferation of hepatocytes "should be considered when evaluating the labeling index of hepatocytes labeled with tritiated thymidine" as the labeling index, defined as the proportion of labeled cells, can serve as a proliferation estimate only if it is assumed that a synthesizing cell will ultimately divide. In tissues, such as the liver, "where cells also accumulate DNA, proliferation estimates based on this index may fail" (Zajicek et al., 1989). The tendency to accumulate DNA is also accompanied by a decreasing probability of a cell to proliferate, since young hepatocytes generally divide after synthesizing DNA, while older cells prefer instead to accumulate DNA. However, polyploidy per se does not preclude cells from dividing (Zajicek et al., 1989). The ploidy level achieved by the cell, no matter how high, does not, in itself, prevent it from going through the next mitotic cycle and the reproduction of hepatocytes in the ploidy classes of 8n and 8n X 2 is common phenomenon (Brodsky and Uryvaeva, 1977). However, along with a reduced capacity to proliferate, Sigal et al. (1999) report that the onset of polyploidy increases the probability of cell death. The proliferative potentials of hepatocytes depend not only on their ploidy, but also on the age of the animals, with liver restoration occurring more slowly in aged animals after partial hepatectomy (Brodsky and Uryvaeva, 1977). Species differences in the ability of hepatocytes to proliferate and respond to a mitogenic stimulus have also been documented (see Section E.3.2, below). The importance of the issues of cellular proliferation vs. DNA accumulation and the differences in ability to respond to a mitogenic stimulus becomes apparent as identification of the cellular targets of toxicity (i.e., diploid vs. polyploidy) and the role of proliferation in proposed modes of action are brought forth. Polyploidization, as discussed above, has been associated with a number of types of toxic injury, disease states, and carcinogenesis by a variety of agents.

E.1.2. Effects of Environment and Age: Variability of Response

The extent of polyploidization of the liver not only changes with age, but structural and functional changes, as well as environmental factors (e.g., polypharmacy), also affect the vulnerability of the liver to toxic insult. In a recent review by Schmucker (2005), several of these factors are discussed. Schmucker (2005) reports that approximately 13% of the population of the United States is over the age of 65 years, that the number will increase substantially over the next 50 years, and that increased age is associated with an overall decline in health and vitality contributing to the consumption of nearly 40% of all drugs by the elderly. Schmucker (2005) estimates that 65% of this population is medicated and many are on polypharmacy regimes with a major consequence of a marked increase in the incidence of adverse drug reactions (ADRs) (i.e., males and females exhibit three- and fourfold increases in ADRs, respectively, when 20- and 60-year-old groups are compared). The percentage of deaths

attributed to liver diseases dramatically increases in humans beyond the age of 45 years with data from California demonstrating a fourfold increase in liver disease-related mortality in both men and women between the ages of 45 and 85 years (Siegel and Kasmin, 1997). Furthermore, Schmucker (2005) cites statistics from the U.S. Department of Health and Human Services to illustrate a loss in potential lifespan prior to 75 years of age due to liver disease (i.e., liver disease reduced lifespan to a greater extent than colorectal and prostatic cancers, to a similar extent as chronic obstructive pulmonary disease, and nearly as much as HIV). Thus, the elderly are predisposed to liver disease.

As stated above, the presence of high polyploidy cell in normal adults, nuclear polyploidization with age, and increase in the mean nuclear volume have been reported in people. Watanabe et al. (1978) reported the results from a cytophotometrical analysis of 35 cases of sudden death including 22 persons over 60 years of age that revealed that although the nuclear size of most hepatocytes in a senile liver remains unchanged, there was an increase in cells with larger nuclei. Variations in both cellular area and nucleocytoplasmic ratio were also analyzed in the study, but the binuclearity of hepatocytes was not considered. No cases with a clinical history of liver disease were included. Common changes in senile liver were reported to include atrophy, fatty metamorphosis of hepatocytes, and occasional collapse of cellular cords in the centrilobular area, slight cellular infiltration and proliferation of Kupffer cells in sinusoids, and elongation of Glisson's triads with slight to moderate fibrosis in association with round cell infiltration. Furthermore, cells with giant nuclei, with each containing two or more prominent nucleoli, and binuclear cells were increased. There was a decrease in diploid populations with age and an increase in tetraploid population and a tendency of polyploidy cells with higher values than hexaploids with age. Cells with greater nuclear size and cellular sizes were observed in livers with greater degrees of atrophy.

Schmucker notes that one of the most documented age-related changes in the liver is a decline in organ volume but also cites a decrease in functional hepatocytes and that other studies have suggested that the size or volume of the liver lobule increases as a function of increasing age. Data are cited for rats suggesting sinusoidal perfusion rate in the rat liver remains stable throughout the lifespan (Vollmar et al., 2002) but evidence in humans shows age-related shifts in the hepatic microcirculation attributable to changes in the sinusoidal endothelium (McLean et al., 2003) (i.e., a 60% thickening of the endothelial cell lining and an 80% decline in the number of endothelial cell fenestrations, or pores, with increasing age in humans) that are similar in baboon liver (Cogger et al., 2003). Such changes could impair sinusoidal blood flow and hepatic perfusion, and the uptake of macromolecules such as lipoproteins from the blood. Schmucker reports that there is a consensus that hepatic volume and blood flow decline with increasing age in humans but that the effects of aging on hepatocyte structure are less clear. In rats, the volume of individual hepatocytes was reported to increase by 60% during development and maturation,

but subsequently decline during senescence yielding hepatocytes of equivalent volumes in senescent and very young animals (Schmucker, 2005).

The smooth surfaced endoplasmic reticulum (SER), which is the site of a variety of enzymes involved in steroid, xenobiotic, lipid, and carbohydrate metabolism, also demonstrated a marked age-related decline rat hepatocytes (Schmucker et al., 1978; Schumucker et al., 1977). Schmucker also notes that several studies have reported that the older rodents have less effective protection against oxidative injury in comparison to the young animals, age-related decline in DNA base excision repair, and increases in the level of oxidatively damaged DNA in the livers of senescent animals in comparison to young animals. Age-related increases in the expression an activity of stress-induced transcription factors (i.e., increased NF-κB binding activity but not expression) were also noted, but that the importance of changes in gene expression to the role of oxidative stress in the aging process remains unsolved. An age-related decline in the proliferative response of rat hepatocytes to growth factors following partial hepatectomy was noted, but despite a slower rate of hepatic regeneration, older livers eventually achieved their original volume with the mechanism responsible for the age-related decline in the posthepatectomy hepatocyte proliferative response unidentified.

As with other tissues, telomere length has been identified as a critical factor in cellular aging with the sequential shortening of telomeres to be a normal process that occurs during cell replication (see Sections E.3.1.1 and E.3.1.5, below). An association in telomere length and strain susceptibility for carcinogenesis in mice has been raised. Herrera et al. (1999) examined susceptibility to disease with telomere shortening in mice. However, this study only cites shorter telomeres for C57BL6 mice in comparison to mixed C57BL6/129sv mice. The actual data are not in this paper and no other strains are cited. Of the differing cell types examined, Takubo and Kaminishi (2001) report that hepatocytes exhibited the next fastest rate of telomere shortening despite being relatively long-lived cells raising the question of whether or not there are correlations between age, hepatocyte telomere length, and the incidence of liver disease (Schmucker, 2005). Aikata et al. (2000) and Takubo et al. (2001) report that the mean telomere length in healthy livers is approximately 10 kilobase (kb) pairs at 80 years of age and these hepatocytes retain their proliferative capacity but that in diseased livers of elderly subjects was approximately 5 kb pairs. Thus, short telomere length may compromise hepatic regeneration and contribute to a poor prognosis in liver disease or as a donor liver (Schmucker, 2005).

Schmucker (2005) reports that interindividual variability in Phase I drug metabolism was so large in human liver microsomes, particularly among older subjects, that the determination of any statistically significant age or gender-related differences were precluded. In fact, Schmucker (2001) notes that "the most remarkable characteristic of liver function in the elderly is the increase in interindividual variability, a feature that may obscure age-related differences." Schumer notes that The National Institute on Aging estimates that only 15% of individuals aged over 65 years exhibit no disease or disability with this percentage diminishing to 11 and 5% for

men and women respectively over 80 years. Thus, the large variability in response and the presence of age-related increases in pharmacological exposures and disease processes are important considerations in predicting potential risk from environmental exposures.

E.2. CHARACTERIZATION OF HAZARD FROM TCE STUDIES

The 2001 Draft assessment of the health risk assessment of TCE (U.S. EPA, 2001) extensively cited the review article by Bull (2000) to describe the liver toxicity associated with TCE exposure in rodent models. Most of the attention has been paid to the study of TCE metabolites, rather than the parent compound, and the review of the TCE studies by Bull (2000) was cursory. In addition, gavage exposure to TCE has been associated with a significant occurrence of gavage-related accidental deaths and vehicle effects, and TCE exposure through drinking water has been reported to decrease palatability and drinking water consumption, and to have significant loss of TCE through volatilization, thus further limiting the TCE database.

In its review of the draft assessment, U.S. EPA's Science Advisory regarding this topic suggested that in its revision, the studies of TCE should be more fully described and characterized, especially those studies considered to be key for the hazard assessment of TCE. Although the database for studies of the parent compound is somewhat limited, a careful review of the rodent studies involving TCE can bring to light the consistency of observations across these studies, and help inform many of the questions regarding potential modes of action of TCE toxicity in the liver. Such information can inform current mode-of-action hypothesis (e.g., such as PPARα activation) as well. Accordingly, the primary acute, subchronic, and chronic studies of TCE will be described and examined in detail below with comments on consistency, major conclusions, and the limitations and uncertainties in their design and conduct. Since all chronic studies were conducted primarily with the goal of ascertaining carcinogenicity, their descriptions focus on that endpoint, however, any noncancer endpoints described by the studies are described as well. For details regarding evidence of hepatotoxicity in humans and associations with increased risk of HCC, please refer to Sections 4.5.1 and 4.5.2. Some of the earlier studies with TCE were contaminated with epichlorhydrin and are discussed in Sections 4.6 and 4.7 of the TCE assessment document.

E.2.1. Acute Toxicity Studies

A number of acute studies have been undertaken to describe the early changes in the liver after TCE administration with the majority using the gavage route of administration. Some have been detailed examinations, while others have reported primarily liver weight changes as a marker of TCE-response. The matching and recording of age, but especially initial and final body weight for control and treatment groups, is of particular importance for studies using liver weight gain as a measure of TCE-response as difference in these parameters affect TCE-induced liver weight gain. Most data are for exposures of at least 10 days.

E.2.1.1. Soni et al. (1998)

Soni et al. (1998) administered TCE in corn oil to male Sprague-Dawley rats (200–250 g, 8–10 weeks old) i.p. at exposure levels of 250, 500, 1,250, and 2,500 mg/kg. Groups (4–6 animals per group) were sacrificed at 0, 6, 12, 24, 36, 48, 72, and 96 hours after administration of TCE or corn oil. Using this paradigm only 50% of rats survived the 2,400 mg/kg i.p. TCE administration with all deaths occurring between days 1 and 3 after TCE administration. Tritiated thymidine was also administered i.p. to rats 2 hours prior to euthanasia. Light microscopic sections of the central lobe in 3–4 sections were examined for each animal. The grading scheme reported by the authors was: 0, no necrosis; +1 minimal, defined as only occasional necrotic cells in any lobule; +2, mild, defined as less than one-third of the lobular structure affected; +3, moderate, defined as between one-third and two-thirds of the lobular structure affected; and +4 severe, defined as greater than two-thirds of the lobular structure affected. At the 2,500 mg/kg dose, histopathology data were obtained for the surviving rats (50%). Lethality studies were done separately in groups of 10 rats. The survival in the groups of rats administered TCE and sacrificed from 0 to 96 hours was given as 30% mortality at 48 hours and 50% mortality by 72 hours.

The authors report that controls and 0-hour groups did not show signs of tissue injury or abnormality. The authors only report a single number with one significant figure for each group of animals with no means or SDs provided. In terms of the extent of necrosis there was no difference between the 250 and 500 mg/kg/treated dose groups though 96 hours with a single +1 given as the maximal amount of hepatocellular necrosis (minimal as defined by occasional necrotic cells in any lobule). At the 1,250 mg/kg dose, the maximal score was achieved 24 hours after TCE administration and was reported as simply +2 (mild, defined as less than one-third of lobular structure affected). The level of necrosis was reported to diminish to a score of 0 by 72 hours after 250 mg/kg TCE with no decrease at 500 mg/kg. At 1,250 mg/kg, the extent of necrosis was reported to diminish from +2 to +1 by 72 hours after administration. At the 2,500 mg/kg dose (LD_{50} for this route) by 48 hours, the surviving rats were reported to have a score of +4 (severe as defined by greater than two thirds of the lobular structure affected). The authors report that:

> The necrosed cells were concentrated mostly in the midzonal areas and the cells around central vein area were unaffected. Extensive necrosis was observed between 24 and 48 hours for both 1250 and 2500 mg/kg groups. Injury was maximal in the group receiving 2500 mg/kg between 36 and 48 hours as evidenced by severe midzonal necrosis, vacuolization, and congestion. Infiltration of polymorphonuclear cell was evident at this time as a mechanism for cleaning dead cells and tissue debris from the lobules. At the highest dose, the injury also started to spread toward the centrilobular areas. At the highest dose, 30 and 50% lethality was observed at 48 and 72 h, respectively. After 48 h, the

number of necrotic cells decreased and the number of mitotic cells increased. The groups receiving 500 and 1250 mg/kg TCE showed relatively higher mitotic activity as evidenced by cells in metaphase compared to other groups.

The authors do not give a quantitative estimate or indication as to the magnitude of the number of cells going through mitosis. Although there was variability in the number of animals dying at 1,250 mg/kg through this route of exposure, no indication of variability in response within these treatment groups was given by the authors in regard to extent of histopathological changes. The authors do not comment on the manner of death using this paradigm or of the effects of i.p. administration regarding potential peritonitis and inflammation.

TCE hepatotoxicity was "assessed by measuring plasma" SDH and ALT after TCE administration with vehicle treated control groups reported to induce no increases in these enzymes. Plasma SDH levels were reported to increase in a linear fashion after 250, 500, and 1,250 mg TCE/kg i.p. administration by 6 hours (i.e., ~3-, 10.5-, 22-, and 24.5-fold in comparison to controls from 250, 500, 1,250, and 2,500 mg/kg TCE, respectively) with little difference between the 1,250 and 250 mg/kg dose. By 12 hours the 250, 500, and 1,250 mg/kg levels had diminished to levels similar to that of the 250 mg/kg dose at 6 hours. The 2,500 mg/kg levels was somewhat diminished from its 6-hour level. By 24 hours after TCE exposure by the i.p. route of administration, all doses were similar to that of the 250-mg/kg-TCE 6-hour level. This pattern was reported to be similar for 5-, 36-, 48-, 72-, and 96-hour time points as well. The results presented were the means and SE for four rats per group. The authors did not indicate which rats were selected for these results from the 4–6 that were exposed in each group. Thus, only SDH levels showed dose-dependence in results at the 6-hour time point, and such increases did not parallel the patterns reported for hepatocellular necrosis from histopathological examination of liver tissues.

For ALT, the pattern of plasma concentrations after i.p. TCE administration differed both from that of SDH and from liver histopathology. Plasma ALT levels were reported to increase in a nonlinear fashion and to a much smaller extent than SDH (i.e., ~2.7-, 1.9-, 2.1-, and 4.0-fold of controls from 250, 500, 1,250, and 2,500 mg/kg TCE, respectively). The patterns for 12, 24, 36, 48, 72, and 96 hours were similar to that of the 6-hour exposure and did not show a dose-response. The authors injected carbon tetrachloride (2.5.mL/kg) into a separate group of rats and then incubated the resulting plasma with unbuffered TCA (TCA; 0, 200, 600, or 600 nmol) with decreases in enzyme activity in vitro at the two higher concentrations. It is not clear whether in vitro unbuffered TCA concentrations of this magnitude, which could precipitate proteins and render the enzymes inactive, are relevant to the patterns observed in the in vivo data. The extent of extinguishing of SDH and ALT activity at the two highest TCA levels in vitro were the same, suggestive of the generalized in vitro pH effect. However, the enzyme activity levels after TCE exposure had different patterns, suggesting that in vitro TCA results are not representative of the

in vivo TCE results. Neither ALT nor SDH levels corresponded to time course or dose-response reported for the histopathology of the liver presented in this study.

Tritiated thymidine results from isolated nuclei in the liver did not show a pattern consistent with either the histopathology or enzyme results. These results were for whole-liver homogenates and were not separated by nuclear size or cell origin. Tritiated thymidine incorporation was assumed by the authors to represent liver regeneration. There was no difference between treated and control animals at 6 hours after i.p. TCE exposure and only a decrease (~50% decrease) in thymidine incorporation after 12 hours of the 2,500 mg/kg TCE exposure level. By 24 hours, there was 5.6- and 2.8-fold tritiated thymidine incorporation at the 500 and 1,250 mg/kg TCE levels, with the 250 and 2,500 mg/kg levels similar to controls. For 36, 48, and 72 hours after i.p. TCE exposure, there continued to be no dose-response and no consistent pattern with enzyme or histopathological lesion patterns. The authors presented "area under the curve" data for tritiated thymidine incorporation for 0–95 hours, which did not include control values. There was a slight elevation at 500 mg/kg TCE and a slight decrease at 2,500 mg/kg from the 250 mg/kg TCE levels. Again, these data did not fit either histopathology or enzyme patterns and also can include the contribution of nonparenchymal cell nuclei as well as changes in ploidy.

The use of an i.p. route of administration is difficult to compare to oral and inhalation routes of exposure given that peritonitis and direct contact with TCE and corn oil with liver surfaces may alter results. Whereas Soni et al. (1998) report the LD_{50} to be 2,500 mg/kg TCE via i.p. administration, both Elcombe et al. (1985) and Melnick et al. (1987) do not report lethality from TCE administered for 10 days at 1,500 mg/kg in corn oil, or up to 4,800 mg/kg-day for 10 days in encapsulated feed. Also, TCE administered via gavage or oral administration through feed will enter the liver through the circulation with periportal areas of the liver the first areas exposed with the entire liver exposed in a fashion dependent on blood concentration levels. However, with i.p. administration, the absorption and distribution pattern of TCE will differ. The lack of concordance with measures of liver toxicity from this study and the lack concordance of patterns and dose-response relationships of toxicity reported from other more environmentally and physiologically relevant routes of exposure make the relevance of these results questionable.

E.2.1.2. Soni et al. (1999)

A similar paradigm and the same results were reported for Soni et al. (1999), in which hepatocellular necrosis, tritiated thymidine incorporation, and in vitro inhibition of SDH and ALT data were presented along with dose-response studies with ally alcohol and a mixture of TCE, thioacetamide, allyl alcohol, and chloroform. The same issues with interpretation present for Soni et al. (1998) also apply to this study as well.

E.2.1.3. Okino et al. (1991)

This study treated adult Wistar male rats (8 weeks of age) with TCE after being on a liquid diet for 3 weeks and either untreated or pretreated with phenobarbital or ethanol. TCE exposure was at 8,000 ppm for 2 hours, 2,000 or 8,000 ppm for 2 hours, and 500 or 2,000 ppm for 8 hours. Each group contained five rats. Livers from rats, that were not pretreated with either ethanol or phenobarbital, were reported to show only a few necrotic hepatocytes around the central vein at 6 and 22 hours after 2 hours of 8,000 ppm TCE exposure. At increased lengths and/or concentrations of TCE exposure, the frequencies of necrotic hepatocytes in the centrilobular area were reported to be increased, but the number of necrotic hepatocytes was still relatively low (out of ~150 hepatocytes the percentages of necrotic pericentral hepatocytes were 0.2 ± 0.4, 0.3 ± 0.4, 2.7 ± 1.0, 0.2 ± 0.4, and 3.5 ± 0.4% for control, 2,000 ppm TCE for 2 hours, 8,000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2,000 ppm TCE for 8 hours, respectively).

"Ballooned" hepatocytes were reported to be zero for controls and all TCE treatments with the exception of 0.3 ± 0.6% ballooned midzonal hepatocytes after 8,000 ppm TCE for 2 hours of exposure. Microsomal protein (mg/g/liver) was increased with TCE exposure concentration and duration, but not reported to be statistically significant (i.e., mg/g/liver microsomal protein was 21.2 ± 4.3, 22.0 ± 1.5, 25.9 ± 1.3, 23.3 ± 0.8, and 24.1 ± 1.0 for control, 2,000 ppm TCE for 2 hours, 8,000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2,000 ppm TCE for 8 hours, respectively).

The metabolic rate of TCE was reported to be increased after exposures over 2,000 ppm TCE (i.e., metabolic rate of TCE in nmol/g/liver/minute was 29.5 ± 5.7, 51.3 ± 6.0, 63.1 ± 16.0, 37.3 ± 3.3, and 69.5 ± 4.3 for control, 2,000 ppm TCE for 2 hours, 8,000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2,000 ppm TCE for 8 hours, respectively). However, the CYP content of the liver was not reported to increase with TCE exposure concentration or duration.

The liver/body weight ratios were reported to increase with all TCE exposures except 500 ppm for 8 hours (i.e., the liver/body weight ratio was 3.18 ± 0.15, 3.35 ± 0.10, 3.39 ± 0.20, 3.15 ± 0.10, and 3.57 ± 0.14% for control, 2,000 ppm TCE for 2 hours, 8,000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2,000 ppm TCE for 8 hours, respectively). These values represent 1.05-, 0.99-, 1.06-, and 1.12-fold of control in the 2,000 ppm TCE for 2 hours, 8,000 ppm TCE for 2 hours, 500 ppm TCE for 8 hours, and 2,000 ppm TCE for 8 hours treatment groups, respectively. A statistically significant difference observed after 8 hours of 2,000 ppm TCE exposure. Initial body weights and those 22 hours after cessation of exposure were not reported, which may have affected liver weight gain. However, these data suggest that TCE-related increases in metabolism and liver weight occurred as early as 22 hours after exposures of this magnitude from 2 to 8 hours of TCE with little concurrent hepatic necrosis.

Ethanol and phenobarbital pretreatment were reported to enhance TCE toxicity. In ethanol-treated rats, a few necrotic hepatocytes were reported to be around the central vein along

with hepatocellular swelling without pyknotic nuclei at 6 hours after TCE exposure with no pathological findings in the midzonal or periportal areas. At 22 hours, centrilobular hepatocytes were reported to have a few necrotic hepatocytes and cell infiltrations around the central vein, but midzonal areas were reported to have ballooned hepatocytes with pyknotic nuclei frequently accompanied by cell infiltrations. In phenobarbital-treated rats 6 hours after TCE exposure, centrilobular hepatocytes showed prenecrotic changes with no pathological changes reported to be observed in the periportal areas. By 22 hours, zonal necrosis was reported in centrilobular areas or in the transition zone between centrilobular and periportal areas. Treatment with phenobarbital or ethanol induced hepatocellular necrosis primarily in centrilobular areas with phenobarbital having a greater effect (89.1 ± 8.5% centrilobular necrosis) at the higher dose and shorter exposure duration (8,000 ppm × 2 hours) with ethanol having a greater effect (16.8 ± 5.3% centrilobular necrosis) at the lower concentration and longer duration of exposure (2,000 ppm × 8 hours).

E.2.1.4. Nunes et al. (2001)

This study was focused on the effects of TCE and lead co-exposure but treated male 75-day-old Sprague-Dawley rats with 2,000 mg/kg TCE for 7 days via corn-oil gavage (n = 10). The rats ranged in weight from 293 to 330 g (~12%) at the beginning of treatment and were pretreated with corn oil for 9 days prior to TCE exposure. TCE was reported to be 99.9% pure. Although the methods section states that rats were exposed to TCE for 7 days, Table 1 of the study reports that TCE exposure was for 9 days. The beginning body weights were not reported specifically for control and treatment groups, but the body weights at the end of exposure were reported to be 342 ± 18 g for control rats and 323 ± 3 g for TCE-exposed rats, and that difference (~6%) to be statistically significant. Because beginning body weights were not reported, it is difficult to distinguish whether differences in body weight after TCE treatment were treatment-related or reflected differences in initial body weights. The liver weights were reported to be 12.7 ± 1.0 g in control rats and 14.0 ± 0.8 g for TCE treated rats with the percent liver/body weight ratios of 3.7 and 4.3%, respectively. The increase in percent liver/body weight ratio represents 1.16-fold of control and was reported to be statistically significant. However, difference in initial body weight could have affected the magnitude of difference in liver weight between control and treatment groups. The authors report no gross pathological changes in rats gavaged with corn oil or with corn oil plus TCE, but observed that one animal in each group had slightly discolored brown kidneys. Histological examinations of "selected tissues" were reported to show an increased incidence of chronic inflammation in the arterial wall of lungs from TCE-dosed animals. There were no descriptions of liver histology given in this report for TCE-exposed animals or corn-oil controls.

E.2.1.5. Tao et al. (2000)

The focus of this study was to assess the effects of methionine on methylation and expression of c-Jun and c-Myc in mouse liver after 5 days of exposure to TCE (1,000 mg/kg in corn oil) and its metabolites. Female 8-week-old $B6C3F_1$ mice (n = 4–6) were administered TCE ("molecular biology or HPLC grade") for 5 days with and without methionine (300 mg/kg i.p.). Data regarding percent liver/body weight was presented as a figure. Of note is the decrease in liver/body weight ratio by methionine treatment alone (~4.6% liver/body weight for control and ~4.0% liver/body weight for control mice with methionine or ~13% difference in liver/body weight ratios between these groups). Neither initial body weights nor body weights after exposure were reported by the authors, so the reported effects of treatment could have reflected differences in initial body weights of the mice. TCE exposure was reported to increase the percent liver/body weight ratio to ~5.8% without methionine and to increase percent liver/body weight ratio to ~5.7% with methionine treatment. These values represent 1.26-fold of control levels from TCE exposure without methionine and 1.43-fold of control from TCE exposure with methionine. The number of animals examined was reported to be 4–6 per group. The authors reported the differences between TCE treated animals and their respective controls to be statistically significant, but did not examine the differences between controls with and without methionine. There were no descriptions of liver histology given in this report for TCE-exposed animals or corn-oil controls.

E.2.1.6. Tucker et al. (1982)

This study describes acute LD_{50}, and 5- and 14-day studies of TCE in a 10% emulphor solution administered by gavage. Screening-level subchronic drinking water experiments with TCE dissolved in 1% emulphor in mice were also conducted but with little detail reported. The authors did describe the strains used (CD-1 and ICR outbred albino) and that they were "weanling mice," but the ages of the mice and their weights were not given. The TCE was described as containing 0.004% diisopropylamine as the preservative and that the stabilizer had not been found carcinogenic or overtly toxic. The authors report that "the highest concentration a mouse would receive during these studies is 0.03 mg/kg/day." The main results are basically an LD_{50} study and a short-term study with limited reporting for 4- and 6-month studies of TCE exposure. Importantly, the authors documented the loss of TCE from drinking water solutions (<20% of the TCE was lost during the 3 or 4 days in the water bottles at 1.0, 2.5, and 5.0 mg/mL concentrations, but in the case of 0.1 mg/mL, up to 45% was lost over a 4-day period). The authors also report that high doses of TCE in drinking water reduced palatability to such an extent that water consumption by the mice was significantly decreased.

The LD_{50} with 95% confidence were reported to be 2,443 mg/kg (1,839–3,779 mg/kg) for female mice and 2,402 mg/kg (2,065–2,771 mg/kg) for male mice. However, the number of mice used in each dosing group was not given by the authors. The deaths occurred within

24 hours of TCE administration with no animals recovering from the initial anesthetic effect of TCE dying during the 14-day observation period. The authors reported that the only gross pathology observed was hyperemia of the stomach of mice dying from lethal doses of TCE, and that mice killed at 14 days showed no gross pathology.

In a separate experiment, male CD-1 mice were exposed to TCE by daily gavage for 14 days at 240 and 24 mg/kg. These two doses did not cause treatment-related deaths and body weight and "most" organ weights were reported by the authors to not be significantly affected but the data were not shown. The only effect noted was increased liver weight, which appeared to be dose dependent but was reported to be significant only at the higher dose. The only significant difference found in hematology was a 5% lower hematocrit in the higher dose group. The number of animals tested in this experiment was not given by the authors.

Male CD-1 mice (n = 11) were given TCE via gavage for 5 days (0.73 g/kg TCE twice on day 0, 1.46 g/kg twice on day 1, 2.91 g/kg twice on day 3, and 1.46 g/kg TCE on days 4 and 5) with only 4 of 11 mice treated with TCE surviving.

In a subchronic study, male and female CD-1 mice received TCE in drinking water at concentrations of 0, 0.1, 1.0, 2.5, and 5 mg/mL in 1% emulphor, and a naïve group received deionized water. There were 140 animals of each sex in the naïve group and in each treatment group, except for 260 mice in the vehicle groups. Thirty mice of each sex and treatment were selected for recording body weights for 6 months. The method of "selection" was not given by the authors. These mice were weighed twice weekly and fluid consumption was measured by weighing the six corresponding water bottles. The authors reported that male mice at the two highest doses of TCE consumed 41 and 66 mL/kg-day less fluid over the 6 months of the study than mice consuming vehicle only and that this same decreased consumption was also seen in the high dose (5 mg/mL) females. They report that weight gain was not affected except at the high dose (5mg/mL) and even though the weight gain for both sexes was lower than the vehicle control group, it was not statistically significant. However, these data were not shown. The authors report that gross pathological examinations performed on mice killed at 4 and 6 months were unremarkable and that a number of mice from all of the dosing regimens had liver abnormalities, such as pale, spotty, or granular livers. They report that 2 of 58 males at 4 months, and 11 of 59 mice at 6 months had granular livers and obvious fatty infiltration, and that mice of both sexes were affected. Animals in the naïve and vehicle groups were reported to infrequently have pale or spotty livers, but exhibit no other observable abnormalities. No quantitation or more detailed descriptions of the incidence of or severity of effects were given in this report.

The average body weight of male mice receiving the highest dose of TCE was reported to be 10% lower at 4 months and 11% lower at 6 months with body weights of female mice at the highest dose also significantly lower. Enlarged livers (as percentage of body weight) were observed after both durations of exposure in males at the three highest doses and in females at

the highest dose. In the 4-month study, brain weights of treated females were significantly increased when compared to vehicle control. However, the authors state:

> This increase is apparently because the values for the vehicle group were low, because the naïve group was also significantly increased when compared to vehicle control. A significant increase in kidney weight occurred at the highest dose in males at 6 months and in females, after both 4 and 6 months of TCE exposure. Urinalysis indicated elevated protein and ketone levels in high-dose females and the two highest dose males after 6 months of exposure (data not shown).

The authors describe differences in hematology to include:

> a decreased erythrocyte count in the high dose males at 4 and 6 months (13% and 16%, respectively); decreased leukocyte counts, particularly in the females at 4 months and altered coagulation values consisting of increased fibrinogen in males at both times and shortened prothrombin time in females at 6 months (data not shown). No treatment-related effects were detected on the types of white cells in peripheral blood.

It must be noted that effects reported from this study may have also been related to decreased water consumption, this study did not include any light microscopic evaluation, and that most of the results described are for data not shown. However, this study does illustrate the difficulties involved in trying to conduct studies of TCE in drinking water, that the LD_{50} values for TCE are relatively high, and that liver weight increases were observed with TCE exposure as early as a few weeks and increased liver weight were sustained through the 6-month study period.

E.2.1.7. Goldsworthy and Popp (1987)

The focus of this study was peroxisomal proliferation activity after exposure to a number of chlorinated solvents. In this study 1,000 mg/kg TCE (99+% epoxide stabilizer free) was administered to male F-344 rats (170–200 g or ~10% difference) and $B6C3F_1$ (20–25 g or ~20% difference) mice for 10 days in corn oil via gavage. The ages of the animals were not given. The TCE-exposed animals were studied in two experiments (experiments #1 and #3). In experiment #2, corn oil and methyl cellulose vehicles were compared. Animals were killed 24 hours after the last exposure. The authors did not show data on body weight, but stated that the administration of test agents (except WY-14,643 to rats which demonstrated no body weight gain) to rats and mice for 10 days "had little or no effect on body weight gain." Thus, differences in initial body weight between treatment and control groups, which could have affected the magnitude of TCE-induced liver weight gain, were not reported. The liver/body weight ratios in corn oil gavaged rats were reported to be 3.68 ± 0.06 and 4.52 ± 0.08% after

TCE treatment, which represented 1.22-fold of control (n = 5). Cyanide-(CN-)insensitive palmitoyl CoA[12] oxidation (PCO) was reported to be 1.8-fold increased after TCE treatment in this same group. In $B6C3F_1$ mice the liver/body weight ratio in corn oil gavaged mice was reported to be 4.55 ± 0.13 and 6.83 ± 0.13% after TCE treatment which represented 1.50-fold of control (n = 7). CN-insensitive PCO activity was reported to be 6.25-fold of control after TCE treatment in this same group. The authors report no effect of vehicle on PCO activity, but do not show the data nor discuss any effects of vehicle on liver weight gain. Similarly, the results for experiment #3 were not shown nor liver weight discussed with the exception of PCO activity reported to be 2.39-fold of control in rat liver and 6.25-fold of control for mouse liver after TCE exposure. The number of animals examined in Experiment #3 was not given by the authors or the variation between enzyme activities. However, there appeared to be a difference in PCO activity in experiments #1 and #3 in rats. There were no descriptions of liver histology given in this report for TCE-exposed animals or corn-oil controls.

E.2.1.8. Elcombe et al. (1985)

In this study, preservative-free TCE was given via gavage to rats and mice for 10 consecutive days with a focus on changes in liver weight, structure, and hepatocellular proliferation induced by TCE. Male Alderley Park rats (Wistar derived) (180–230 g), male Osborne-Mendel rats (240–280 g), and male $B6C3F_1$ or male Alderley Park Mice (Swiss) weighing 30–35 g were administered 99.9% pure TCE dissolved in corn oil via gavage. The ages of the animals were not given by the authors. The animals were exposed to 0, 500, 1,000, or 1,500 mg/kg body weight TCE for 10 consecutive days. The number of mice and rats varied widely between experiments and treatment groups and between various analyses. In some experiments, animals were injected with tritiated thymidine approximately 24 hours following the final dose of TCE and killed 1 hour later. The number of hepatocytes undergoing mitosis was identified in 25 random high-power fields (X40) for each animal with 5,000 hepatocyte per animal examined. There was no indication by the authors that zonal differences in mitotic index were analyzed. Sections of the liver were examined by light and electron microscopy by conventional staining techniques. Tissues selected for electron microscopy included central vein and portal tract so that zonal differences could be elucidated. Morphometric analysis of peroxisomes was performed "according to general principles of Weibel et al. (1964) on electronphotomicrographs from pericentral hepatocytes." DNA content of samples and peroxisomal enzyme activities were determined in homogenized liver (catalase and PCO activity).

The authors reported that TCE treatment had no significant effect on body-weight gain in either strain of rat or mouse during the 10-day exposure period. However, marked increases (up

[12]CoA = coenzyme A.

to 175% of control value) in the percent liver/body weight ratio were observed in TCE-treated mice. Smaller increases (up to 130% of control) in relative liver weight were observed in TCE-treated rats. No significant effects of TCE on hepatic water content were seen, so the liver weight did not represent increased water retention.

An interesting feature of this study was that it was conducted in treatment blocks at separate times with separate control groups of mice for each experimental block. Therefore, there were three control groups of $B6C3F_1$ mice (n = 10 for each control group) and three control groups for Alderley Park (n = 9–10 for each control group) mice that were studied concurrently with each TCE treatment group. However, the percent liver/body weight ratios were not the same between the respective control groups. There was no indication from the authors as to how controls were selected or matched with their respective experimental groups. The authors did not give liver weights for the animals, so the actual changes in liver weights were not given. The body weights of the control and treated animals were also not given by the authors. Therefore, if there were differences in body weight between the control groups or treatment groups, the liver/body weight ratios could also have been affected by such differences. The percentage increase over control could also have been affected by what control group each treatment group was compared to. There was a difference in the mean percent liver/body weight ratio in the control groups, which ranged from 4.32 to 4.59% in the $B6C3F_1$ mice (~6% difference) and from 5.12 to 5.44% in the Alderley Park mice (~6% difference). The difference in average percent liver/body weight ratio for untreated mice between the two strains was ~16%. Because the ages of the mice were not given, the apparent differences between strains may have been due to both age or to strain.

After TCE exposure, the mean percent liver/body weight ratios were reported to be 5.53% for 500 mg/kg, 6.50% for 1,000 mg/kg, and 6.74% for 1,500 mg/kg TCE-exposed $B6C3F_1$ mice. This resulted in 1.20-, 1.50-, and 1.47-fold values of control in percent liver weight/body weight for $B6C3F_1$ mice. For Alderley Park mice, the percent liver/body weight ratios were reported to be 7.31, 8.50, and 9.54% for 500, 1,000, and 1,500 mg/kg TCE treatment, respectively. This resulted in 1.43-, 1.56-, and 1.75-fold of control values. Thus, there appeared to be more of a consistent dose-related increase in liver/body weight ratios in the Alderley Park mice than the $B6C3F_1$ mice after TCE treatment. However, the variability in control values may have distorted the dose-response relationship in the $B6C3F_1$ mice. The SDs for liver/body weight ratio were as much as 0.52% for the treated $B6C3F_1$ mice and 0.91% for the Alderley Park treated mice. In regard to the correspondence of the magnitude of the TCE-induced increases in percent liver/body weight with the magnitude of difference in TCE exposure concentrations, in the $B6C3F_1$, mice the increases were similar (approximately twofold) between the 500 and 1,000 mg/kg TCE exposure groups. For the Alderley Park mice, the increases in TCE exposure concentrations were slightly less than the magnitude of increases in percent liver/body ratios between all of the concentrations (i.e., ~1.3-fold of control vs. 2-fold for 500

and 1,000 mg/kg TCE dose and 1.3-fold of control vs. 1.5-fold for the 1,000 and 1,500 mg/kg TCE dose).

The DNA content of the liver varied greatly between control animal groups. For $B6C3F_1$ mice it ranged from 2.71 to 2.91 mg/g liver. For Alderley Park mice, it ranged from 1.57 to 2.76 mg/g liver. The authors do not discuss this large variability in baseline levels of DNA content. The DNA content in $B6C3F_1$ mice was mildly depressed by TCE treatment in a nondose-dependent manner. DNA concentration decrease from control ranged from 20 to 25% between all three TCE exposure levels in $B6C3F_1$ mice. For Alderley Park mice there was also nondose related decrease in DNA content from controls that ranged from 18 to 34%. Thus, the extent of decrease in DNA content of the liver from TCE treatment in $B6C3F_1$ mice was similar to the variability between control groups. The lack of dose-response for apparent treatment-related effects in $B6C3F_1$ mice and especially in the Alderley Park mice was confounded by the large variability in the control animals. The changes in liver weight after TCE exposure for the AP mice did not correlate with changes in DNA content further, raising doubt about the validity of the DNA content measures. However, a small difference in DNA content due to TCE treatment in all groups was reported for both strains and this is consistent with hepatocellular hypertrophy.

The reported results for incorporation of tritiated thymidine in liver DNA showed large variation in control groups and SDs that were especially evident in the Alderley Park mice. For $B6C3F_1$ mice, mean control levels were reported to range from 5,559 to 7,767 dpm/mg DNA with SDs ranging from 1,268 to 1,645 dpm/mg DNA. In Alderley Park mice, mean control levels were reported to range from 6,680 to 10,460 dpm/mg DNA with SDs ranging from 308 to 5,235 dpm/mg DNA. For $B6C3F_1$ mice, TCE treatment was reported to induce an increase in tritiated thymidine incorporation with a very large SD, indicating large variation between animals. For the 500 mg/kg TCE treatment group, the values were reported as 12,334 ± 4,038, for the 1,000 mg/kg TCE treatment group, 21,909 ± 13,386, and for the 1,500 mg/kg treatment TCE group, 26,583 ± 10,797 dpm/mg DNA. In Alderley Park mice, TCE treatment was reported to give an increase in tritiated thymidine incorporation also with a very large SD. For 500 mg/kg TCE, the values were reported as 19,315 ± 12,280; for 1,000 mg/kg, TCE 21,197 ± 8,126; and for 1,500 mg/kg TCE, 38,370 ± 13,961. As a percentage of concurrent control, the increase in tritiated thymidine was reported to be 2.11-, 2.82-, and 4.78-fold of control in $B6C3F_1$ mice, and 2.09-, 2.03-, and 5.74-fold of control in Alderley Park mice. Accordingly, the change in tritiated thymidine incorporation did show a treatment related increase but not a dose-response.

Similar to the DNA content of the liver, the large variability in measurements between control groups and variability between animals limit quantitative interpretation of these data. The increase in tritiated thymidine, seen most consistently only at the highest exposure level in both strains of mice, could have resulted from either a change in ploidy of the hepatocytes or cell number. However, the large change in volume in the liver (75%) in the Alderley Park mice,

could not have resulted from only a fourfold of control in cell proliferation even if all tritiated thymidine incorporation had resulted from changes in hepatocellular proliferation. As mentioned in Section E.1.1 above, the baseline level of hepatocellular proliferation in mature control mice is very low and represents a very small percentage of hepatocytes.

In the experiments with male rats, the same issues discussed above, associated with the experimental design, applied to the rat experiments with the additional concern that the numbers of animals examined varied greatly (i.e., 6–10) between the treatment groups. In Osborne-Mendel rats, the control liver/body weight ratio was reported to vary from 4.26 to 4.36% with the SDs varying between 0.22 and 0.27%. For the Alderley Park rats, the liver/body weight ratios were reported to vary between 4.76 and 4.96% (in control groups) with SDs varying between 0.24 and 0.47%. TCE treatment was reported to induce a dose-related increase in liver/body weight ratio in Osborne-Mendel rats with mean values of 5.16, 5.35, and 5.53% in 500, 1,000, and 1,500 mg/kg TCE treated groups, respectively. This resulted in 1.18-, 1.26-, and 1.30-fold values of control. In Alderley Park rats, TCE treatment was reported to result in increased liver weights of 5.45, 5.83, and 5.65% for 500, 1,000, and 1,500 mg/kg TCE respectively. This resulted in 1.14-, 1.17-, and 1.17-fold values of control. Again, the variability in control values may have distorted the nature of the dose-response relationships in Alderley Park rats. TCE treatment was reported to result in SDs that ranged from 0.31 to 0.48% for Osborne-Mendel rats and from 0.24 to 0.38% for Alderley Park rats. What is clear from these experiments is that TCE exposure was associated with increased liver/body weight in rats.

The reported mean hepatic DNA concentrations and SDs varied greatly in control rat liver as it did in mice. The variation in DNA concentration in the liver varied more between control groups than the changes induced by TCE treatment. For Osborne-Mendel rats, the mean control levels of mg DNA/g liver were reported to range from 1.99 to 2.63 mg DNA/liver with SDs varying from 0.17 to 0.33 mg DNA/g. For Alderley Park rats, the mean control levels of mg DNA/g liver were reported to be 2.12–3.16 mg DNA/g with SD ranging from 0.06 to 1.04 mg DNA/g. TCE treatment decreased the liver DNA concentration in all treatment groups. For Osborne-Mendel rats, the decrease ranged from 8 to 13% from concurrent control values and for Alderley Park rats the decrease ranged from 8 to 17%. There was no apparent dose response in the decreases in DNA content, with all TCE treatment levels giving a similar decrease from controls and the same limitations discussed above for the mouse data apply here. The magnitude of increases in liver/body ratios shown by TCE treatment were not correlated with the changes in DNA content. However, as with the mouse data, the small differences in DNA content due to TCE treatment in all groups and in both strains were consistent with hepatocellular hypertrophy.

Incorporation of tritiated thymidine was reported to be even more variable between control groups of rats than it was for mice and was reported to be especially variable between control groups (i.e., 2.7-fold difference between control groups within strain) and differed between the strains (average of 2.5-fold between strains). For Osborne-Mendel rats, the mean

control levels were reported to range from 13,315 to 33,125 dpm/mg DNA, while for Alderley Park rats, tritiated thymidine incorporation ranged from 26,613 to 69,331 dpm/mg DNA for controls. The SDs were also very large (i.e., for control groups of Osborne-Mendel rats, they were reported to range from 8,159 to 13,581 dpm/mg DNA, while for Alderley Park rats, they ranged from 9,992 to 45,789 dpm/mg DNA). TCE treatment was reported to induce increases over controls of 110, 118, and 106% for 500, 1,000, and 1,500 mg/kg TCE-exposed groups, respectively, in Osborne-Mendel rats with large SDs for these treatment groups as well. In Alderley Park rats, the increases over controls were reported to be 206, 140, and 105% for 500, 1,000, and 1,500 mg/kg TCE, respectively. In general, these data do indicate that TCE treatment appeared to give a mild increase in tritiated thymidine incorporation but the lack of dose-response can be attributable to the highly variable measurements of tritiated thymidine incorporation in control animal groups. The variation in the number of animals examined between groups and small numbers of animals examined additionally decrease the likelihood of being able to discern the magnitude of difference between species- or strain-related effects for this parameter. Again, given the very low level of hepatocyte turnover in control rats, this does not represent a large population of cells in the liver that may be undergoing proliferation and cannot be separated from changes in ploidy.

The authors report that the reversibility of these phenomena was examined after the administration of TCE to Alderley Park mice for 10 consecutive days. Effects upon liver weight, DNA concentration, and tritiated thymidine incorporation 24 and 48 hours after the last dose of TCE were reported to be still apparent. However, 6 days following the last dose of TCE, all of these parameters were reported to return to control values with the authors not showing the data to support this assertion. Thus, cessation of TCE exposure would have resulted in a 75% reduction in liver weight by one week in mice exposed to the highest TCE concentration.

Analyses of hepatic peroxisomal enzyme activities were reported for catalase and β-oxidation (PCO activity) following administration of TCE to $B6C3F_1$ mice and Alderley Park rats exposed to 1,000 mg/kg TCE for 10 days. The authors only used five control and five exposed animals for these tests. An 8-fold of control value for PCO activity and a 1.5-fold of control value for catalase activity were reported for $B6C3F_1$ mice exposed to 1,000 mg/kg TCE. In the Alderley Park rats, no significant changed occurred. It is unclear which mice or rats were selected from the previous experiments for these analyses and what role selection bias may have played in these results. The reduced number of animals chosen for this analysis also reduces the power of the analysis to detect a change. In rats, there was a reported 13% increase in PCO; however, the variation between the TCE-treated rats was more than double that of the control animals in this group and the other limitations described above limit the ability to detect a response. There was no discussion given by the authors as to why only one dose was tested in half of the animals exposed to TCE or why the strain with the lowest liver weight change due to TCE exposure was chosen as the strain to test for peroxisomal proliferative activity.

The authors provided a description of the histopathology at the light microscopy level in $B6C3F_1$ mice, Alderley Park mice, Osborne-Mendel rats, and Alderley Park rats, but did not provide a quantitative analysis or specific information regarding the variability of response between animals within groups. There appeared to be 20 animals examined in the 1,000 mg/kg TCE exposed group of $B6C3F_1$ mice but no explanation as to why there were only 10 animals examined in analyses for liver weight changes, DNA concentration, and tritiated thymidine incorporation. There was no indication by the authors regarding how many rats were examined by light microscopy.

Apart from a few inflammatory foci in occasional animals, hematoxylin and eosin (H&E) section from $B6C3F_1$ control mice were reported to show no abnormalities. The authors suggest that this is a normal finding in the livers of mice kept under "non-SPF conditions." A stain for neutral lipid was reported to not be included routinely in these studies, but subsequent electron microscopic examination of lipid was reported to show increases in the livers of corn-oil treated control animals. The individual fat droplets were described as "generally extremely fine and are not therefore detectable in conventionally process H&E stained sections, since both glycogen and lipid are removed during this procedure." Thus, this study documents effects of using corn oil gavage in background levels of lipid accumulation in the liver.

The finding of little evidence of gross hepatotoxicity in TCE-treated mice was reported, even at a dose of 1,500 mg/kg. Specifically,

> Of 19 animals examined receiving 1500 mg/kg body weight TCE, only 6 showed any evidence of hepatocyte necrosis, and this pathology was restricted to single small foci or isolated single cells, frequently occurring in a subcapsular location. Examination of 20 animals receiving 1000 mg/kg body wt TCE demonstrated no hepatocyte necrosis. Of 20 animals examined receiving 500 mg/kg body wt TCE, 1 showed necrosis of single isolated hepatocytes; however, this change was not a treatment-related finding.

TCE-treated mice were reported to show:

> a change in staining characteristic of the hepatocytes immediately adjacent to the central vein of the hepatocyte lobules, giving rise to a marked 'patchiness' of the liver sections. Often this change consisted of increased eosinophilia of the central cells. There was some evidence of cell hypertrophy in the centrilobular regions. These changes were evident in most of the TCE treated animals, but there was a dose-related trend, relatively few of the 500 mg/kg animals being affected, while the majority of the 1,500 mg/kg animals showed central change. No other significant abnormalities were seen in the liver of TCE treated mice compared to controls apart from occasional mitotic figures and the appearance of isolated nuclei with an unusual chromatin pattern. This pattern generally consisted of a course granular appearance with a prominent rim of chromatin around the

periphery of the nucleus. These nuclei may have been in the very early stages of mitosis. Similar changes were not seen in control mice.

The authors briefly commented on the findings in the Alderley Park mice stating that:

> H& E sections from Alderley Park mice gave similar results as for $B6C3F_1$ mice. No evidence of hepatotoxicity was seen at a dose of 500 mg/kg body wt TCE. However, a few animals at the higher doses showed some necrosis and other degenerative changes. This change was very mild in nature, being restricted to isolated necrotic cells or small foci, frequently in subcapsular position. Hypertrophy and increased eosinophilia were also noticed in the centrilobular regions at higher doses.

Thus, from the brief description given by the authors, the centrilobular region is identified as the location of hepatocellular hypertrophy due to TCE exposure in mice, and for it to be dose-related with little evidence of accompanying hepatotoxicity.

The description of histopathology for rats was even more abbreviated than for the mouse. H& E sections from Osborne-Mendel rats showed that:

> livers from control rats contained large quantities of glycogen and isolated inflammatory foci, but were otherwise normal. The majority of rats receiving 1,500 mg/kg body weight TCE showed slight changes in centrilobular hepatocytes. The hepatocytes were more eosinophilic and contained little glycogen. At lower doses, these effects were less marked and were restricted to fewer animals. No evidence of treatment-related hepatotoxicity (as exemplified by single cell or focal necrosis) was seen in any rat receiving TCE. H& E sections from Alderley Park Rats showed no signs of treatment-related hepatotoxicity after administration of TCE. However, some signs of dose-related increase in centrilobular eosinophilia were noted.

Thus, both mice and rats exhibited pericentral hypertrophy and eosinophilia as noted from the histopathological examination.

The study did report a quantitative analysis of the effects of TCE on the number of mitotic figures in livers of mice. Few if any control mice exhibited mitotic figures. But, the authors report:

> a considerable increase in both the numbers of figures per section was noted after administration of TCE." The numbers of animals examined for mitotic figures ranged from 75 (all control groups were pooled for mice) to 9 in mice, and ranged from 15 animals in control rat groups to as low as 5 animals in the TCE treatment groups. The range of mitotic figures found in 25 high-power fields was reported and is equivalent to the number of mitotic figures per 5,000 hepatocytes examined in random fields.

Thus, the predominance of mitotic figures in any zone of the liver cannot be ascertained.

For $B6C3F_1$ mice, the number of animals with mitotic figures was reported to be 0/75, 3/20, 7/20, and 5/20 for control, 500, 1,000, and 1,500 mg/kg TCE exposed mice, respectively. The range of the number of mitotic figures seen in 5,000 hepatocytes was reported to be 0, 0–1, 0–5, and 0–5 for those same groups with group means of 0, 0.15 ± 0.36, 0.6 ± 1.1, and 0.5 ± 1.2. These results demonstrate a very small and highly variable response due to TCE treatment in $B6C3F_1$ mice in regard to mitosis. Thus, the highest percentage of cells undergoing mitosis within the window of observation would be on average 0.012% with a SD twice that value. The data presented for mitotic figures also indicated no differences in results between 1,000 and 1,500 mg/kg treated $B6C3F_1$ mice in regard to mitotic figure detection. However, the tritiated thymidine incorporation data indicated that thymidine incorporation was approximately twofold greater at 1,500 than 1,000 mg/kg TCE in $B6C3F_1$ mice. For Alderley Park mice, the number of animals with mitotic figures was reported to be 1/15, 0/9, 4/9, and 2/9 for control, 500, 1,000, and 1,500 mg/kg TCE exposed mice. The range of the number of mitotic figures seen in 5,000 hepatocytes was 0–1, 0, 0–2, and 0–1 for those same groups with group means of 0.06 ± 0.25, 0.7 ± 0.9, and 0.2 ± 0.4. These results reveal the detection of, at the most, two mitotic figures in 5,000 hepatocytes for any mouse an any treatment group and no dose-related increased after TCE treatment in Alderley Park mice. Thus, the highest percentage of cells with a mitotic figure would be on average 0.014% with a SD twice that value. The small number of animals examined reduces the power of the experiment to draw any conclusions as to a dose-response.

Similar to the $B6C3F_1$ mice, there did not appear to be concordance between mitotic figure detection and thymidine incorporation for Alderley Park mice. Thymidine incorporation showed a 2-fold increase over control for 500 and 1,000 mg/kg TCE and a 5.7-fold increase for 1,500 mg/kg TCE treated animals. However, in regard to mitotic figure detection, there were fewer mitotic figures in 500 mg/kg TCE treated mice than controls, and fewer animals with mitotic figures and fewer numbers of figures in the 1,500 mg/kg dose than the 1,000 mg/kg exposed group. The inconsistencies between mitotic index data and thymidine incorporation data in both strains of mice suggest that either thymidine incorporation is representative of only DNA synthesis and not mitosis, an indication of changes in ploidy rather than proliferation, or that this experimental design is incapable of discerning the magnitude of these changes accurately. Data from both mouse strains show very little, if any, hepatocyte proliferation due to TCE exposure with the mitotic figure index data having that advantage of being specific for hepatocytes and to not to also include nonparenchymal cells or inflammatory cells in the liver.

The results for rats were similar to those for mice and even more limited by the varying and low number of animals examined. For Osborne-Mendel rats, the numbers of animals with mitotic figures were reported to be 8/15, 2/9, 0/7, and 0/6 for control, 500, 1,000, and 1,500 mg/kg TCE exposed rats groups, respectively, with the respective ranges of the number of mitotic figures seen in 5,000 hepatocytes to be 0–8, 0–3, 0, and 0. The group means were 1.5 ±

2.0, 0.4 ± 1.0, 0, and 0 for these groups. It would appear from these results that there are fewer mitotic figures after TCE treatment with the highest percentage of cells undergoing mitosis to be on average 0.03% in control rats. However, thymidine incorporation studies show a modest increase at all treatment levels over controls in Osborne-Mendel rats rather than a decrease from controls. For Alderley Park rats, the numbers of animals with mitotic figures were reported to be 13/15, 5/9, 9/9, and 4/9 for control, 500, 1,000, and 1,500 mg/kg TCE exposed rat groups with the ranges of the number of mitotic figures seen in 5,000 hepatocytes to be 0–26, 0–5, 1–7, and 0–9. The group means were 7.2 ± 4.7, 1.6 ± 4.3, 3.8 ± 3.4, and 1.8 ± 2.9 for these groups.

It would appear that there are fewer mitotic figures after TCE treatment with the highest percentage of cells to an average of 0.14% in control rats. However, thymidine incorporation studies show twofold greater level at 500 mg/kg TCE than for control animals and a 40 and 5% increase at 1,000 and 1,500 mg/kg TCE exposure groups, respectively. Similar to the results reported in mice, results in both rat strains show an inconsistency in mitotic index and thymidine incorporation. The control rats appear to have a much greater mitotic index than any of the mouse groups (treated or untreated) or the TCE-treatment groups. However, it is the mice that were exhibiting the largest increased in liver weight after TCE exposure. By either thymidine incorporation or mitosis, these data do provide a consistent result that at 10 days of exposure, very little sustained hepatocellular proliferation is occurring in either mouse or rat and neither is correlated well with the concurrent changes in liver weight observed from TCE exposure.

This study provided a qualitative discussion and quantitative analysis of structural changes using electron microscopy. The qualitative discussion was limited and included statements about increased observances without quantitative data shown other than the morphometric analysis. The authors reported that:

> the ultrastructure of control mouse liver was essentially normal, although mild dilatation of RER and SER was a frequent finding. Lipid droplets were also usually present in the cell cytoplasm. The ultrastructural changes seen in mouse liver following administration of up to 1,500 mg/kg body wt TCE for 10 days were essentially similar in the $B6C3F_1$ mouse and the Alderley Park mouse. The most notable change in both strains of mouse was a dramatic increase in the number of peroxisomes. This change was only apparent in the cells immediately surrounding the central veins. Peroxisome proliferation was not noticeable in periportal cells. The induced peroxisomes were generally small and very electron dense and frequently lacked the characteristic nucleoid core found in peroxisomes of control livers.

The authors conclude that:

> morphometric analysis showed evidence of a dose-related response, peroxisomal induction appearing to reach a maximum at 1,000 mg/kg in $B6C3F_1$ mice…Lipid was increased in the livers of treated mice at all doses and was present both as

free droplets in the cytoplasm and as liposomes (small lipid droplets in ER cisternae). The centrilobular cell, which showed the greatest increase in numbers of peroxisomes, showed no evidence of this lipid accumulation: fatty change was more prominent in those cells away from the central vein (i.e., zone 2 of the liver acinus). Accumulation of lipid, particularly in liposomes, was less marked in Alderley Park mouse than in B6C3F$_1$ mouse. Mild proliferation of smooth endoplasmic reticulum was seen in both strains and both rough and smooth endoplasmic reticulum was generally more dilated than in control mice.

Electron microscopic results for rat liver were reported

to show similar changes in Osborne-Mendel and Alderley Park rat treated with TCE…Rats receiving either 1,000 or 1,500 mg/kg TCE for 10 days generally showed mild proliferation of SER in centrilobular hepatocytes. The cisternae of RER were frequently dilated, giving rise to a rather disorganized appearance in contrast to the parallel stacks seen in control livers, although no detachment of ribosomes was evident. The SER was also dilated. In contrast to mice, peroxisomes were only very slightly and not significantly, increased in the liver of TCE –treated rats. Morphometric analysis confirmed this observation, with the volume density of peroxisomes in the cytoplasm of centrilobular hepatocytes being only slightly increased in rats of both strains receiving 1,000 or 1,500 mg/kg body wt TCE…Lipid droplets were occasionally increased in some livers obtained from rats receiving TCE, but the degree of fatty change generally appeared similar to that found in control rats receiving corn oil. There were no changes in membrane –bound liposomes, other organelles, or Golgi condensing vesicles. Centrilobular glycogen was somewhat depleted in male rats receiving 1,500 mg/kg TCE. Periportal cells were ultrastructurally normal in all rats.

For the morphometric analysis, the number of mice examined ranged from seven in the control group to eight in the 1,500 mg/kg TCE exposed group. The authors did not indicate which control animals were used for the morphometric analysis from the 75 animals examined for mitotic index, the 20 examined by light microscopy, or the 30 mice used as concurrent controls in the liver weight, DNA concentration, and tritiated thymidine incorporation studies. The authors stated that morphometry was performed on three randomly selected photomicrographs from each of three randomly selected pericentral hepatocytes for each animal (i.e., nine photomicrographs per animal). A mean value representing the exposure group was reported with the variability between photomicrographs per animal or the variation between animals unclear. The morphometric analysis did not examine all treatment groups (e.g., only the control and 500 mg/kg TCE group were examined in Alderley Park mice).

The percent cytoplasmic volume of the peroxisomal compartment (mean ± SD) was reported to be 0.6 ± 0.6% for controls, 4.8 ± 3.3% for 500 mg/kg TCE, 6.7 ± 1.9% for 1,000 mg/kg TCE, and 6.4 ± 2.5% for 1,500 mg/kg TCE in B6C3F$_1$ mice. In Alderley Park mice, only 12 control and 12,500 mg/kg TCE exposed mice were examined and, similarly, their

selection criteria was not given. The percent cytoplasmic volume of the peroxisomal compartment was 1.2 ± 0.4% for control and 4.7 ± 2.8% for 500 mg/kg TCE exposed mice.

For Osborne-Mendel rats, control rats (n = 9) were reported to have a percent cytoplasmic volume of the peroxisomal compartment of 1.8 ± 0.4%; 1,000 mg/kg TCE (n = 5), 2.3 ± 1.6%, and 1,500 mg/kg exposed rats (n = 7), 2.3 ± 2.0%. For Alderley Park rats, only two groups were examined (control and 1,000 mg/kg TCE exposure). The percent cytoplasmic volume of the peroxisomal compartment for control rats (n = 15) was reported to be 1.8 ± 0.8% and for 1,000 mg/kg TCE (n = 16), 2.4 ± 1.2%. The varying numbers of animals examined, the varying and inconsistent number of treatment groups examined, the limited number of photomicrographs per animal, and the potential selection bias for animals examined make quantitative conclusions regarding this analysis difficult. Although control levels differed by a factor of 2 between the two strains of mice examined, as well as the number of control animals examined (7 vs. 12), it appears that the 500-mg/kg TCE-exposed $B6C3F_1$ and Alderley Park mice had similar percentages of peroxisomal compartment in the pericentral cells examined (~4.8%). There also appeared to be little difference between 1,000 mg/kg TCE treated Osborne-Mendel and Alderley Park rats for this parameter (~2.4%). Although few animals were examined, there was little difference reported between 500, 1,000, and 1,500 mg/kg TCE exposure groups in regard to percentages of peroxisomal compartment in $B6C3F_1$ mice (4.8–6.7%). For the few rats of the Osborne-Mendel strain examined, there also did not appear to be a difference between 1,000 and 1,500 mg/kg TCE exposure for this parameter (2.3%).

Based on peroxisome compartment volume data, one would expect there to be little difference between TCE exposure groups in mice or rats in regard to enzyme activity or other "associated events." However, such comparisons are difficult due to limited power to detect differences and the possibility of bias in selection of animals in differing assays. For the $B6C3F_1$ mice, only 5 animals per group were examined for enzyme analysis, 7–8 animals for morphometric analysis, 75 animals in control, and 20 animals in 1,000 mg/kg TCE-exposed groups for mitotic figure identification, and 10 animals per group for thymidine incorporation. Since only a few animals were tested for enzyme activity, the comparison between peroxisomal compartment volume and that parameter is very limited. There was a reported 47% increase in catalase activity between control (n = 5) and 1,000 mg/kg TCE exposed $B6C3F_1$ mice (n = 5) and a 7.8-fold increase in PCO activity. The percent peroxisome compartment was reported to be 10.6-fold greater (0.6 vs. 6.4%). However, the $B6C3F_1$ control percent volume of peroxisomal compartment was reported to be half that of the Alderley Park mouse control. An accurate determination of the quantitative differences in peroxisomal proliferation would be dependent on an accurate and stable control value. For Alderley Park rats, there was an 8% decrease in catalase activity between control (n = 5) and 1,000 mg/kg TCE exposed rats (n = 5), and a 13% increase in PCO activity. The percent peroxisome compartment was reported to be 33% greater in the TCE-exposed than control group. Thus, for the very limited data that were

available to compare peroxisomal compartment volume with enzyme activity, there was consistency in result.

However, were such increases in peroxisomes associated with other events reported in this study? Mouse peroxisome proliferation associated enzyme activities in $B6C3F_1$ mice at 1,000 mg/kg TCE were reported to be 8-fold over control values in mice after 10 days of treatment. However, this increase in activity was not accompanied by a similar increase in thymidine incorporation (2.8-fold of control) or concordant with increases in mitotic figures (7/20 mice having any mitotic figures at all with a range of 0–5 and a mean of 0.014% of cells undergoing mitosis for 1,000 mg/kg TCE vs. 0 for control).

Although results reported in the rat showed discordance between thymidine incorporation and detection of mitotic figures, there was also discordance with these indices and those for peroxisomal proliferation. In comparison to controls, there was a reported 13% increase in PCO activity in Alderley Park rats exposed to 1,000 mg/kg TCE, a group mean of mitotic figures half that in the TCE treated animals vs. controls, and increase in thymidine incorporation of 40%. Thus, these results are not consistent with TCE induction of peroxisome enzyme activity to be correlated with hepatocellular proliferation by either mitotic index or thymidine incorporation. Thymidine incorporation in liver DNA seen with TCE exposure also did not correlate with mitotic index activity in hepatocytes and suggests that this parameter may be a reflection of polyploidization rather than hepatocyte proliferation. More importantly, these data show that hepatocyte proliferation, indicated by either measure, is confined to a very small population of cells in the liver after 10 days of TCE exposure. Hepatocellular hypertrophy in the centrilobular region appears to be responsible for the liver weight gains seen in both rats and mice rather than increases in cell number. These results at 10 days do not preclude the possibility that a greater level of hepatocyte proliferation did not occur earlier and then had subsided by 10 days, as is characteristic of many mitogens. Thymidine incorporation represents the status of the liver at one time point rather than over a period of whole week, and thus, would not capture the earlier bouts of proliferation. However, there is no evidence of a sustained proliferative response, as measured at the 10-day time period, in hepatocytes in response to TCE indicated from these data.

In regards to weight gain, although the volume of the peroxisomal compartment was reported to be similar at 500 mg/kg TCE in $B6C3F_1$ and Alderley Park mice (4.3%), the liver weight/body weight gain in comparison to control was 20% higher in $B6C3F_1$ mice vs. 43% higher in Alderley Park mice after 10 days of exposure. The liver/body weight ratio was 5.53% in the $B6C3F_1$ mice and 7.31% in the Alderley Park mice at 500 mg/kg TCE for 10 days.

Similarly, although the peroxisomal compartment was similar at 1,000 mg/kg TCE in Osborne-Mendel (2.3%) and Alderley Park rats (2.4%), the liver weight/body weight gain was 26% in Osborne-Mendel rats but 17% in Alderley Park rats at this level of TCE exposure. The liver/body weight ratio was 5.35% in the Osborne-Mendel rats and 5.83% in the Alderley Park mice at 1,000 mg/kg TCE for 10 days. Although there are several limitations regarding the

quantitative interpretation of the data, as discussed above, the data suggest that liver weight and weight gain after TCE treatment was not just a function of peroxisome proliferation. This study does clearly demonstrate TCE-induced changes at the lowest level tested in several parameters without toxicity and without evidence of regenerative hyperplasia or sustained hepatocellular proliferation. In regards to susceptibility to liver cancer induction in more susceptible ($B6C3F_1$) vs. less susceptible (Alderley Park/Swiss) strains of mice (Maltoni et al., 1988), there was a greater baseline level of liver weight/body weight ratio change, a greater baseline level of thymidine incorporation as well as greater responses for those endpoints due to TCE exposure in the "less susceptible" strain. However, both strains showed a hepatocarcinogenic response to TCE induction and the limitations of being able to make quantitative conclusions regarding species and strain susceptibility TCE toxicity from this study have been described in detail above.

E.2.1.9. Dees and Travis (1993)

The focus of this study was to evaluate the nature of DNA synthesis induced by TCE exposure in mice. The mitotic rate of liver cells was extrapolated using tritiated thymidine uptake into DNA of male and female mice treated with HPLC grade (99 + pure) TCE. Male and female hybrid $B6C3F_1$ mice 8 weeks of age (male mice weighed 24–27 g [~12% difference] and females weighing 18–21 g [~4% difference]) were dosed orally by gavage for 10 days with 100, 250, 500, and 1,000 mg/kg body weight TCE in corn oil (n = 4 per treatment group). Sixteen hours after the last daily dose of TCE, mice received tritiated thymidine and were sacrificed 6 hours later. Hepatic DNA was extracted from whole liver and standard histopathology was also performed. Hepatic DNA content and cellular distributions were also determined for thymidine uptake using autoradiography of tissue sections. Tritiated thymidine incorporation into DNA was determined by microscopic observations of autoradiography slides and reported as positive cells per 100 (200× power) fields.

Changes in the treatment groups were reported to:

> include an increase in eosinophilic cytoplasmic staining of hepatocytes located near central veins, accompanied by loss of cytoplasmic vacuolization. Intermediate zones appeared normal and no changes were noted in portal triad areas. Male and female mice given 1,000 mg/kg body weight TCE exhibited apoptosis located near central veins. No evidence of cellular proliferation was seen in the portal areas. No evidence of increased lipofuscin was seen in liver sections from male and female mice treated with TCE. Evaluation of cell death in male and female mice receiving TCE was performed by enumerating apoptosis.

The apoptosis "did not appear to be in proportion to the applied TCE dose given to male or female mice." The mean number of apoptosis per 100 (400×) fields in each group of 4 animals (male mice) was 0, 0, 0, 1, and 8 for control, 100, 250, 500, and 1,000 mg/kg TCE

treated groups, respectively. Variations in number of apoptosis between mice were not given by the authors. Feulgen stain was <1 for all doses except for 9 at 1,000 mg/kg.

Mitotic figure were reported to be:

> frequently seen in liver sections from both male and female mice treated with TCE. Dividing cells were most often found in the intermediate zone and resembled mature hepatocytes. Incorporation of the radiolabel into cells located near the portal triad areas was rare. In general, mitotic figures were very rare, but when found they were usually located in the intermediate zone. Little or no incorporation of label was seen in areas near the bile duct epithelia or in areas close to the portal triad.

No quantitative description of mitotic index was reported by the authors, but this description is consistent with there being replication of mature hepatocytes induced by TCE.

The distribution of tritiated thymidine was given for specific cell types in the livers of five animals per treatment group and radiolabel was reported to be predominantly associated with perisinusoidal cell in control mice. The authors state that the label was more often found in cells resembling mature hepatocytes. The mean number of labeled cells in autoradiographs per 100 (200× power) fields was reported to be ~125 and ~150 labeled perisinusoidal cells in controls male and female mice, respectively. The authors do not give any SDs for the female perisinusoidal data except for the 1,000-mg/kg exposure group. For mature hepatocytes, the mean baseline level of cell labeling for control male and female mice were reported to be ~65 and ~90 labeled cells, respectively. Although the baseline levels of hepatocyte labeling were reported to differ between male and female mice, the mean peak level of labeling was similar at ~250 labeled cells for male and female mice treated with TCE. In male mouse liver, the number of labeled cells increased approximately twofold of control levels after 500 and 1,000 mg/kg TCE and in female mouse liver increased approximately fourfold of control levels after 250, 500, and 1,000 mg/kg TCE over their respective control levels.

Incorporation of tritiated thymidine into DNA extracted from whole liver in male and female mice was reported to be significantly elevated after TCE treatment but, unlike the autoradiographic data, there was no difference between genders and the mean peak level of tritiated thymidine incorporation occurred at 250 mg/kg TCE treatment and remained constant for the 500 and 1,000 mg/kg treated groups. Increased thymidine incorporation into DNA extracted from liver of male and female mice were reported to show a very large SD with TCE treatment (e.g., at 100 mg/kg TCE exposure, male mice had a mean of ~130 dpm tritiated thymidine/µg DNA with the upper bound of the SD to be 225 dpm). The increased thymidine incorporation peaked at a level that was a little <2-fold of control level. Thus, for both male and female mice both autoradiographs and total hepatic DNA were reported to show that male and

female mice had similar peaks of increased thymidine incorporation after TCE exposure that reached a plateau at the 250 mg/kg TCE exposure level and did not increase with increasing exposure concentration. These data also indicate a very small population undergoing mitosis due to TCE exposure after 10 days of exposure. If higher levels of hepatocyte replication had occurred earlier, such levels were not sustained by 10 days of TCE exposure. More importantly, these data suggest that tritiated thymidine levels were targeted to mature hepatocytes and in areas of the liver where greater levels of polyploidization occur. The ages and weights of the mice were described by these authors, unlike Elcombe et al. (1985), and a different strain was used. However, these results are consistent with those of Elcombe in regard to the magnitude of thymidine incorporation induced by TCE treatment and the lack of a dose response once a relative low level of exposure has been exceeded.

The total liver DNA content of male and female mice treated with TCE were also determined with the total micrograms DNA/g liver reported to be ~4 μg/g for female control mice and ~2 μg/g for male control mice. Although not statistically significant, the total DNA concentration dropped from ~4 to ~3 at 100 mg/kg through 1,000 mg/kg exposure to TCE in female mice. For male mice, the total DNA rose slightly in the 250- and 500-mg/kg groups to ~3 μg/g and was similar to control levels at the 100 and 1,000 mg/kg TCE treatment groups. The SD in male mice was very large and the number of animals small making quantitative judgments regarding this parameter difficult. The slight decrease reported for female mice would be consistent with the results of Elcombe et al. (1985) who describe a slight decrease in hepatic DNA in male mice. However, the reported slight increase in hepatic DNA in male mice in this study is not consistent. Given the small number of animals and the large deviations for female and male mice in the TCE treated groups, this study may not have had the sensitivity to detect slight decreases reported by Elcombe et al. (1985).

In regard to clinical evaluation and weight analyses, both male and female mice given TCE were reported "to appear clinically ill. These mice showed reduced activity and failed to groom. Control mice showed no adverse effects. Female mice were markedly more affected by TCE than their male counterparts. Several deaths of female mice occurred during the course of the TCE treatment regimen." The authors do not give cause of deaths but state that two female mice died in the group receiving 250 mg/kg TCE and one in the group receiving 1,000 mg/kg during the gavage regimen of the female mice. This appears to be similar gavage error or "accidental death" reported in NTP studies chronic studies of TCE (see below).

The authors report:

> no significant difference in the absolute body weight of male and female mice were noted in control groups. Body weight gain in female and males mice treated with TCE was not significantly different from that of control mice. Liver weights in male mice given 500 or 1,000 mg/kg and corrected for total body weight were

significantly elevated. The corrected liver weights of female mice increase proportionally with the applied dose of TCE.

For male mice, liver weights were reported to be 1.40 ± 0.16, 1.38 ± 1.23, 1.48 ± 0.09, 1.61 ± 0.07, and 1.63 ± 0.11 g for control, 100, 250, 500, and 1,000 mg/kg TCE in male mice (n = 5), respectively. Body weights were smaller for the 100 mg/kg TCE treatment group although not statistically significant. The liver weights after treatment had a much larger reported SD (1.23 g for 100 mg/kg group vs. <0.16 for all other groups). The percent liver/body weight ratios were reported to be 5.40, 5.41, 5.42, 5.71, and 6.34% for the same groups in male mice. This represents 1.06- and 1.17-fold of control at the 500 and 1,000 mg/kg dose. The authors report a statistically significant increase in percent liver/body weight ratio only for the 500 mg/kg (i.e., 1.06-fold of control) and 1,000 mg/kg (i.e., 1.17-fold of control) TCE exposure groups.

The results for female mice liver weights were reported in Table III of the paper, which was mistakenly labeled as for male mice. The reported values for liver weight were 1.03 ± 0.07, 1.05 ± 0.10, 1.15 ± 0.98, 1.21 ± 0.18, and 1.34 ± 0.08 g for control, 100, 250, 500, and 1,000 mg/kg TCE in female mice (n = 5, except for 250 and 1,000 mg/kg groups), respectively. The percent liver/body weight ratios were 5.26, 5.44, 5.68, 6.24, and 6.57% for the same groups. These values represent 1.03-, 1.08-, 1.19-, and 1.25-fold of controls in percent liver/body weight. The magnitude of increase in TCE-induced percent liver/body weight ratio in female mice is reflective of the magnitude of the difference in dose up to 1,000 mg/kg where it is slightly lower. The female mice were reported to have statistically significant increases in percent liver/body ratios at the lowest dose tested (100 mg/kg TCE) after 10 days of TCE exposure that also increased proportionately with dose. Male mice were not reported to have a significant increase in percent liver/body weight until 500 mg/kg TCE but a statistically significant increase in liver weight at 250 mg/kg TCE. Male mice had a much larger variation in initial body weight than did female mice (range of means of 24.86–27.84 g between groups for males or ~11% difference and range of means of 19.48–20.27 g for females or ~4%), which may contribute to an apparent lack of effect for a parameter that is dependent on body weight. Only five mice were used in each group so the power to detect a change was relatively small.

The results from this experiment are consistent with those of Elcombe et al. (1985) in showing a slight increase in thymidine incorporation (approximately twofold of control) and mitotic figures that are rare after TCE exposure. This study also records a lack of apoptosis with TCE treatment except at the highest exposure level (i.e., 1,000 mg/kg). The increases in liver weight induced by TCE were reported to be dose-related, especially in female mice where baseline body weights were more consistent. However, the incorporation of tritiated thymidine reached a plateau at 250 mg/kg TCE in the DNA of both genders of mice. This study specifically identified where thymidine incorporation and mitotic figures were occurring in

TCE-treated livers and noted that the mature hepatocyte that appeared to be primarily affected, as well as in the portion of the liver where mature hepatocytes with higher ploidy are found. The authors note that the "lack of thymidine incorporation in the periportal area, where the liver stem cells are reside," suggesting that the mature hepatocyte is the target of TCE effects on DNA synthesis. This finding is consistent with a change in ploidy accompanying hepatocellular hypertrophy and not just cell proliferation after 10 days of TCE exposure. Like Elcombe et al. (1985), these data represent "a snapshot in time," which does not show whether increased cell proliferation may have happened at an earlier time point and then subsided by 10 days. However, like Elcombe et al. (1985), it suggests that sustained proliferation is not a feature of TCE exposure and that the level of DNA synthesis (which is very low in quiescent control liver) is increased in a small population of hepatocytes due to TCE exposure that is not dose-dependent (only twofold increase over control in animals exposed from 250 to 1,000 mg/kg TCE). In regards to toxicity, no evidence of increased lipid peroxidation in TCE-treated animals was reported using histopathologic sections stained to enhance observation of lipofuscin. No necrosis is noted by these authors and the deaths in female mice are likely due to gavage error.

E.2.1.10. Nakajima et al. (2000)

This study focused on the effect of TCE treatment on PPARα-null mice in terms of peroxisome proliferation but also included information on differences in liver weight between null and wild-type mice, as well as gender-related effects. SV129 wild-type and PPARα-null mice (10 weeks of age) were treated with corn oil or 750 mg/kg TCE in corn oil daily for 2 weeks via gavage (n = 6 per group). A small portion of the liver was removed for histopathological examination but the lobe used was not specified by the authors. Liver peroxisome proliferation was reported to be evaluated morphologically using 3,3'-diamino-benzidine (DAB) staining of sections and electron photomicroscopy to detect the volume density of peroxisomes (percent of cytoplasm) in 15 micrographs of the pericentral area per liver. A number of β-oxidation enzymes and P450s were analyzed by immunoblot of liver homogenates.

The final body weights, liver weights, and percent liver/body weight ratios were reported for all treatment groups. For male mice, vehicle treated PPARα-null mice had slightly lower mean body weights (24.5 ± 1.8 vs. 25.4 ± 1.9 g [SD]), slightly larger liver weights (1.14 ± 0.13 vs. 1.05 ± 0.15 g or ~9%), and slightly higher percent liver/body weight ratios (4.12 ± 0.32 vs. 4.10 ± 0.37%) than wild-type mice. The mean values for final body weights of the groups of mice in this study were reported and were similar which, as demonstrated by the inhalation studies by Kjellstrand et al. (1983b) (see Section E.2.2.5), is particularly important for determining the effects of TCE treatment on percent liver/body weight ratios. For both groups of male mice, 2 weeks of TCE treatment significantly increased both liver weight and percent liver/body weight ratios. For male wild-type mice, the increase in percent liver/body weight was

1.50-fold of vehicle control and for male PPARα-null mice the increase was 1.26-fold of control after 2 weeks of TCE treatment.

For female mice, vehicle-treated PPARα-null mice had slightly higher mean body weights (22.7 ± 2.1 vs. 22.4 ± 2.0 g), slightly larger liver weights (0.98 ± 0.15 vs. 0.95 ± 0.14 g or ~3%), and slightly higher percent liver/body weight ratios (4.32 ± 0.35 vs. 4.24 ± 0.41%) than wild-type mice. For both groups of female mice, 2 weeks of TCE treatment significantly increased percent liver/body weight ratios. For liver weights, there was a reporting error for PPARα-null female treated with TCE so that liver weight changes due to TCE treatment cannot be determined for this group. For female wild-type mice, the increase in percent liver/body weight was 1.24-fold of vehicle control and for female PPARα-null mice, the increase was 1.26-fold of control after 2 weeks of TCE treatment.

Thus, for both wild-type and PPARα-null mice, TCE exposure resulted in increased percent liver/body weight over controls that was statistically significant after 2 weeks of gavage exposure using corn oil as the vehicle. For male mice, there was a greater TCE-induced increase in percent liver/body weight in wild-type than PPARα-null mice (1.50- vs. 1.26-fold of control) that was statistically significant, but for female mice, the induction of increased liver weight was statistically increased but the same in wild-type and PPARα-null mice (i.e., both were ~1.25-fold of control). These date indicate that TCE-induced increases in mouse liver weight were not dependent on a functional PPARα receptor in female mice and suggest that some portion may be in male mice.

In regard to light and electron microscopic results, the numbers of peroxisomes in hepatocytes of wild-type mice were reported to be increased, especially in the pericentral area of the hepatic lobule, to a similar extent in both males and females (15 micrographs, n = 4 mice). TCE exposure was reported to increase the volume density of peroxisomes twofold of control in the pericentral area with no evident change in peroxisomes in the periportal areas, but data were not shown for that area of the liver lobule. In contrast, no increase in peroxisomes was reported to be observed in PPARα-null mice. Therefore, increases in liver weight observed in PPARα-null mice after TCE treatment did not result from peroxisome proliferation. Similarly, the small twofold increase in peroxisome volume from 2 to 4% of cytoplasmic volume in the pericentral area of the liver lobule in wild-type mice could not have been responsible for the 50% increase liver weight observed in male wild-type mice.

Although no difference was reported between male and female wild-type mice in regard to TCE-induced peroxisome proliferation in wild-type mice, the levels of hepatic enzymes associated with peroxisomes (acyl-CoA [AOX], peroxisomal bifunctional protein [PH], peroxisomal thiolase [PT], very long chain acyl-CoA synthetase, and D-type peroxisomal bifunctional protein [DBF], cytosolic enzyme [cytosolic thioesterase II (CTEII)], mitochondrial enzymes [mitochondrial trifunctional protein α subunits α and β(TPα and TPβ)], and microsomal enzymes [CYP 4A1 (CYP4A1)]) as measured by immunoblot analysis were significantly

elevated in male wild-type mice (n = 4) by a factor of ~2–3, but except for a slight elevation in PH and PT, were reported to not be elevated in female wild-type mice (n = 4). The magnitude of increase in peroxisomal enzymes was similar to that of peroxisomal volume in male mice. No TCE-induced increases in any of these enzymes were reported in male or female PPARα-null mice by the authors. For CYP4A1, an enzyme reported to be induced by peroxisomal proliferators, TCE exposure resulted in a much lower amount in female than male wild-type mice (i.e., 2% of the level induced by TCE in males). However, the expression of catalase was reported to be "nearly constant in all samples" (at most ~30% change), which the authors suggested resulted from induction by TCE that was independent of PPARα. The basis for selection of four mice for this comparison out of the six studied per group was not given by the authors. A comparison of control wild-type and PPARα-null mice showed that in males background levels of the enzymes examined were generally similar except for DBF in which the null mice had values ~50% of the wild-type controls. A similar decrease was reported for female PPARα-null mice. With regard to gender differences in wild-type mice, females had similar values as males with the exceptions of TPα, TPβ, and CYP2E1, which were in untreated female wild-type mice at a 3.06-, 2.38-, and 1.63-fold for l TPα, TPβ, and CYP2E1 levels over males, respectively. Female PPARα-null mice had increases of 2.50-, 1.54-, and 2.07-fold over male wild-type mice.

With regard to the induction of TCE metabolizing enzymes (CYP1A2, CYP2E1, and ALDH), CYP1A2 was reported to be decreased by TCE treatment of both male and female wild-type mice but liver CYP2E1 reported to be increased in male mice and constant in female mice which resulted in similar expression level in both genders after TCE treatment. There was no gender difference in ALDH activity reported after TCE exposure and activity was reported to be independent of PPARα. The authors concluded that TCE metabolizing abilities of the liver of male and female mice were similar, and therefore, poor induction of peroxisomal related enzymes was not due to gender-related differences in TCE metabolism.

To investigate whether the a gender-related difference peroxisomal enzymes after TCE exposure was due to a lower levels of PPARα and RXRα receptors, western blotting was employed (n = 3). The level of PPARα protein was reported to be increased in both male wild-type mice with less induction in females (control vs. TCE, 1.00 ± 0.20 vs. 2.17 ± 0.24 in males and 0.95 ± 0.25 vs. 1.44 ± 0.09 in females) after TCE treatment. The hepatic level of RXRα was also reported to be increased in the same manner as PPARα (control vs. TCE, 1.00 ± 0.33 vs. 1.92 ± 0.04 in males 0.81 ± 0.16 vs. 1.14 ± 0.10 in females). Northern blot analysis of hepatic PPARα mRNA was reported to show greater TCE induction in male (2.6-fold of control) than in female (1.5-fold of control) wild-type mice. Thus, males appeared to have higher induction of the two receptor proteins as well as a greater response in peroxisomal enzymes and CYP4A1, even though TCE-induced increases in peroxisomal volume was similar between male and female mice. The increased response in males for induction of the two receptor proteins is

consistent with liver weight data that shows some portion of the induction of increased liver weight response in male mice using this paradigm may be due to gender-specific differences in PPARα response. However, as noted below (see Section E.2.2), corn oil vehicle has liver effects alone, especially in the male liver, that have also been associated with PPARα responses.

E.2.1.11. Berman et al. (1995)

This study included TCE in a suite of compounds used to compare endpoints for toxicological screening methods. Female F344 rats of 77 days of age (n = 8 per group) were administered TCE in corn oil for 1 day (0, 150, 500, 1,500, or 5,000 mg/kg-day) or for 14 days (0, 50, 150, 500, or 1,500 mg/kg-day). Blood samples were taken 24 hours after the last dose and livers were weighed and H&E sections were examined for evidence of parenchymal cell degeneration, necrosis, or hypertrophy. No details were provided by the authors for the extent or severity of the liver affects by histopathological examination. The serum chemistry analysis included LDH, ALP, ALT, AST, total bilirubin, creatine, and BUN. The starting and ending body weights of the animals or the absolute liver weights were not reported by the authors.

The results of a multivariate analysis were reported to show a lowest effective dose of 1,500 mg/kg after 1 day of TCE exposure and 150 mg/kg after 14 days of TCE exposure that was statistically significant. Liver weight and liver weight changes were not reported by the authors but the percent liver to body weight ratios were. For the two control groups, there was a difference in percent liver/body weight of ~8% (3.43 ± 0.74% for the 1-day control group and 3.16 ± 0.41% for the 14-day control group, mean ± SEM). For the 1-day groups, only the 5,000 mg/kg group was reported to show a statistically significant difference in percent liver/body weight between control and TCE treatment (i.e., ~1.08-fold increase). Hepatocellular necrosis was noted to occur in the 1,500 and 5,000 mg/kg groups in 6/7 and 6/8 female rats, respectively, but not to occur in lower doses. The extent of necrosis was not noted by the authors for the two groups exhibiting a response after 1 day of exposure. Serum enzymes indicative of liver necrosis were not presented and because only positive results were presented in the paper, were presumed to be negative. Therefore, the extent of necrosis was not of a magnitude to affect serum enzyme markers of cellular leakage.

After 14 days of TCE exposure, there was a dose-related increase reported for percent liver/body weight ratios that was statistically significant at all TCE dose levels although the multivariate analysis indicated the lowest effective dose to be 150 mg/kg. The percent liver/body weight ratio was 3.16 ± 0.41, 3.38 ± 0.56, 3.49 ± 0.69, 3.82 ± 0.76, and 4.47 ± 0.66% for control, 50, 150, 500, and 1,500 mg/kg TCE exposure levels, respectively, after 14 days of exposure. No hepatocellular necrosis was reported at any dose and hepatocellular hypertrophy was reported only at the 1,500 mg/kg dose and in all rats. These rat liver weights were 1.07-, 1.10-, 1.21-, and 1.41-fold of controls for the 50, 150, 500, and 1,500 mg/kg TCE dose groups, respectively. The 7% increase in liver weight at the 50 mg/kg dose was approximately the same

difference between the two control groups for days 1 and 14 treatments. Without the data for starting and final body weights and an examination of whether the control animals had similar body weight, it is impossible to discern whether the reported effects at the low dose of TCE were also reflected differences between the control groups. No serum enzyme levels changes were reported after 14 days of exposure to TCE for any group.

The authors note that their study provided evidence of liver effects at lower levels than other studies citing Elcombe et al. (1985) and Goldsworthy and Popp (1987). They suggest that the differences in sensitivity to TCE between their results and those of these two studies may reflect differences in strain or gender of the rats examined. However, they did not study male rats of this strain concurrently so that differences in gender may have reflected differences between experiments. The increase in liver weight without reporting increases in hepatocellular hypertrophy as well as the lack of necrosis as low doses is consistent with the results of Melnick et al. (1987) in male Fischer rats given TCE orally (see Section E.2.1.12).

E.2.1.12. Melnick et al. (1987)

The focus of this study was to assess microencapsulation as a way to expose rodents to substances such as TCE that have issues related to volatilization in drinking water or apparent gavage-related deaths. In this study, liver weight changes, extent of focalized necrosis, and indicators of peroxisome proliferation were reported as metrics of TCE toxicity. TCE (99+ %) was encapsulated in gelatin-sorbitol microcapsules and was 44.1% TCE w/w. The TCE microcapsules were administered to male F344 rats (6-week-old and weighing between 89 and 92 g or ~3% difference) in the diet (0, 0.55, 1.10, 2.21, and 4.42% TCE in the diet) for 14 days. The number of animals in each group was 10. A parallel group of animals was administered TCE in corn oil gavage for 14 consecutive days (corn oil control, 0.6, 1.2, and 2.8 g/kg-day TCE). The dosage levels of TCE in the gavage study were reported to be "adjusted 5 times during the 14-day" treatment period to be similar to the dosage levels of TCE in the feed study. The TWA dosage levels of TCE in the feed study were reported to be 0.6, 1.3, 2.2, and 4.8 g/kg-day.

There was less food consumption reported in the 2.2 and 4.8 g/kg-day dose feed groups, which the authors attribute to either palatability or toxicity. There were no deaths in any of the groups treated with microencapsulated TCE while, similar to many other gavage studies of TCE reported in the literature, there were four deaths in the high-dose gavage group. Mean body weight gains of the two highest dose groups of the feed study and of the highest dose group of the gavage study were reported to be significantly lower than the mean body weight gains of the respective control groups (i.e., ~22 and ~35% reduction at 2.2 and 4.8 g/kg-day in the feed study, respectively, and ~33% reduction at 2.8 g/kg-day TCE in the gavage study).

After 14 days of treatment, liver weights were reported to be 8.1 ± 0.8, 8.4 ± 0.8, 9.5 ± 0.5, 10.1 ± 1.2, 8.9 ± 1.3, and 7.4 ± 0.5 g for untreated control, placebo control, 0.6, 1.3, 2.2, and

4.8 g/kg TCE exposed feed groups, respectively. The corresponding percent liver/body weight ratios were reported to be 5.2 ± 0.3, 5.3 ± 0.2, 6.0 ± 0.3, 6.5 ± 0.5, 7.0 ± 0.9, and 7.1 ± 0.5% for untreated control, placebo control, 0.6, 1.3, 2.2, and 4.8 g/kg TCE exposed groups, respectively. The increased percent liver/body weight ratio represents 1.13-, 1.23-, 1.32-, and 1.34-fold of placebo controls, respectively.

For the gavage experiment, after 14 days of treatment, liver weights were reported to be 7.1 ± 1.3, 9.3 ± 1.2, 9.1 ± 0.9, and 7.7 ± 0.4 g for corn oil control, 0.6, 1.2, and 2.8 g/kg TCE exposed groups, respectively. The corresponding percent liver/body weight ratios were reported to be 5.0 ± 0.4, 6.0 ± 0.4, 6.1 ± 0.3, and 7.3 ± 0.5% for corn oil control, 0.6, 1.2, and 2.8 g/kg TCE exposed groups, respectively. The percent liver/body weight ratios represent 1.20-, 1.22-, and 1.46-fold of corn oil controls, respectively. The 2.8 g/kg TCE gavage results are reflective of the 6 surviving animals in the group rather than 10 animals in the rest of the groups. There was no explanation given by the authors for the lower liver weights in the control gavage group than the placebo control in the feed group (i.e., 20% difference), although the initial and final body weights appeared to be similar. The decreased body weights in the feed and gavage study are reflective if TCE systemic toxicity and appeared to affect the TCE-induced liver weight increases in those groups.

The authors reported that the only treatment-related lesion observed microscopically in rats from either dosed-feed or gavage groups was individual cell necrosis of the liver with the frequency and severity of this lesion similar at each dosage levels of TCE administered microencapsulated in the feed or in corn oil. Using a scale of minimal = 1–3 necrotic hepatocytes/10 microscopic 200× fields, mild = 4–7 necrotic hepatocytes/10 microscopic 200× fields, and moderate = 8–12 necrotic hepatocytes/10 microscopic 200× fields, the frequency of lesion was 0–1/10 for controls, 2/10 for 0.6 and 1.3 g/kg, and 9/10 for 2.2 and 4.8 g/kg feed groups. The mean severity was reported to be 0.0–0.1 for controls, 0.3–0.4 for 0.6 and 1.3 g/kg, and 2.0–2.5 for 2.2 and 4.8 g/kg feed groups. For the corn oil gavage study, the corn oil control and 0.6 g/kg groups were reported to have a frequency of 0 lesions/10 animals; the 1.2 g/kg group had a frequency of 1/10 animals, while the 2.8 g/kg group had a frequency of 5/6 animals. The mean severity score was reported to be 0 for the control and 0.6 g/kg groups, 0.1 for the 1.2 g/kg groups, and 1.8 for the remaining six animals in the 2.8 g/kg group. The individual cell necrosis was reported to be randomly distributed throughout the liver lobule with the change to not be accompanied by an inflammatory response. The authors also report that there was no histologic evidence of cellular hypertrophy or edema in hepatic parenchymal cells. Thus, although there appeared to be TCE-treatment-related increases in focal necrosis after 14 days of exposure, the extent was, even at the highest doses, mild and involved few hepatocytes.

Microsomal NADPH cytochrome c-reductase was reported to be elevated in the 2.2 and 4.8 g/kg feed groups and in the 1.2 and 2.8 g/kg gavage groups. CYP levels were reported to be elevated only in the two highest dose groups of the feed study. The authors reported a dose-

related increase in peroxisome PCO and catalase activities in liver homogenates from rats treated with TCE microcapsules or by gavage, and that treatment with corn oil alone, but not placebo capsules, caused a slight increase in PCO activity.

After 14 days of treatment, PCO activities were reported to be 270 ± 12, 242 ± 17, 298 ± 64, 424 ± 55, 651 ± 148, and 999 ± 266 nmol hydrogen peroxide (H_2O_2) produced/minute/g liver for untreated control, placebo control, 0.6, 1.3, 2.2, and 4.8 g/kg TCE exposed feed groups, respectively. This represents 1.23-, 1.75-, 2.69-, and 4.13-fold of placebo controls, respectively. After 14 days of treatment, catalase activities were reported to be 8.49 ± 0.81, 7.98 ± 1.62, 8.49 ± 1.92, 8.59 ± 1.31, 13.03 ± 2.01, and 15.76 ± 1.11 nmol H_2O_2 produced/minute/g liver for untreated control, placebo control, 0.6, 1.3, 2.2, and 4.8 g/kg TCE exposed groups, respectively. This represents 1.06-, 1.07-, 1.63-, and 1.97-fold of placebo controls, respectively. Thus, although reported to be dose related, only the two highest exposure levels of TCE increased catalase activity and to a smaller extent than PCO activity in microencapsulated TCE fed rats.

For the gavage experiment, after 14 days of treatment, PCO activities were reported to be 318 ± 27, 369 ± 26, 413 ± 40, and 1,002 ± 271 nmol H_2O_2 produced/minute/g liver for corn oil control, 0.6, 1.2, and 2.8 g/kg TCE exposed groups, respectively. This represents 1.16-, 1.29-, and 3.15-fold of corn oil controls. After 14 days of treatment, catalase activities were reported to be 8.59 ± 0.91, 10.10 ± 1.82, 12.83 ± 3.43, and 13.54 ± 2.32 nmol H_2O_2 produced/minute/g liver for corn oil control, 0.6, 1.2, and 2.8 g/kg TCE exposed groups, respectively. This represents 1.18-, 1.49-, and 1.58-fold of corn oil controls. As stated by the authors, the corn oil vehicle appeared to elevate catalase activities and PCO activities.

In regard to dose-response, liver and body weight were affected by decreased body weight gain in the higher dosed animals in this experiment (i.e., 2.2 g/kg-day TCE exposure and above) and by gavage related deaths in the highest-dosed group. The lower liver weight in the gavage control group also may have affected the determination of the magnitude of TCE-related liver weight gain at that dose. At the two doses, below which body weight gain was affected, there appeared to be an approximately 20% increase in percent liver/body weight ratio in the gavage study and a 13 and 23% weight increase in the feed study.

The extent of PCO activity appeared to increase more steeply with dose in the feed study than did liver weight gain (i.e., a 1.23-fold of liver/body weight ratio at 1.3 g/kg-day corresponded with a 1.75-fold PCO activity over control). At the two highest doses in the feed study, the increase in PCO activity was 2.69- and 4.13-fold of control, but the increase in liver weight was not more than 34%. For the gavage study, there was also a steeper increase in PCO activity than liver weight gain. For catalase activity, the increase was slightly less than that of liver/body weight ratio percent for the two doses that did not decrease body weight gain in the feed study. In the gavage study, they were about the same. In regard to what the cause of liver weight gain was, the authors report that there was no histologic evidence of cellular hypertrophy or edema in hepatic parenchymal cells and do not describe indicators of hepatocellular

proliferation or increased polyploidy. Accordingly, the cause of liver weight gain after TCE exposure in this paradigm is not readily apparent.

E.2.1.13. Laughter et al. (2004)

Although the focus of the study was an exploration of potential modes of action for TCE effects through macroarray transcript profiling (see Section E.3.1.2 for discussions of limitations of this approach and especially the need for phenotypic anchoring, Section E.3.4.1.3 for use of PPARα knockout mice, and Section E.3.4.2.2 for discussion of genetic profiling data for TCE), information was reported regarding changes in the liver weight of PPARα-null mouse and their background strains. SV129 wild-type and PPARα-null male mice (9 ± 1.5 weeks of age) were treated with three daily doses of TCE in 0.1% methyl cellulose for either 3 days or 3 weeks (n = 4–5/group). Thus, this paradigm does not use corn oil, which has been noted to affect toxicity (see Section E.2.2 below), but is not comparable to other paradigms that administer the total dose in one daily gavage administration rather than to give the same cumulative dose but in three daily doses of lower concentration. The initial or final body weights of the mice were not reported. Thus, the effects of systemic toxicity from TCE exposure on body weight and the influence of differences in initial body weight on percent liver/body weight determinations cannot be made.

For the 3-day study, mice were administered 1,500 mg/kg TCE or vehicle control. For the 3-week study, mice were administered 0, 10, 50, 125, 500, 1,000, or 1,500 mg/kg TCE 5 days/week except for 4 days/week on the last week of the experiment. In a separate study, mice were given TCA or DCA at 0.25, 0.5, 1, or 2 g/L (pH ~7) in the drinking water for 7 days. For each animal, a block of the left, anterior right, and median liver lobes was reported to be fixed in formalin with five sections stained for H&E and examined by light microscopy. The remaining liver samples were combined and used as homogenates for transcript arrays. In the 3-week study, bromodeoxyuridine (BrdU) was administered via miniosmotic pump on day 1 of week 3 and sections of the liver assessed for BrdU incorporation in at least 1,000 cells per animal in 10–15 fields.

Although initial body weights, final body weights, and the liver weights were not reported, the percent liver/body ratios were. In the 3-day study, control wild-type and PPARα-null mice were reported to have similar percent liver/body weight ratios of ~4.5%. These animals were ~10 weeks of age upon sacrifice. However, at the end of the 3-week experiment, the percent liver/body weight ratios were increased in the PPARα-null male mice and were 5.1%. There was also a slight difference in the percent liver/body weight ratios in the 1-week study (4.3 ± 0.4 vs. 4.6 ± 0.2% for wild-type and PPARα-null mice, respectively). These results are consistent with an increasing baseline of hepatic steatosis with age in the PPARα-null mice and increase in liver weight.

In the 3-day study, the mean report for the percent liver/body ratio was 1.4-fold of the wild type animals tested with TCE in comparison to the control level. In the PPARα-null mice, there was a 1.07-fold of control level reported by the authors to not be statistically significant. However, given the low number of animals tested (the authors give only that four to five animals were tested per group without identification as to which groups had four animals and which had five), the ability of this study to discern a statistically significant difference is limited.

In the 3-week study, wild-type mice exposed to various concentrations of TCE had percent liver/body weights that were within ~2% of control values except for the 1,000 mg/kg and 1,500 mg/kg groups that were ~1.18- and 1.30-fold of control levels, respectively. For the PPARα-null mice exposed to TCE for 3 weeks, the variability in percent liver/body weight was greater than that of the wild-type mice in most of the groups. The baseline level percent liver/body weight was 1.16-fold in the PPARα-null mice in comparison to wild-type mice. At the 1,500 mg/kg TCE exposure level, percent liver/body weights were not recorded because of the death of the null mice at this level. The authors reported that at the 1,500 mg/kg level, all PPARα-null mice were moribund and had to be removed from the study. However, at the 1,000 mg/kg TCE exposure level, there was a 1.10-fold of control percent liver/body weight value that was reported to not be statistically significant. As noted above, the power of the study was limited due to low numbers of animals and increased variability in the null mice groups. The percent liver/body weight reported in this study was actually greater in the null mice than the wild-type male mice at the 1,000 mg/kg TCE exposure level (5.6 ± 0.4 vs. 5.2 ± 0.5%, for null and wild-type mice, respectively).

Thus, at 1 and 3 weeks, TCE appeared to induce increases in liver weight in PPARα-null mice, although not reaching statistical significance in this study, with concurrent background of increased liver weight reported in the knockout mice. At 1,000 mg/kg TCE exposure for 3 weeks, percent liver/body weight was reported to be 1.18-fold in wild-type and 1.10-fold in null mice of control values. As discussed above, Nakajima et al. (2000) reported statistically significant increased liver weight in both wild-type and PPARα-null mice after 2 weeks of exposure with less TCE-induced liver weight increases in the knockout mice (see Section E.2.1.10). They also used more mice, carefully matched to weights of their mice, and used a single dose of TCE each day with corn oil gavage.

The authors noted that inspection of the livers and kidneys of the moribund null mice, who were removed from the 3-week study, "did not reveal any overt signs of toxicity in this dose group that would lead to morbidity" but did not show the data and did not indicate when the animals were affected and removed. For the wild-type mice exposed to the same concentration (1,500 mg/kg) but whose survival was not affected by TCE exposure, the authors reported that these mice exhibited mild granuloma formation with calcification or mild hepatocyte degeneration, but gave no other details or quantitative information as to the extent of the lesions or what parts of the liver lobule were affected. The authors noted that "wild-type mice

administered 1000 and 1500 mg/kg exhibited centrilobular hypertrophy" and that "the mice in the other groups did not exhibit any gross pathological changes after TCE exposure." Thus, the hepatocellular hypertrophy reported in this study for TCE appeared to be correlated with increases in percent liver/body weight in wild-type mice. In regard to the PPARα-null mice, the authors stated that "differences in the liver to body weights in the control PPARα-null mice [between Study 1 and 2 the 3-day and 3-week studies] were noted and may be due to differences in the degree of steatosis that commonly occurs in this strain." Further mention of the background pathology due to knockout of the PPARα was not discussed. The increased percent liver/body weight reported between control and 1,000 mg/kg TCE exposed mice (5.1 vs. 5.6%) was not accompanied by any discussion of pathological changes that could have accounted for the change.

Direct comparisons of the effects of TCE, DCA, and TCA cannot be made from this study as they were not studied for similar durations of exposure. However, while TCE induced increased in percent liver/body weight ratios after 3 days and 3 weeks of exposure in wild-type mice at the highest dose levels, for TCA exposure, percent liver/body weight after 1 week exposure in drinking water was slightly elevated at all dose levels with no dose-response (~10% increase), and for DCA exposure in drinking water, a similar elevation in percent liver/body weight was also reported for the 0.25, 0.5, and 1.0 g/L dose levels (~11%) and that was increased at the 2.0 g/L level by ~25% reaching statistical significance. The authors interpret these data to show no TCA-related changes in wild-type mice but the limited power of the study makes quantitative conclusions difficult.

For PPARα-null mice, there was a slight decrease in percent liver/body weight between control and TCA treated mice at the doses tested (~2%). For DCA-treated mice, all treatment levels of DCA were reported to induce a higher percent liver/body weight ratio of at least ~5% with a 13% increase at the 2.0 g/L level. Again, the limited power of the study and the lack of data for TCE at similar durations of exposure as those studied for TCA and DCA makes quantitative conclusions difficult and comparisons between the chemicals difficult. However, the pattern of increased percent liver/body weight appears to be more similar between TCE and DCA than TCA in both wild-type and PPARα-null mice.

In terms of histological description of effects, the authors note that "livers from the 2 g/L DCA-treated wild-type and PPARα-null mice had hepatocyte cytoplasmic rarefication probably due to an increase in glycogen accumulation." However, no special procedures of staining were performed to validate the assumption in this experiment. No other pathological descriptions of the DCA treatment groups were provided. In regard to TCA, the authors noted that "the livers from wild-type but not PPARα-null mice exposed to 2.0g/L TCA exhibited centrilobular hepatocyte hypertrophy." No quantitative estimate of this effect was given and although the extent of increase of percent liver/body weight was similar for all dose levels of TCA, there is no indication from the study that lower concentrations of TCA also increased hepatocellular

hypertrophy or why there was no concurrent increase in liver weight at the highest dose of TCA in which hepatocellular hypertrophy was reported. Thus, reports of hepatocellular hypertrophy for DCA and TCA in the 1-week study were not correlated with changes in percent liver/body weight.

For control animals, BrdU incorporation in the last week of the 3-week study was reported to be at a higher baseline level in PPARα-null mice than wild-type mice (~2.5-fold). For wild-type mice the authors reported a statistically significant increase at 500 and 1,000 mg/kg TCE at levels of ~1 and ~4.5% hepatocytes incorporating the label after 5 days of BrdU incorporation. Whether this measure of DNA synthesis is representative of cellular proliferation or of polyploidization was not examined by the authors. Even at 1,000 mg/kg TCE, the percent of cells that had incorporated BrdU was <5% of hepatocytes in wild-type mice. The magnitude percent liver/body weight ratio change at this exposure level was fourfold greater than that of hepatocytes undergoing DNA synthesis (16% increase in percent liver/body weight ratio vs. 4% increase in DNA synthesis). The ~1% of hepatocytes undergoing DNA synthesis at the 500 mg/kg TCE level, reported to be statistically significant by the authors, was not correlated with a concurrent increase in percent liver/body weight ratio. Thus, TCE-induced changes in liver weight were not correlated with increases in DNA synthesis in wild-type mice after 3 weeks of TCE exposure.

For PPARα-null mice, there was an approximately threefold of control value for the percent of hepatocytes undergoing DNA synthesis at the 1,000 mg/kg TCE exposure level. The higher baseline level in the null mouse, large variability in response at this exposure level, and low power of this experimental design limited the ability to detect statistical significance of this effect, although the level was greater than that reported for the 500 mg/kg TCE exposure in wild-type mice that was statistically significant. Thus, TCE appeared to induce an increase in DNA synthesis in PPARα-null mice, albeit at a lower level than wild-type mice. However, the ~2% increase in percent of hepatocytes undergoing DNA synthesis during the 3rd week of a 3-week exposure to 1,000 mg/kg TCE in PPARα-null mice was insufficient to account for the ~10% observed increase in liver weight. For wild-type and PPARα-null mice, the magnitude of TCE-induced increases in liver weight were four- to fivefold higher than that of increases in DNA-synthesis under this paradigm and in both types of mice, a relatively small portion of hepatocytes were undergoing DNA synthesis during the last week of a 3-week exposure duration. Whether the increases in liver weight could have resulted from an early burst of DNA synthesis as well as whether the DNA synthesis results reported here represents either proliferation or polyploidization, cannot be determined from this experiment. Because of the differences in exposure protocol (i.e., use of three daily doses in methylcellulose rather than one dose in corn oil), the time course of the transient increase in DNA synthesis reported cannot be assumed to be the same for this experiment and others.

Not only were PPARα-null mice different than wild-type mice in terms of background levels of liver weights, and hepatic steatosis, but this study also reported that background levels of PCO activity to be highly variable and, in some instances, different between wild-type and null mice. There was reported to be approximately sixfold PCO activity in PPARα-null control mice in comparison to wild-type control mice in the 1-week DCA/TCA experiment (~0.15 vs. 0.85 units of activity/g protein). However, in the same figure, a second set of data are reported for control mice for comparison to WY-14,643 treatment in which PCO activity was slightly decreased in PPARα-null control mice vs. wild-type controls (~0.40 vs. 0.65 units of activity/g protein). In the experimental design description of the paper, WY-14,643 treatment and a separate control were not described as part of the 1-week DCA/TCA experiment. For the only experiment in which PCO activity was compared between wild-type and PPARα-null mice exposed to TCE (i.e., 3-day exposure study), there was a reported increased over the control value of ~2.5-fold that was reported to be statistically significant at 1,500 mg/kg TCE (1.5 vs. 0.60 units of activity/g protein). For control mice in the 3-day TCE experiment, there was an increase in this activity in PPARα-null mice in comparison to wild-type mice (~0.60 vs. 0.35 units of activity/g protein). While not statistically significant, there appeared to be a slight increase in PCO activity after 1,500 mg/kg TCE exposure for 3 days in PPARα-null mice of ~30%. However, as noted above, the background levels of this enzyme activity varied widely between the experiments with not only values for control animals varying as much as sixfold (i.e., for PPARα-null mice), but also for WY-14,643 administration. There was a 6.6-fold difference in PCO results for WY-14,643 in PPARα-null mice at the same concentration of WY-14,643 in the 3-day and 1-week experiment, and a 1.44-fold difference in results in wild-type mice in these two data sets.

E.2.1.14. Ramdhan et al. (2008)

Ramdhan et al. (2008) examined the role of CYP2E1 in TCE-induced hepatotoxicity, using CYP2E1 +/+ (wild-type) and CYP2E1 -/- (null) Sv/129 male mice (6/group) that were exposed for 7 days to 0, 1,000, or 2,000 ppm TCE by inhalation for 8 hours/day. The exposure concentrations are noted by the authors to be much higher than occupational exposures and to have increased liver toxicity after 8 hours of exposure as measured by plasma AST levels. To put this exposure concentration into perspective, the Kjellstrand et al. (1983a; 1983b) inhalation studies for 30 days showed that these levels were well above the 150-ppm exposure levels in male mice that induced systemic toxicity. Nunes also reported hepatic necrosis up to 4% in rats at 2,000 ppm for just 8 hours not 7 days. AST and ALT were measured at sacrifice. Histological changes were scored using a qualitative scale of 0 = no necrosis, 1 = minimal as defined as only occasional necrotic cells in any lobule, 2 = mild as defined as less than one-third of the lobule structure affected, 3 = moderate as defined as between one-third and two-thirds of the lobule structure affected, and 4 = severe defined as greater than two-thirds of the lobule structure

affected. Real-time polymerase chain reaction (PCR) was reported for mRNA encoding a number of receptors and proteins. Total RNA and Western Blot analysis was obtained from whole-liver homogenates. The changes in mRNA expression were reported as means for six mice per group after normalization to a level of β-actin mRNA expression and were shown relative to the control level in the CYP2E1 wild-type mice.

The deletion of the CYP2E1 gene in the null mouse had profound effects on liver weight. The body were was significantly increased in control CYP2E1 -/- mice in comparison to wild-type controls (24.48 ± 1.44 g for null mice vs. 23.66 ± 2.44 g, m ± SD). This represents a 3.5% increase over wild-type mice. However, the liver weight was reported in the CYP2E1 -/- mice to be 1.32-fold of that of CYP2E1 +/+ mice (1.45 ± 0.10 g vs. 1.10 ± 0.14 g). The percent liver/body weight ratio was 5.47 vs. 4.63% or 1.18-fold of wild-type control for the null mice.

The authors report that 1,000 and 2,000 ppm TCE treatment did induce a statistically significant change body weight for null or wild-type mice. However, there was an increase in body weight in the wild-type mice (i.e., 23.66 ± 2.44, 24.52 ± 1.17, and 24.99 ± 1.78 for control, 1,000, and 2,000 ppm groups, respectively) and an increase in the variability in response in the null mice (i.e., 24.48 ± 1.44, 24.55 ± 2.26, and 24.99 ± 4.05, for control, 1,000, and 2,000 ppm exposure groups, respectively). The percent liver/body weight was 5.47 ± 0.23, 5.51 ± 0.27, and 5.58 ± 0.70% for control, 1,000, and 2,000 ppm the CYP2E1 -/- mice, respectively. The percent liver/body weight was 4.63 ± 0.13, 6.62 ± 0.40, and 7.24 ± 0.84% for control, 1,000, and 2,000 ppm wild-type mice, respectively. Therefore, while there appeared to be little difference in the TCE and control exposures for percent liver/body weights in the CYP2E1 -/- mice (2%), there was a 1.56-fold of control level after 2,000 ppm in the wild-type mice after 7 days of inhalation exposure.

The authors reported that "in general, the urinary TCE level in CYP2E1 -/- mice was less than half that in CYP2E1 +/+ mice: urinary TCA levels in the former were about one-fourth those in the latter." Of note is the large variability in urinary TCE detected in the 2,000-ppm TCE exposed wild-type mice, especially after day 4, and that, in general, the amount of TCE in the urine appeared to be greatest after the 1st day of exposure and steadily declined between 1 and 7 days (i.e., ~45% decline at 2,000 ppm and a ~70% decline at 1,000 ppm) in the wild-type mice. The amount of TCE in the urine was proportional to the difference in dose at days 1 and 5 (i.e., a twofold difference in dose resulted in a twofold difference in TCE detected in the urine). As the detection of TCE in the urine declined with time, the amount of TCA was reported to steadily increase between days 1 and 7 (e.g., from ~3 mg TCA after the 1st day to ~5.5 mg after 7 days after 2,000 ppm exposure in wild-type mice). However, unlike TCE, there was a much smaller differences in response between the two TCE exposure levels (i.e., a 12–44% or 1.12–1.44-fold difference in TCA levels in the urine at days 1–7 for exposure concentrations that differ by a factor of 2). This could be indicative of saturation in metabolism and TCA clearance into urine at these high concentrations levels. The authors note that their results suggest that the

metabolism of TCE in both null and wild-type mice may have reached saturation at 1,000 ppm TCE.

For ALT and AST activities in CYP2E1 -/- or CYP2E1 +/+ mice, both liver enzymes were significantly elevated only at the 2,000 ppm level in CYP2E1 +/+ mice. Although the increases in excreted TCA in the urine differed by only ~33% between the 1,000 and 2,000 ppm levels, liver enzyme levels in plasma differed by a much greater extent after 7 days exposure between the 1,000 and 2,000 ppm groups of CYP2E1 +/+ mice (i.e., 1.26- and 1.83-fold of control [ALT] and 1.40- and 2.20-fold of control [AST] for 1,000 and 2,000 ppm TCE exposure levels, respectively). The authors reported a correlation between plasma ALT and both TCE (r = 0.7331) and TCA (r = 0.8169) levels but do not report details of what data were included in the correlation (i.e., were data from CYP2E1 +/+ mice combined with those of the CYP2E1 -/- mice and were control values included with treated values?).

The authors show photomicrograph of a section of liver from control CYP2E1 +/+ and CYP2E1 -/- mice and describe the histological structure of the liver to appear normal. This raises the question as to the cause of the hepatomegaly for the CYP2E1 mice in which the liver weight was increased by a third.

The qualitative scoring for each of the six animals per group showed that none of the CYP2E1 -/- control or treated mice showed evidence of necrosis. For the CYP2E1 +/+ mice, there was no necrosis reported in the control mice and in three of six mice treated with 1,000 ppm TCE. Of the three mice that were reported to have necrosis, the score was reported as 1–2 for two mice and 1 for the third. It is not clear what a score of 1–2 represented given the criteria for each score given by the authors, which defined a score of 1 as minimal and 2 as mild. For the 2,000 ppm TCE-exposed mice, all mice were reported to have at least minimal necrosis (i.e., four mice were reported to have scores of 1–2, one mouse a score of 3, and one mouse a score of 1).

What is clear from the histopathology data are that there appeared to be great heterogeneity of response between the six animals in each TCE-exposure group in CYP2E1 +/+ mice and that there was a greater necrotic response in the 2,000 ppm exposed mice than the 1,000 ppm mice. These results are consistent with the liver enzyme data but not consistent with the small difference between the 1,000 and 2,000 ppm exposure groups for TCA content in urine and, by analogy, metabolism of TCE to TCA. A strength of this study is that it reports the histological data for each animal so that the heterogeneity of liver response can be observed (e.g., the extent of liver necrosis was reported to range from only occasional necrotic cells in any lobule to between one-third and two-thirds of the lobular structure affected after 2,000 ppm TCE exposure for 7 days). Immunohistochemical analysis was reported to show that CYP2E1 was expressed mainly around the centrilobular area in CYP2E1 +/+ mice where necrotic changes were observed after TCE treatment.

Given the large variability in response within the liver after TCE exposure in CYP2E1 mice, phenotypic anchoring becomes especially important for the interpretation of mRNA expression studies (see Sections E.1.1 and E.3.1.2 for macroarray transcript profiling limitations and the need for phenotypic anchoring). However, the data for mRNA expression of PPARα, peroxisomal bifunctional protein (hydratase+3-hydroxyacyl-CoA dehydrogenase),very long chain acyl-CoA dehydrogenase (VLCAD), CYP4A10, NFκB (p65, P50, P52), and IκBα was reported at the means ± SD for six mice per group and represented total liver homogenates. A strength of the study was that they did not pool their RNA and can show means and SDs between treatment groups. The low numbers of animals tested, however, limits the ability to detect statistically significance of the response. By reporting the means, differences in the responses within dose groups was limited and reflected differential response and involvement for different portions of the liver lobule and for the responses of the heterogeneous group of liver cells populating the liver.

The authors reported that they normalized values to the level of β-actin mRNA in the same preparation with a value of 1 assigned as the mean from each control group. The values for mRNA and protein expression reported in the figures appeared to have all been normalized to the control values for the CYP2E1 -/- mice. Although all of the CYP2E1 -/- control values were reported as a value of 1, the control values for the CYP2E1+/+ mice differed with the greatest difference being presented for the CYP4A10-mRNA (i.e., the control level of CYP4A10 mRNA was approximately threefold higher in the CYP2E1+/+ mice than the CYP2E1 -/- mice). Further characterization of the CYP2E1 mouse model was not provided by the authors.

The mean expression of PPARα mRNA was reported slightly reduced after TCE treatment in CYP2E1 -/- mice (i.e., 0.72- and 0.78-fold of control after 1,000 and 2,000 ppm TCE exposure, respectively). The CYP2E1 -/- mice had a higher baseline of PPARα mRNA expression than the CYP2E1+/+ mice (i.e., the control level of the CYP2E1 -/- mice was 1.5-fold of the CYP2E1+/+ mice). After TCE exposure, the CYP2E1 +/+ had a similar increase in PPARα mRNA (~2.3-fold) at both 1,000 and 2,000 ppm TCE. Thus, without the presence of CYP2E1, there did not appear to be increased PPARα mRNA expression. For PPARα protein expression, there was a similar pattern with ~1.6-fold of control levels of protein in the CYP2E1 -/- mice after both 1,000 and 2,000 ppm TCE exposures.

In the CYP2E1 +/+ mice, the control level of PPARα protein was reported to be ~1.5-fold of the CYP2E1 -/- control level. Thus, while the mRNA expression was less, the protein level was greater. After TCE treatment, there was a 2.9-fold of control level of protein at 1,000 ppm TCE and a 3.1-fold of control level of protein at 2,000 ppm. Thus, the magnitude of mRNA increase was similar to that of protein expression for PPARα in CYP2E1 +/+ mice. The magnitude of both was threefold or less over control after TCE exposure. This pattern was similar to that of TCA concentration formed in the liver where there was very little difference between the 1,000 and 2,000 ppm exposure groups in CYP2E1 +/+ mice. However, this pattern

was not consistent with the liver enzyme and histopathology of the liver that showed a much greater response after 2,000 ppm exposure than 1,000 ppm TCE. In addition, where the mean enzyme markers of liver injury and individual animals displayed marked heterogeneity in response to TCE exposure, there was a much smaller degree of variability in the mean mRNA expression and protein levels of PPARα.

For peroxisomal bifunctional protein, there was a greater increase after 1,000 ppm TCE-treated exposure than after 2,000 ppm TCE-treatment for both the CYP2E1 -/- and CYP2E1 +/+ mice (i.e., there was a 2:1 ratio of mRNA expression in the 1,000 vs. 2,000 ppm exposed groups). The CYP2E1 +/+ mice had a much greater response than the CYP2E1 -/- mice (i.e., the CYP2E1 -/- mice had a 2-fold of control and the CYP2E1 +/+ mice had a 7.8-fold of control level after 1,000 ppm TCE treatment). For peroxisomal bifunctional protein expression, the magnitude of protein induction after TCE exposure was much greater than the magnitude of increase in mRNA expression. In the CYP2E1 -/- mice, 1,000 ppm TCE exposure resulted in a 6.9-fold of control level of protein, while the 2,000 ppm TCE group had a 2.3-fold level. CYP2E1 +/+ mice had a ~50% higher control level than CYP2E1 mice and after TCE exposure, the level of peroxisomal bifunctional protein expression was 44-fold of control at 1,000 ppm TCE and 40-fold of control at 2,000 ppm. Thus, CYP2E1 -/- mice were reported to have less mRNA expression and peroxisomal bifunctional protein formed than CYP2E1 +/+ mice after TCE exposure. However, there appeared to be more mRNA expression after 1,000 than 2,000 ppm TCE in both groups and protein expression in the CYP2E1 -/- mice. After 2,000 ppm TCE, there was similar peroxisomal bifunctional protein expression between the 1,000 and 2,000 ppm TCE treated CYP2E1 +/+ mice. Again, this pattern was more similar to that of TCA detection in the urine—not that of liver injury.

For VLCAD, the expression of mRNA was similar between control and treated CYP2E1 -/- mice. For CYP2E1 +/+ mice, the control level of VLCAD mRNA expression was half that of the CYP2E1 -/- mice. After 1,000 ppm TCE, the mRNA level was 3.7-fold of control and after 2,000 ppm TCE the mRNA level was 3.1-fold of control. For VLCAD, protein expression was 1.8-fold of control after 1,000 ppm and 1.6-fold of control after 2,000 ppm in CYP2E1 -/- mice. The control level of VLCAD protein in CYP2E1 +/+ mice appeared to be 1.2-fold control CYP2E1 -/- mice. After 1,000-ppm TCE treatment, the CYP2E1 -/- mice were reported to have 3.8-fold of control VLCAD protein levels and after 2,000-ppm TCE treatment, 3.9-fold of control protein levels. Thus, although showing no increase in mRNA, there was an increase in VLCAD protein levels that was similar between the two TCE exposure groups in CYP2E1 -/- mice. Both VLCAD mRNA and protein levels were greater in CYP2E1 +/+ mice than CYP2E1 -/- mice after TCE exposure. This was not the case for peroxisomal bifunctional protein. The magnitudes of TCE-induced increases in mRNA and protein increases were similar between the 1,000 and 2,000 ppm TCE exposure concentrations, a pattern more similar to TCA detection in the urine but not that of liver injury.

Finally, for CYP4A10 mRNA expression, there was an increase in expression after TCE treatment of threefold for 1,000 ppm and fivefold after 2,000 ppm in CYP2E1 -/- mice. Thus, although the enzyme assumed to be primarily responsible for TCE metabolism to TCA was missing, there was still a response for the mRNA of this enzyme commonly associated with PPARα activation. Of note is that urinary concentrations of TCA were not zero after TCE exposure in CYP2E1 -/- mice. Both 1,000 and 2,000 ppm TCE exposure resulted in ~0.44 mg TCA after 1 day or about 15–22% of that observed in CYP2E1 +/+ mice. Thus, some metabolism of TCE to TCA is taking place in the null mice, albeit at a reduced rate. For CYP2E1 +/+ mice, 1,000 ppm TCE resulted in an 8.3-fold of control level of CYP4A10 mRNA and 2,000 ppm TCE resulted in a 9.3-fold of control level.

The authors did not perform an analysis of CYP4A10 protein. The authors state that "in particular, the mRNA levels of microsomal enzyme CYP4A10 significantly increased in CYP2E1+/+ mice after TCE exposure in a dose-dependent manner." However, the twofold difference in TCE exposure concentrations did not result in a similar difference in response as shown above. Both resulted in approximately ninefold of control response in CYP2E1 +/+ mice. As with PPARα, peroxisomal bifunctional protein, and VLCAD, the response was more similar to that of TCA detection in the urine and not measured of hepatic toxicity. These data show that CYP2E1 metabolism of TCE is important in the manifestation of TCE liver toxicity; however, data suggest that effects other than TCA concentration and indicators of PPARα are responsible for acute hepatotoxicity resulting from very high concentrations of TCE.

The NFκB family and IκBα were also examined for mRNA and protein expression. These cell signaling molecules are involved in inflammation and carcinogenesis and are discussed in Sections E.3.3.3.3 and E.3.4.1.4. Given that presence of hepatocellular necrosis in some of the CYP2E1 +/+ mice to varying degrees, inflammatory cytokines and cell signaling pathways would be expected to be activated. The authors reported that:

> overall, TCE exposure did not significantly increase the expression of p65 and p50 mRNAs in either CYP2E1+/+ or CYP2E1 -/- mice… However, p52 mRNA expression significantly increased in the 2,000 ppm group of CYP2E1+/+ mice, and correlation analysis showed that a significant positive relationship existed between the expression of NFκB p52 mRNA and plasma ALT activity.., while no correlation was seen between NFκB p64 or p50 and ALT activity (data not shown).

The authors also note that TCE treatments "did not increase the expression of TNFR1 and TNFR2 mRNA in CYP2E1+/+ and CYP2E1 -/- mice (data not shown)."

A more detailed examination of the data reveals that there was a similar increases in p65, p50, and p52 mRNA expression increases with TCE treatment in CYP2E1 +/+ mice at both TCE exposure levels. However, only p52 levels for the 2,000 ppm exposed mice were reported to be

statistically significant (see comment above about the statistical power of the experimental design and variability between animals). For 1,000 ppm TCE exposure, the levels of p65, p50, and p52 mRNA expression were 1.5-, 1.8-, and 2.0-fold of control. For 2,000 ppm TCE, the levels of p65, p50, and p52 mRNA expression were 1.8-, 1.8-, and 2.1-fold of control. Thus, there was generally a similar response in all of these indicators of NFκB mRNA expression in CYP2E1 +/+ mice that was mild with little to no difference between the 1,000 and 2,000 ppm TCE exposure levels. For IκBα mRNA expression, there was no difference between control and treatment groups for either type of mice. For CYP2E1 -/- mice, there appeared to be a ~50% decrease in P52 mRNA expression in mice treated with both exposure concentrations of TCE. The authors plotted the relationship between p52 mRNA and plasma ALT concentration for both CYP2E1 -/- and CYP2E1 +/+ mice together and claimed that the correlation coefficient (r = 0.5075) was significant. However, of note is that none of the CYP2E1 -/- mice were reported to have either hepatic necrosis or significant increases in ALT detection.

For protein expression, the authors showed results for p50 and p42 proteins. The control CYP2E1 -/- mice appeared to have a slightly lower level of p50 protein expression (~30%) with a much larger increase in p52 protein expression (i.e., 2.1-fold) than CYP2E1 +/+ mice. There appeared to be a 2-fold increase in p50 protein expression after both 1,000 and 2,000 ppm TCE exposures in the CYP2E1 +/+ mice and a similar increase in p52 protein levels (i.e., 1.9- and 2.5-fold of control for 1,000- and 2,000-ppm TCE exposures, respectively). Thus, the magnitude of mRNA and protein levels were similar for p50 and p52 in CYP2E1 +/+ mice and there was no difference between the 1,000 and 2,000 ppm treatments. For the CYP2E1 -/- mice, there was a modest increase in p50 protein after TCE exposure (1.1- and 1.3-fold of control for 1,000 and 2,000 ppm respectively) and a slight decrease in p52 protein (0.76- and 0.79-fold of control). There was little evidence that the patterns of either expression or protein production of NFκB family and IκBα corresponded to the markers of hepatic toxicity or that they exhibited a dose-response. The authors note that although he expression of p50 protein increased in CYP2E1 +/+ mice, "the relationship between p50 protein and ALT levels was not significant (data not shown)." For TNFR1, there appeared to be less protein expression in the CYP2E1 +/+ mice than the CYP2E1 -/- mice (i.e., the null mice levels were 1.8-fold of the wild-type mice levels). Treatment with TCE resulted in mild decrease of protein levels in the CYP2E1 -/- mice and a 1.4- and 1.7-fold of control level in the CYP2E1 +/+ mice for 1,000 and 2,000 ppm levels, respectively. For p65, although TCE treatment-related effects were reported, of note is that the levels of protein were 2.4 higher in the CYP2E1 +/+ mice than the CYP2E1 -/- mice. Thus, protein levels of the NFκB family appeared to have been altered in the knockout mice. Also, as noted in Section E.3.4.1.4, the origin of the NF-κB is crucial as to its effect in the liver and the results of this report are for whole-liver homogenates that contain parenchymal as well as nonparenchymal cell and have been drawn from liver that are heterogeneous in the magnitude of hepatic necrosis. The authors suggest that "TCA may act as a defense against hepatotoxicity

cause by TCE-delivered reactive metabolite(s) via PPARα in CYP2E1+/+ mice." However, the data from this do not support such an assertion.

E.2.1.15. Ramdhan et al. (2010)

Ramdhan et al. (2010) examined the role of mouse and human PPARα in TCE-induced hepatic steatosis and toxicity using male wild type, PPARα-null and PPARα-null mice with human PPARα inserted (hPPARα) (Cheung et al., 2004) on Sv/129 male mice (6/group), which were exposed for 7 days to 0, 1,000, or 2,000 ppm TCE by inhalation for 8 hours/day. This was a similar paradigm as that used in Ramdhan et al. with results between wild type mice directly comparable. The expression of human PPARα cDNA in the humanized mice was limited to hepatocytes under the control of tetracycline regulatory system.

Plasma aminotransferase activities (AST and ALT) were measured in plasma as well as triglycerides. Hepatic triglyceride levels were measured as well. Urinary metabolites were measured similarly to Ramdhan et al. (2008). Hepatic steatosis was identified based on the presence of vacuoles consistent with lipid accumulation and classified as microvesicular steatosis if the nucleus remained in the center of the hepatocyte. Hepatocyte proliferation was classified based on the presence of large hepatocytes with prominent eosinophilic cytoplasm. Histopathology findings were scored in 20 randomly selected 200x microscopic fields per section with steatotic scores of 0–3: none, mild 5–44% of parenchymal involvement of steatosis), moderate (33–66%), or severe (>66%). Necrotic cells were scored as 0–4: no necrosis, minimal (only occasional necrotic cells in any lobule), mild (<one-third of the lobular structure affected), moderate (one-third to two-thirds of lobular structure affected), or severe (>two-thirds of the lobular structure affected). Hepatocyte proliferation was scored as 0 (absent) or 1 (present).

Real-time PCR analysis was performed on total RNA from whole liver. Western Blot analysis was also performed on whole liver (derived from both hepatocytes and non-parenchymal cells) for NFκB, p65, p50, p52, and PPARα.

Significant differences were observed among control mice for each genotype. The mean body weight of hPPARα mice was 14 and 8.5% less than wild type mouse and PPARα-null mice, respectively. The mean liver weight of hPPARα mice was 11% less than PPARα-null mice and the liver/body weight ratio of PPARα-null mice was 11% higher than wild type mice. TCE, at both 1,000 and 2,000 ppm, significantly increased liver weight in the three mouse lines to a similar extent (i.e., 38 and 49% in wild type mice, 20 and 37% in PPAR-null mice, and 28 and 32% in hPPARα mice). The increases were not statistically significant between doses within each strain. Liver/body weight ratios were also significantly increased with TCE exposure at 1,000 and 2,000 ppm relative to controls (i.e., 38 and 43% in wild type mice, 24 and 36% in PPARα-null mice, and 27 and 39% in hPPARα mice, respectively). The difference between 2.000 and 1.000 ppm TCE exposure was statistically significant in PPARα-null mice.

The authors reported no differences in urinary volume by genotype or exposure but did not show the data. TCA and TCOH were detected in all exposed mice with no significant differences between the 1,000 and 2,000 ppm TCE levels. TCA concentrations were reported to be significantly lower and TCOH levels significantly higher in PPARα-null mice relative to wild type mice with no differences in genotype between the sum of total TCA and TCOH concentrations between genotypes.

AST and ALT liver injury biomarkers were reported to vary <10% among control mice of each strain and to be significantly increased in all exposed mice relative to controls (41–74% and 36–79% higher, respectively) with mean levels within each group higher, though not statistically significantly different, with exposure to 2,000 vs. 1,000 ppm TCE.

Higher levels of plasma triglycerides were reported in untreated hPPARα mice than wild-type mice (52%). Significantly higher liver triglyceride levels were reported in untreated hPPARα mice than wild type mice or PPARα-null mice (77 and 30%, respectively) and between untreated PPARα-null mice and wild-type mice (36%). Exposure to 2,000 ppm TCE was reported to induce an even greater difference between the wild type and PPARα-null mice (113%). Exposure to 1,000 ppm TCE was reported to induce greater liver triglyceride level in hPPARα mice (50%) compared to wild type mice as well as 2,000 ppm TCE (87%). There were no significant difference in mean plasma or liver triglyceride levels between the 2,000 and 1,000 ppm TCE treatment groups within each genotype. Hepatic triglyceride levels were reported to be significantly correlated with liver/body weight ratios of all mice used in the study ($r = 0.54$).

Neither necrosis nor inflammatory cells were reported in liver sections from unexposed mice. The authors reported small cytoplasmic vacuoles in sections from unexposed PPARα-null mice and hPPARα mice that resulted in steatosis scores >0. Steatosis was reported to be absent in unexposed wild type mice and significantly increased in exposed vs. unexposed PPARα-null and hPPARα mice. Steatosis scores were reported to be significantly higher in the 2,000 vs. 1,000 ppm TCE exposures to PPARα-null mice. The authors reported steatosis scored to be significantly correlated with liver triglyceride levels of all mice examined in the study ($r = 0.75$). Macrovesicular steatosis was reported to occur more frequently in hPPARα than PPARα-null mice. Necrosis scores were reported to be significantly higher in TCE exposed mice relative to controls in all three genotype mice and to be significantly higher with 2,000 vs. 1,000 ppm TCE exposure in wild type mice and hPPARα mice. Inflammation scores were reported to be significantly higher with exposed group than control with 2,000 ppm TCE exposure than controls for each genotype group with a difference between the 2,000 and 1,000 ppm exposure groups in wild type mice. Hepatocyte proliferation was reported to be significantly increased with 2,000 ppm TCE exposure in wild-type mice, but not in the other genotypes or exposure concentrations. Of note, the criteria for "proliferation" did not employ quantitative methods of

DNA synthesis but phenotypic descriptions of enlarged hepatocytes that may be indicative of polyploidy.

Background expression levels of several genes were reported to differ significantly between strains in control mice. VLCAD, medium chain acyl-CoA dehydrogenase (MCAD), peroxisomal bifunctional protein (hydratase+3-hydroxyacyl-CoA dehydrogenase) (PH), peroxisomal thiolase (PT), diacylflicerol acyltransferase 1 (DGAT1), and p52 mRNA levels were reported to be higher in untreated hPPARα mice than wild type mice and PPARα-null mice. PPARα, proliferation cell nuclear antigen (PCNA), p50, and tumor necrosis factor alpha (TNFα) mRNA levels were reported to be higher in untreated hPPARα mice than PPARα-null mice. VLCAD, PH, and PT mRNA levels were reported to be significantly lower in untreated PPARα-null mice than wild type mice and p50, p52, PPARγ, and TNFα were higher in untreated PPARα-null mice than wild type mice.

Exposure to TCE was reported to not increase the expression of human PPARα mRNA in hPPARα mice but 2,000 ppm TCE exposure did significantly increase mouse PPARα mRNA in wild type mice. PCNA mRNA expression and mRNA expression of VLCAD, MCAD, PH, and PT was increased in TCE exposed vs. control wild type mice and hPPARα mice. More pronounced induction of PH and PT mRNA was reported for exposed wild type mice. Significant differences were not reported in gene expression between 1,000 and 2,000 ppm TCE exposures.

DGAT1 and DGAT2 mRNA was reported to be significantly increased in hPPARα mice exposed to 2,000 ppm TCE and PPARα-null mice exposed to 1,000 and 2,000 ppm TCE in comparison to respective control mice. Exposure to 1,000 and 2,000 ppm TCE was reported to significantly increase PPARγ mRNA in PPARα-null and hPPARα mice. DGAT1 and DGAT2, PPARγ mRNA levels were not changed with TCE exposure in wild type mice.

NFκB p65 mRNA was reported to be significantly increase after TCE exposure in PPARα-null and hPPARα mice but not wild type mice. NFκB p50 mRNA expression was reported to be significantly increased with exposure to TCE in PPARα-null mice only but NFκB p52 and TNFα mRNA expression was increased significantly with exposure in all strains. The authors reported that NFκB p52 mRNA levels were significantly correlated with plasma ALT levels in all mice used in the study ($r = 0.54$).

Protein expression levels were reported to differ between the genotypes of untreated mice. PPARα levels were 10.4 times higher in untreated hPPARα mice than wild type mice. VLCAD, PT, acyl-CoA(ACOX) A, and ACOX B proteins were reported to be significantly higher in untreated hPPARα mice than wild type and PPARα-null mice and NFκB p65 to be lower in hPPARα mice than PPARα-null mice. VLCAD, MCAD, PH, PT, ACOX A, and ACOX B expression was reported to be slightly lower and p65 and p52 expression slightly higher in untreated PPARα-null mice vs. wild type mice.

TCE exposure was reported to increase VLCAD, PH, PT, ACOX A, and ACOX B in wild type and hPPARα mice but not to induce PPARα protein expression. MCAD protein was significantly increased after TCE exposure in hPPARα mice only. PCNA protein was increased in TCE exposed mice in comparison to controls in all strains. NFκB p52 and TNFα proteins were also increased from TCE exposure in all strains but NFκB p50 and p65 proteins were increased in TCE-exposed PPARα-null mice only. 4-Hydroxy-2- nonenal protein (a marker of oxidative stress) was increased by 1,000 ppm TCE exposure in PPARα-null mice and by 2,000 ppm TCE exposure in wild type and hPPARα mice.

The authors reported that they measured hepatic protein expression of CYP2E1 and ALDH2 enzymes and did not observe a significant difference among controls (data not shown) and that TCE exposure did not alter hepatic CYP2E1 expression but did decrease ALDH2 expression to a comparable extent in all mouse lines (data not shown). Thus, changes in urinary TCA levels in the differing strains were not related to changes in expression of these metabolic enzymes.

While the authors of the paper suggested that the increased susceptibility of PPARα-null mice and hPPATα mice to TCE toxicity is indicative of "protection" by having intact and normal PPARα expression in mice, the disturbances they reported in these genotypes without treatment shows that an already compromised animal is more susceptible to additional insult by high levels of TCE exposure. This study provides an extensive set of parameters altered in the PPARα-null and hPPARα mice by such genetic manipulation alone. In particular, insertion of human PPAR in the null mice did not return the mice to a normal state. The authors noted that hepatic triglyceride levels were the highest in untreated hPPARα among the three strains suggesting that human PPARα insertion did not restore proper lipid regulation in the liver. The humanized mice in particular exhibited a ≥10-fold expression of PPAR in an untreated state. Functional differences between the human and rodent versions of PPAR are difficult to ascertain from this study given the large differences in PPAR protein expression between wild type and humanized mice and the presence of human PPAR only in the hepatocytes in this model. The authors noted that the replacement of human PPARα in the humanized mouse may not have been sufficient to prevent steatosis and that the differences in responses between wild type and humanized mice may reflect functional consequences related to the use of an artificial construct of the reinserted gene without normal control elements in addition to or instead of any differences between human or mouse PPARα. They stated that because they used genetically modified mice with underlying dysregulation, and evaluated very high TCE exposures, their findings may not directly reveal the differences in human PPARα function between mice and humans. The increased toxicity from overexpression of human PPARα in this model is also acknowledged as leading to greater background toxicity in unexposed humanized mice.

Responses reported for gene expression are for liver homogenates so that NFκB and TNFα mRNA expression changes could not be distinguished between Kupffer cell or

hepatocytes origin. The authors noted the similarity of TCE induced hepatomegaly in PPARα null mice in this study and that of Nakajima et al. (2000). They noted that TCE induction of PCNA protein (cell proliferation marker) was increased in all three group but using their phenotypic marker of increased cell size of evidence of increased hepatocyte proliferation in wild type mice.

The authors noted differences in this study and their study of similar design (Ramdhan et al., 2008) for gene expression induced by TCE exposure in wild type mice. Differences in TCE-induced effects between the two studies include less pronounced induction of PPARα, more pronounced increases in PH protein and VLCAD mRNA expression, and ALT and AST levels for this study than the previous one for wild type mice. They stated that urinary TCA levels in wild type mice were incorrectly reported by Ramdhan et al. (2008) but have been corrected in this study. They also noted discrepancies in mRNA and protein expression for some genes in this study. Finally, the authors acknowledged that the small number of mice examined in each group limits the power to identify statistically significant biological effects.

E.2.2. Subchronic and Chronic Studies of TCE

For the purposes of this discussion, studies of duration of ≥4 weeks are considered subchronic. Like those of shorter duration, there is variation in the depth of study of liver changes induced by TCE with many of the longer duration studies focused on the induction of liver cancer. Many subchronic studies were conducted a high doses of TCE that caused toxicity with limited reporting of effects. Similar to acute studies, some of the subchronic and chronic studies have detailed examinations of the TCE-induced liver effects while others have reported primarily liver weight changes as a marker of TCE-response. Similar issues also arise with the impact of differences in initial and final body weights between control and treatment groups on the interpretation of liver weight gain as a measure of TCE-response.

For many of the subchronic inhalation studies, issues associated with whole-body exposures make determination of dose levels difficult. For gavage experiments, death from gavage dosing, especially at higher TCE exposures, is a recurring problem and, unlike inhalation exposures, the effects of vehicle can also be at issue for background liver effects. Concerns regarding effects of oil vehicles, especially corn oil, have been raised with Kim et al. (1990a) noting that a large oil bolus will not only produce physiological effects, but alter the absorption, target organ dose, and toxicity of VOCs. Charbonneau et al. (1991) reported that corn oil potentiates liver toxicity from acetone administration that is not related to differences in acetone concentration. Several oral studies, in particular, document that the use of corn oil gavage induces a different pattern of toxicity, especially in male rodents (see Merrick et al., 1989, Section E.2.2.1 below). Several studies listed below report the effects of hepatocellular DNA synthesis and indices of lipid peroxidation (i.e., Channel et al., 1998) are especially subject to background vehicle effects. Rusyn et al. (1999) report that a single dose of dietary corn oil

increases hepatocyte DNA synthesis 24 hours after treatment by ~3.5-fold, activation of NF-κB to a similar extent ~2 hours after treatment almost exclusively in Kupffer cells, a ~3–4-fold increase in hepatocytes after 8 hours, and increased in TNFα mRNA between 8 and 24 hours after a single dose in female rats. In regard to studies that have used the i.p. route of administration, as noted by Kawamoto et al. (1988b) (see Section E.2.2.11), injection of TCE may result in paralytic ileus and peritonitis and that subcutaneous treatment paradigm will result in TCE not immediately being metabolized but retained in the fatty tissue. Wang and Stacey (1990) state that "intraperitoneal injection is not particularly relevant to humans" and that intestinal interactions require consideration in responses such as increase serum bile acid (see Section E.2.6).

E.2.2.1. Merrick et al. (1989)

The focus of this study was the examination of potential differences in toxicity or orally gavaged TCE administered in corn oil an aqueous vehicle in B6C3F$_1$ mice. As reported by Melnick et al. (1987) above, corn oil administration appeared to have an effect on peroxisomal enzyme induction. TCE (99.5% purity) was administered in corn oil or an aqueous solution of 20% Emulphor to 14–17-week-old mice (n = 12/group) at 0, 600, 1,200, and 2,400 mg/kg-day (males) and 0, 450, 900, and 1,800 mg/kg-day (females) 5 times/week for 4 weeks. The authors stated that due to "varying lethality in the study, 10 animals per dose group were randomly selected (where possible) among survivors for histological analysis." Hepatocellular lesions were characterized:

> as a collection of approximately 3–5 necrotic hepatocytes surrounded by macrophages and polymorphonuclear cells and histopathological grading was reported as based on the number of necrotic lesions observed in the tissue sections: 0 = normal; 1 = isolated lesions scattered throughout the section; 2 = one to five scattered clusters of necrotic lesions; 3 = more than five scattered clusters of necrotic lesions; and 4 = clusters of necrotic lesions observed throughout the entire section."

The authors described lipid scoring of each histological section as "0 = no Oil-Red O staining present; 1 = <10% staining; 2 = 10–25% staining; 3 = 25–30% staining; and 4 = ≥50% staining.

The authors reported dose-related increases in lethality in both males and females exposed to TCE in Emulphor with all male animals dying at 2,400 mg/kg-day with 8/12 females dying at 1,800 mg/kg-day. In both males and females, 2/12 animals also died at the next highest dose as well with no unscheduled deaths in control or lowest dose animals. For corn oil gavaged mice, there were 1–2 animals in each TCE treatment groups of male mice that died while there were no unscheduled deaths in female mice.

The authors stated that lethality occurred within the first week after chemical exposure. The authors presented data for final body weight and liver/body weight values for 4 weeks of exposure and listed the number of animals per group to be 10–12 for corn oil gavaged animals. The reduced number of animals in the Emulphor gavaged animals are reflective of lethality and limit the usefulness of this measure at the highest doses (i.e., 1,800 mg/kg-day for female mice). In mice treated with TCE in Emulphor gavage, the final body weight of control male animals appeared to be lower than those that were treated with TCE while for female mice the final body weights were similar between treated and control groups. For male mice treated with Emulphor, body weights were 22.8 ± 0.8, 25.3 ± 0.5, and 24.3 ± 0.4 g for control, 600, and 1,200 mg/kg-day and for female mice body weights were 20.7 ± 0.4, 21.4 ± 0.3, and 20.5 ± 0.3 g for control, 450, and 900 mg/kg-day of TCE.

For percent liver/body weight ratios, male mice were reported to have 5.6 ± 0.2, 6.6 ± 0.1, and 7.2 ± 0.2% for control, 600, and 1,200 mg/kg-day and for female mice were 5.1 ± 0.1, 5.8 ± 0.1, and 6.5 ± 0.2% for control, 450 and 900 mg/kg-day of TCE. These values represent 1.11- and 1.07-fold of control for final body weight in males exposed to 600 and 1,200 mg/kg-day and 1.18- and 1.29-fold of control for percent liver/body weight, respectively. For females, they represent 1.04- and 0.99-fold of control for final body weights in female exposed to 450mg/kg-day and 900 mg/kg-day and 1.14- and 1.27-fold of control for percent liver/body weight, respectively.

In mice treated with corn oil gavage, the final body weight of control male mice was similar to the TCE treatment groups and higher than the control value for male mice given Emulphor vehicle (i.e., 22.8 ± 0.8 g for Emulphor control vs. 24.3 ± 0.6 g for corn oil gavage controls or a difference of ~7%). The final body weights of female mice were reported to be similar between the vehicles and TCE treatment groups. The baseline percent liver/body weight was also lower for the corn oil gavage control male mice (i.e., 5.6% for Emulphor vs. 4.7% for corn oil gavage or a difference of ~19% that was statistically significant). Although the final body weights were similar in the female control groups, the percent liver/body weight was greater in the Emulphor vehicle group (5.1 ± 0.1% in Emulphor vehicle group vs. 4.7 ± 0.1% for corn oil gavage or a difference of ~9%, which was statistically significant). For male mice treated with corn oil, final body weights were 24.3 ± 0.6, 24.3 ± 0.4, 25.2 ± 0.6, and 25.4 ± 0.5 g for control, 600, 1,200, and 2,400 mg/kg-day, and for female mice, body weights were 20.2 ± 0.3, 20.8 ± 0.5, 21.8 ± 0.3, and 22.6 ± 0.3 g for control, 450, 900, and 1,800 mg/kg-day of TCE.

For percent liver/body weight ratios, male mice were reported to have 4.7 ± 0.1, 6.4 ± 0.1, 7.7 ± 0.1, and 8.5 ± 0.2% for control, 600, 1,200, and 2,400 mg/kg-day and for female mice were reported to have 4.7 ± 0.1, 5.5 ± 0.1, 6.0 ± 0.2, and 7.2 ± 0.1% for control, 450, 900, and 1,800 mg/kg-day of TCE. These values represent 1.0-, 1.04-, and 1.04-fold of control for final body weight in males exposed to 600, 1,200, and 2,400 mg/kg-day TCE and 1.36-, 1.64-, and

1.81-fold of control for percent liver/body weight, respectively. For females, they represent 1.03-, 1.08-, and 1.12-fold of control for body weight for 450, 900, and 1,800 mg/kg-day and 1.17-, 1.28-, and 1.53-fold of control for percent liver/body weight, respectively.

Because of premature mortality, the difference in TCE treatment between the highest doses that are vehicle-related cannot be determined. The decreased final body weight and increased percent liver/body weight ratios in the Emulphor control animals make comparisons of the exact magnitude of change in these parameters due to TCE exposure difficult to determine as well as differences between the vehicles. The authors did not present data for age-matched controls, which did not receive vehicle so that the effects of the vehicles cannot be determined (i.e., which vehicle control values were most similar to untreated controls given that there was a difference between the vehicle controls).

A comparison of the percent liver/body weight ratios at comparable doses between the two vehicles shows little difference in TCE-induced liver weight increases in female mice. However, the corn oil vehicle group was reported to have a greater increase in comparison to controls for male mice treated with TCE at the two lower dosage groups. Given that the control values were approximately 19% higher for the Emulphor group, the apparent differences in TCE-dose response may have reflected the differences in the control values rather than TCE exposure. Because controls without vehicle were not examined, it cannot be determined whether the difference in control values was due to vehicle administration or whether a smaller or younger group of animals was studied on one of the control groups. The body weight of the animals was also not reported by the authors at the beginning of the study, so that the impact of initial differences between groups vs. treatment cannot be accurately determined.

Serum enzyme activities for ALT, AST, and LDH (markers of liver toxicity) showed that there was no difference between vehicle groups at comparable TCE exposure levels for male or female mice. Enzyme levels appeared to be elevated in male mice at the higher doses (i.e., 1,200 and 2,400 mg/kg-day for ALT and 2,400 mg/kg-day for AST), with corn oil gavage inducing similar increases in LDH levels at 600, 1,200, and 2,400 mg/kg-day TCE. For ALT and AST, there appeared to be a dose-related increase in male mice with the 2,400 mg/kg-day treatment group having much greater levels than the 1,200 mg/kg-day group. In Emulphor treatment groups there was a similar increase in ALT levels in males treated with 1,200 mg/kg TCE as with those treated with corn oil and those increases were significantly elevated over control levels. For LDH levels, there were similar increase at 1,200 mg/kg-day TCE for male mice treated using either Emulphor or corn oil.

The authors report that visible necrosis was observed in 30–40% of male mice administered TCE in corn oil, but not that there did not appear to be a dose-response (i.e., the score for severity of necrosis was reported to be 0, 4, 3, and 4 for corn oil control, 600, 1,200, and 2,400 mg/kg-day treatment groups from 10 male mice in each group). No information in regard to variation between animals was given by the authors. For male mice given Emulphor

gavage the extent of necrosis was reported to be 0, 0, and 1 for 0, 600, and 1,200 mg/kg-day TCE exposure, respectively. For female mice, the extent of necrosis was reported to be 0 for all control and TCE treatment groups using either vehicle.

Thus, except for LDH levels in male mice exposed to TCE in corn oil, there was not a correlation with the extent of necrosis and the increases in ALT and AST enzyme levels. Similarly, there was an increase in ALT levels in male mice treated with 1,200 mg/kg-day exposure to TCE in Emulphor that did not correspond to increased necrosis.

For Oil-Red O staining, there was a score of 2 in the Emulphor-treated control male and female mice, while 600 mg/kg-day TCE exposure in Emulphor gavaged male mice and 900 mg/kg-day TCE in corn oil gavaged female mice had a score of 0, along with the corn oil gavage controls in male mice. For female control mice treated with corn oil gavage, the staining was reported to have a score of 3. Thus, there did not appear to be a dose-response in Oil-Red oil staining, although the authors claimed that there appeared to be a dose-related increase with TCE exposure.

The authors described lesions produced by TCE exposure as:

> focal and were surrounded by normal parenchymal tissue. Necrotic areas were not localized in any particular regions of the lobule. Lesions consisted of central necrotic cells encompassed by hepatocytes with dark eosinophilic staining cytoplasm, which progressed to normal-appearing cells. Areas of necrosis were accompanied by localized inflammation consisting of macrophages and polymorphonuclear cells.

No specific descriptions of histopathology of mice given Emulphor were provided in terms of effects of the vehicle or TCE treatment. The scores for necrosis were reported to be only a 1 for the 1,200 mg/kg-day concentration of TCE in male mice gavaged with Emulphor, but 3 for male mice given the same concentration of TCE in corn oil. However, enzyme levels of ALT, AST, and LDH were similarly elevated in both treatment groups.

These results do indicate that administration of TCE for 4 weeks via gavage using Emulphor resulted in mortality of all of the male mice and most of the female mice at a dose in corn oil that resulted in few deaths. Not only was there a difference in mortality, but vehicle also affected the extent of necrosis and enzyme release in the liver (i.e., Emulphor vehicle caused mortality as the highest dose of TCE in male and female mice that was not apparent from corn oil gavage, but Emulphor and TCE exposure induced little, if any, focal necrosis in males at concentrations of TCE in corn oil gavage that caused significant focal necrosis). In regard to liver weight and body weight changes, TCE exposure in both vehicles at nonlethal doses induced increased percent liver/body weight changes male and female mice that increased with TCE exposure level. The difference in baseline control levels between the two vehicle groups

(especially in males) make a determination of the quantitative difference that the vehicle had on liver weight gain problematic, although the extent of liver weight increase appeared to be similar between male and female mice given TCE via Emulphor and female mice given TCE via corn oil. In general, enzymatic markers of liver toxicity and results for focal hepatocellular necrosis were not consistent and did not reflect dose-responses in liver weight increases. The extent of necrosis did not correlate with liver weight increases and was not elevated by TCE treatment in female mice treated with TCE in either vehicle, or in male mice treated with Emulphor. There was a reported difference in the extent of necrosis in male mice given TCE via corn oil and female mice given TCE via corn oil, but the necrosis did not appear to have a dose-response in male mice. Female mice given corn oil and male and female mice given TCE in Emulphor had no to negligible necrosis, although they had increased liver weight from TCE exposure.

E.2.2.2. Goel et al. (1992)

The focus of this study was the description of TCE exposure-related changes in mice after 28 days of exposure with regard to TCE-induced pathological and liver weight change. Male Swiss mice (20–22 g body weight or 9% difference) were exposed to 0, 500, 1,000, or 2,000 mg/kg-day TCE (BDH analytical grade) by gavage in groundnut oil (n = 6 per group) 5 days/week for 28 days. The ages of the mice were not given by the authors. Livers were examined for "free -SH contents," total proteins, catalase activity, acid phosphatase activity, and "protein specific for peroxisomal origin of approx, 80 kd."

The authors report no statistically significant change in body weight with TCE treatment but a significant increase in liver weight. Body weight (mean ± SE) was reported to be 32.67 ± 1.54, 31.67 ± 0.61, 33.00 ± 1.48, and 27.80 ± 1.65 g from exposure to oil control, 500, 1,000, and 2,000 mg/kg-day TCE, respectively. There was a 15% decrease in body weight at the highest exposure concentration of TCE that was not statistically significant, but the low number of animals examined limits the power to detect a significant change. The percent relative liver/body weight was reported to be 5.29 ± 0.48, 7.00 ± 0.36, 7.40 ± 0.39, and 7.30 ± 0.48% from exposure to oil control, 500, 1,000, and 2,000 mg/kg-day TCE, respectively. This represents 1.32-, 1.41-, and 1.38-fold of control in percent liver/body weight for 500, 1,000, and 2,000 mg/kg-day TCE, respectively.

The "free –SH content" in μmol –SH/g tissue was reported to be 5.47 ± 0.17, 7.46 ± 0.21, 7.84 ± 0.34, and 7.10 ± 0.34 from exposure to oil control, 500, 1,000, and 2,000 mg/kg-day TCE, respectively. This represents 1.37-, 1.44-, and 1.30-fold of control in –SH/g tissue weight for 500, 1,000, and 2,000 mg/kg-day TCE, respectively. Total protein content in the liver in mg/g tissue was reported to be 170 ± 3, 183 ± 5, 192 ± 7, and 188 ± 3 from exposure to oil control, 500, 1,000, and 2,000 mg/kg-day TCE, respectively. This represents 1.08-, 1.13-, and 1.11-fold of control in total protein content for 500, 1,000, and 2,000 mg/kg-day TCE, respectively. Thus, the increases in liver weight, "free -SH content," and protein content were generally parallel and

all suggest that liver weight increases had reached a plateau at the 1,000 mg/kg-day exposure concentration, perhaps reflecting toxicity at the highest dose as demonstrated by decreased body weight in this study.

The enzyme activities of δ-ALA dehydrogenase ("a key enzyme in heme biosynthesis"), catalase, and acid phosphatase were assayed in liver homogenates. Treatment with TCE decreased δ-ALA dehydrogenase activity to a similar extent at all exposure levels (32–35% reduction). For catalase the activity as units of catalase/mg, protein was reported to be 25.01 ± 1.81, 32.46 ± 2.59, 41.11 ± 5.37, and 33.96 ± 3.00 from exposure to oil control, 500, 1,000, and 2,000 mg/kg-day TCE, respectively. This represents 1.30-, 1.64-, and 1.36-fold in catalase activity for 500, 1,000, and 2,000 mg/kg-day TCE, respectively. The increasing variability in response with TCE exposure concentration is readily apparent from these data as is the decrease at the highest dose, perhaps reflective of toxicity. For acid phosphatase activity in the liver, there was a slight increase (5–11%) with TCE exposure that did not appear to be dose-related.

The authors report that histologically, "the liver exhibits swelling, vacuolization, widespread degeneration/necrosis of hepatocytes as well as marked proliferation of endothelial cells of hepatic sinusoids at 1,000 and 2,000 mg/kg TCE doses." Only one figure is given at the light microscopic level in which it is impossible to distinguish endothelial cells from Kupffer cells and no quantitative measures or proliferation were examined or reported to support the conclusion that endothelial cells are proliferating in response to TCE treatment. Similarly, no quantitation regarding the extent or location of hepatocellular necrosis is given. The presence or absence of inflammatory cells was not noted by the authors. In terms of white blood cell count, the authors noted that it was slightly increased at 500 mg/kg-day but decreased at 1,000 and 2,000 mg/kg-day TCE, perhaps indicating macrophage recruitment from blood to liver and kidney, which was also noted to have pathology at these concentrations of TCE.

E.2.2.3. Kjellstrand et al. (1981b)

This study was conducted in mice, rats, and gerbils and focused on the effects of 150 ppm TCE exposure via inhalation on body and organ weight. No other endpoints other than organ weights were examined in this study and the design of the study is such that quantitative determinations of the magnitude of TCE response are very limited. NMRI mice (weighing ~30 g with age not given), Sprague-Dawley rats (weighing ~200 g with age not given), and Mongolian gerbils (weighing ~60 g with age not given) were exposed to 150-ppm TCE continuously. Mice were exposed for 2, 5, 9, 16, and 30 days with the number of exposed animals and controls in the 2, 5, 9, and 16 days groups being 10. For 30-day treatments, there were two groups of mice containing 20 mice per group and one group containing 12 mice per group. In addition, there was a group of mice (n = 15) exposed to TCE for 30 days and then examined 5 days after cessation of exposure and another group (n = 20) exposed to TCE for 30 days and then examined

30 days after cessation of exposure. For rats, there were three groups exposed to TCE for 30 days, which contained 24, 12, and 10 animals per group. For gerbils, there were three groups exposed to TCE for 30 days, which contained 24, 8, and 8 animals per group. The groups were reported to consist of equal numbers of males and females but for the mice exposed to TCE for 30 days and then examined 5 days later, the number was 10 males and 5 females. Body weights were reported to be recorded before and after the exposure period. However, the authors state "for technical reasons the animals within a group were not individually identified, i.e., we did not know which initial weight in the group corresponded to which final one." They authors stated that this design presented problems in assessing the precision of the estimate. They go on to state that rats and gerbils were partially identifiable as the animals were housed three to a cage and cage averages could be estimated. Not only were mice in one group housed together, but:

> even worse: at the start of the experiment, the mice in M2 [group exposed for 2 days] and M9 [group exposed for 9 days] were housed together, and similarly M5 [group exposed for 5 days] and M16 [group exposed for 16 days]. Thus, we had, e.g., 10 initial weights for exposed female mice in M2 and M9 where we could not identify those 5 that were M2 weights. Owing to this bad design (forced upon us by the lack of exposure units), we could not study weight gains for mice and so we had to make do with an analysis of final weights.

The problems with the design of this study are obvious from the description given by the authors themselves. The authors stated that they assumed that the larger the animal, the larger the weight of its organs so that all organ weights were converted into relative weights as percentage of body weight. The fallacy of this assumption is obvious, especially if there was toxicity that decreased body weight and body fat but at the same time caused increased liver weight, as has been observed in many studies at higher doses of TCE. In fact, Kjellstrand et al. (1983b) reported that a 150 ppm TCE exposure for 30 days does significantly decreases body weight while elevating liver weight in a group of 10 male NMRI mice. Thus, the body weight estimates from this study are inappropriate for comparison to those in studies where body weights were actually measured. The liver/body weight ratios that would be derived from such estimates of body weights would be meaningless.

The group averages for body weight reported for female mice at the beginning of the 30-day exposure varied significantly and ranged from 23.2 to 30.2 g (~24%). For males, the group averages ranged from 27.3 to 31.4 g (~14%). For male mice, there was no weight estimate for the animals that were exposed for 30 days and then examined 30 days after cessation of exposure.

The authors only report relative organ weight at the end of the experiment rather than the liver weights for individual animals. Thus, these values represent extrapolations based on what body weight may have been. For mice that were exposed to TCE for 30 days and examined after

30 days of exposure, male mice were reported to have "relative organ weight" for liver of 4.70 ± 0.10 vs. 4.27 ± 0.13% for controls. However, there were no initial body weights reported for these male mice, and the body weights are extrapolated values. Female mice exposed for 30 days and examined 30 days after cessation of exposure were reported to have "relative organ weights" for liver of 4.42 ± 0.11 vs. 3.62 ± 0.09% for controls. The group average of initial body weights for this group was reported by the authors.

Although the initial body weight for female control mice as a group average was reported to be similar between the female group exposed to 30 days of TCE and sacrificed 30 days later and those exposed for 30 days and sacrificed 5 days later (30.0 vs. 30.8 g), the liver/body weight ratio varied significantly in these controls (4.25 ± 0.19 vs. 3.62 ± 0.09) as did the number of animals studied (5 female mice in the animals sacrificed after 5 days exposure vs. 10 female mice in the group sacrificed after 30 days exposure). In addition, although there were differences between the three groups of mice exposed to TCE for 30 days and then sacrificed immediately, the authors present the data for extrapolated liver/body weight as pooled results between the three groups. In comparison to control values, the authors report 1.14-, 1.35-, 1.58-, 1.47-, and 1.75-fold of control for percent liver/body weight using body weight extrapolated values in male mice at 2, 5, 9, 16, and 30 days of TCE exposure, respectively. For females, they report 1.27-, 1.28-, 1.49-, 1.41-, and 1.74-fold of control at 2, 5, 9, 16, and 30 days of TCE, respectively.

Although the authors combine female and male relative increases in liver weight in a figure, assign error bars around these data point, and attempt to draw assign a time-response curve to it, it is clear that these data, especially for female mice, do not display time-dependent increase in liver/body weight from 5 to 16 days of exposure and that a comparison of results between 5 and 26 animals is very limited in interpretation. Of note is the wide variation in the control values for relative liver/body weight.

For male mice, there did not seem to be a consistent pattern with increasing duration of the experiment, with values of 4.61, 5.15, 5.05, 4.93, and 4.04% for 2-, 5-, 9-, 16-, and 30-day exposure groups. This represented a difference of ~27%. For female mice, the relative liver/body weight was 4.14, 4.58, 4.61, 4.70, and 3.99% for 2-, 5-, 9-, 16-, and 30-day exposure groups. Thus, it appears that the average relative liver/body weight percent was higher in the 5-, 9-, and 16-day treatment group for both genders than that in the 30-day group and was consistent between these days. There is no apparent reason for there to be such large difference between the 16- and 30-day treatment groups due to increasing age of the animals. Of note is that for the control groups paired with animals treated for 30 days and then examined 30 days later, the male mice had increased relative liver/body weights (4.27 vs. 4.04%), but that the females had decreases (3.62 vs. 3.99%). Such variation between controls does not appear to be age or size related, but rather due to variations in measure or extrapolations, which can affect comparisons between treated and untreated groups and add more uncertainty to the estimates. In addition, the

number of mice in the groups exposed to 2–16 days were only 5 animals for each gender in each group, while the number of animals reported in the 30-day exposure group numbered 26 for each gender.

For animals exposed to 30 days and then examined after 5 or 30 days, male mice were reported to have percent liver/body weight 1.26- and 1.10-fold of control after 5 and 30 days cessation of exposure, while female mice were reported to have values of 1.14- and 1.22-fold of control after 5 and 30 days cessation of exposure, respectively. Again, the male mice exposed for 30 days and then examined after 30 days of cessation of exposure did not have reported initial body weights, giving this value a great deal of uncertainty. Thus, while liver weights appeared to increase during 30 days of exposure to TCE and decrease after cessation of exposure in both genders of mice, the magnitudes of the increases and decreases cannot be determined from this experimental design. Of note is that liver weights appeared to still be elevated after 30 days of cessation exposure.

In regard to initial weights, the authors reported that the initial weights of the rats were different in the three experiments they conducted with them and state that "in those 2 where differences were found in females, their initial weights were about 200 g and 220 g, respectively, while the corresponding weights were only about 160 g in that experiment where no differences were found." The differences in initial body weight of the rat groups were significant. In females, group averages were 198, 158, and 224 g, for groups 1, 2, and 3, respectively, and for males, group averages were 222, 166, and 248 g for groups 1, 2, and 3 respectively. This represents as much as a 50% difference in initial body weights between these TCE treatment groups. Control values varied as well with group averages for controls ranging from 167 g for group 2 to 246 g for group 3 at the start of exposure. For female rats, control groups ranged from 158 to 219 g at the start of the experiment.

The number of animals in each group varied greatly as well, making quantitative comparison even more difficult with the numbers varying between 5 and 12 for each gender in rats exposed for 30 days to TCE. The authors pooled the results for these very disparate groups of rats in their reporting of relative organ weights. They reported 1.26- and 1.21-fold of control in male and female rat percent relative liver/body weight after 30 days of TCE exposure. However, as stated above, these estimates are limited in their ability to provide a quantitative estimate of liver weight increase due to TCE.

There were evidently differences between the groups of gerbils in response to TCE with one group reported to have larger weight gain than control and the other two groups reported to not show a difference by the authors. Of the three groups of gerbils, group 1 contained 12 animals per gender but groups 2 and 3 only 4 animals per gender. As with the rat experiments, the initial average weights for the groups varied significantly (30% in females and males). The authors pooled the results for these very disparate groups of gerbils in their reporting of relative organ weights as well. They reported a nearly identical increase in relative

liver/body weight increase for gerbils (1.22-fold of control value in males and 1.25-fold in females) as for the rats after 30 days of TCE exposure. However, similar caveats should be applied in the confidence in this experimental design to determine the magnitudes of response to TCE exposure.

E.2.2.4. Woolhiser et al. (2006)

An unpublished report by Woolhiser et al. (2006) was received by the U.S. EPA to fill the "priority data needed" for the immunotoxicity of TCE as identified by the ATSDR and designed to satisfy U.S. EPA OPPTS 870.7800 Immunotoxicity Test Guidelines. The study was conducted on behalf of the Halogenated Solvents Industry Alliance and has been submitted to the U.S. EPA but not published. Although conducted as an immunotoxicity study, it does contain information regarding liver weight increases in female Sprague-Dawley female rats exposed to 0, 100, 300, and 1,000 ppm TCE for 6 hours/day, 5 days/week for 4 weeks. The rats were 7 weeks of age at the start of the study. The report gives data for body weight and food weight for 16 animals per exposure group and the mean body weights ranged between 181.8 and 185.5 g on the first day of the experiment. Animals were weighed pre-exposure, twice during the first week, and then "at least weekly throughout the study." All rats were immunized with a single i.v. injection of SRBCs via the tail vein at day 25. Liver weights were taken and samples of liver retained "should histopathological examination have been deemed necessary." But, histopathological analysis was not conducted on the liver.

The effect on body weight gain by TCE inhalation exposure was shown by 5 days and continued for 10 days of exposure in the 300 and 1,000 ppm groups. By day 28, the mean body weight for the control group was reported to be 245.7 g, but 234.4, 232.4, and 232.4 g for the 100, 300, and 1,000 ppm groups, respectively. Food consumption was reported to be decreased in the day 1–5 measurement period for the 300 and 1,000 ppm exposure groups and in the 5–10-day measurement period for the 100 ppm group.

Although body weight and food consumption data are available for 16 animals per exposure group, for organ and organ/body weight summary data, the report gives information for only eight rats per group. The report gives individual animal data in its appendix so that the data for the eight animals in each group examined for organ weight changes could be examined separately. The final body weights were reported to be 217.2, 212.4, 203.9, and 206.9 g for the control, 100, 300, and 1,000 ppm exposure groups containing only eight animals. For the 8-animal exposure groups, the mean initial body weights were 186.6, 183.7, 181.6, and 181.9 g for the control, 100, 300, and 1,000 ppm groups. Thus, there was a difference from the initial and final body weight values given for the groups containing 16 rats and those containing 8 rats. The ranges of initial body weights for the eight animals were 169.8–204.3, 162.0–191.2, 169.0–201.5, and 168.2–193.7 g for the control, 100-, 300 -, and 1,000-ppm groups. Thus, the control

group began with a larger mean value and large range of values (20% difference between highest and lowest weight rat) than the other groups.

In terms of the percent liver/body weight ratios, an increase due to TCE exposure is reported in female rats, although body weights were larger in the control group and the two higher exposure groups did not gain body weight to the same extent as controls. The mean percent liver/body weight ratios were 3.23, 3.39, 3.44, and 3.65%, respectively, for the control, 100, 300, and 1,000 ppm exposure groups. This represented 1.05-, 1.07-, and 1.13-fold of control percent liver/body weight changes in the 100, 300, and 1,000 ppm groups. However, the small number of animals and the variation in initial animal weight limit the ability of this study to determine statistically significant increases and the authors report that only the 1,000 ppm group had statistically significant liver weight increases.

E.2.2.5. Kjellstrand et al. (1983b)

This study examined seven strains of mice (wild, C57BL, DBA, B6CBA, A/sn, NZB, and NMRI) after continuous inhalation exposure to 150 ppm TCE for 30 days. "Wild" mice were reported to be composed of "three different strains: 1. Hairless (HR) from the original strain, 2. Swiss (outbred), and 3. Furtype Black Pelage (of unknown strain)." The authors did not state the age of the animals prior to TCE exposure, but stated that weight-matched controls were exposed to air only chambers. The authors stated that "the exposure methods" have been described earlier (Kjellstrand et al., 1980) but the only reference provided was (Kjellstrand et al., 1981b). In both this study (Kjellstrand et al., 1983b) and the 1981 study, animals were continuously exposed with only a few hours of cessation of exposure noted each week, for a change of food and bedding. Under this paradigm, there is the possibility of additional oral exposure to TCE due to grooming and consumption of TCE on food in the chamber.

The study was reported to be composed of two independent experiments with the exception of strain NMRI, which had been studied in Kjellstrand et al. (1983a; 1981b). The number of animals examined in this study ranged from three to six in each treatment group. The authors reported "significant difference between the animals intended for TCE exposure and the matched controls intended for air-exposure were seen in four cases (Table 1)," and stated that the grouping effects developed during the 7-day adaptation period. Premature mortality was attributed to an accident for one TCE-exposed DBA male and fighting to the deaths of two TCE-exposed NZB females and one B6CBA male in each air exposed chamber. Given the small number of animals examined in this study in each group, such losses significantly decrease the power of the study to detect TCE-induced changes. The range of initial body weights between the groups of male mice for all strains was between 18 g (as mean value for the A/sn strain) and 32 g (as mean value for the B6CBA strain) or ~44%. For females, the range of initial body weights between groups for all strains was 15 g (as mean value for the A/sn strain) and 24 g (as mean value for the DBA strain) or ~38%.

Rather than reporting percent liver/body weight ratios or an extrapolated value, as was done in Kjellstrand et al. (1981b), this study only reported actual liver weights for treated and exposed groups at the end of 30 days of exposure. The authors reported final body weight changes in comparison to matched control groups at the end of the exposure periods but not the changes in body weight for individual animals. They reported the results from statistical analyses of the difference in values between TCE and air-exposed groups.

A statistically significant decrease in body weight was reported between TCE-exposed and control mice in experiment 1 of the C57BL male mice (~20% reduction in body weight due to TCE exposure). This group also had a slight but statistically significant difference in body weight at the beginning of exposure, with the control group having a ~5% difference in starting weight. There was also a statistically significant decrease in body weight of 20% reported after TCE exposure in one group of male B6CBA mice that did not have a difference in body weight at the beginning of the experiment between treatment and control groups. One group of female and both groups of male A/sn mice had statistically significant decreases in body weight after TCE exposure (10% for the females, and 22 and 26% decreases in the two male groups) in comparison to untreated mice of the same strain. The magnitude of body weight decrease in this strain after TCE treatment also reflects differences in initial body weight as there were also differences in initial body weight between the two groups of both treated and untreated A/sn males that were statistically significant, 17 and 10% respectively. One group of male NZB mice had a significant increase in body weight after TCE exposure of 14% compared to untreated animals. A female group from the same strain treated with TCE was reported to have a nonsignificant 7% increase in final body weight in comparison to its untreated group. The one group of male NMRI mice (n = 10) in this study was reported to have a statistically significant 12% decrease in body weight compared to controls.

For the groups of animals with reported TCE exposure-related changes in final body weight compared to untreated animals, such body weight changes may also have affected the liver weights changes reported. The authors did not explicitly state that they did not record liver and body weights specifically for each animal, and thus, would be unable to determine liver/body weight ratios for each. However, they did state that the animals were housed 4–6 in each cage and placed in exposure chambers together. The authors only present data for body and liver weights as the means for a cage group in the reporting of their results. While this approach lends more certainty in their measurements than the approach taken by Kjellstrand et al. (1981b) as described above, the relative liver/body weights cannot be determined for individual animals.

It appears that the authors tried to carefully match the body weights of the control and exposed mice at the beginning of the experiment to minimize the effects of initial body weight differences and distinguish the effects of treatment on body weight and liver weight. However, there was no ability to determine liver/body weight ratios and adjust for difference in initial body weight from changes due to TCE exposure. For the groups in which there was no change in

body weight after TCE treatment and in which there was no difference in initial body weight between controls and TCE-exposed groups, the reporting of liver weight changes due to TCE exposure is a clearer reflection of TCE-induced effects and the magnitude of such effects. Nevertheless, the small number of animals examined in each group is still a limitation on the ability to determine the magnitude of such responses and there statistical significance.

In wild-type mice, there were no reported significant differences in the initial and final body weight of male or female mice before or after 30 days of TCE exposure. For these groups there was 1.76- and 1.80-fold of control values for liver weight in groups 1 and 2 for female mice, and for males 1.84- and 1.62-fold of control values for groups 1 and 2, respectively. For DBA mice, there were no reported significant differences in the initial and final body weight of male or female mice before or after 30 days of TCE exposure. For DBA mice, there was 1.87- and 1.88-fold of control for liver weight in groups 1 and 2 for female mice, and 1.45- and 2.00-fold of control for group 1 and 2 males, respectively. These groups represent the most accurate data for TCE-induced changes in liver weight not affected by initial differences in body weight or systemic effects of TCE, which resulted in decreased body weight gain. These results suggest that there is more variability in TCE-induced liver weight gain between groups of male than female mice.

The C57BL, B6CBA, NZB, and NMRI groups all had at least one group of male mice with changes in body weight due to TCE exposure. The A/sn group had not only decreased body weight in both male groups after TCE exposure (along with differences between exposed and control groups at the initiation of exposure), but also decreased body weight in one of the female groups. Thus, the results for TCE-induced liver weight change in these male groups also reflected changes in body weight. These results suggest a strain-related increased sensitivity to TCE toxicity as reflected by decreased body weight.

For C57BL mice, there was 1.65- and 1.60-fold of control for liver weight after TCE exposure was reported in groups 1 and 2 for female mice, and for males, 1.28-fold (the group with decreased body weight) and 1.82-fold of control values for groups 1 and 2, respectively. For B6CBA mice there was 1.70- and 1.69-fold of controls values for liver weight after TCE exposure in groups 1 and 2 for female mice, and for males, 1.21-fold (the group with decreased body weight) and 1.47-fold of control values reported for groups 1 and 2, respectively. For the NZB mice, there was 2.09-fold (n = 3) and 2.08-fold of control values for liver weight after TCE exposure in groups 1 and 2 for female mice, and for males, 2.34- and 3.57-fold (the group with increased body weight) of control values reported for groups 1 and 2, respectively. For the NMRI mice, whose results were reported for one group with 10 mice, there was 1.66-fold of control value for liver weight after TCE exposure for female mice, and for males, 1.68-fold of control value reported (a group with decreased body weight). Finally, for the A/sn strain that had decreased body weight in all groups but one after TCE exposure and significantly smaller body weights in the control groups before TCE exposure in both male groups, the results still show

TCE-related liver weight increases. For the As/n mice, there was 1.56- and 1.72-fold (a group with decreased body weight) of control value for liver weight in groups 1 and 2 for female mice, and for males, 1.62-fold (a group with decreased body weight) and 1.58-fold (a group with decreased body weight) of control values reported for groups 1 and 2, respectively.

The consistency between groups of female mice of the same strain for TCE-induced liver weight gain, regardless of strain examined, is striking. The largest difference within female strain groups occurred in the only strain in which there was a decrease in TCE-induced body weight. For males, even in strains that did not show TCE-related changes in body weight, there was greater variation between groups than in females. For strains in which one group had TCE-related changes in body weight and another did not, the group with the body weight decrease always had a lower liver weight as well. Groups that had increased body weight after TCE exposure also had an increased liver weight in comparison to the groups without a body weight change. These results demonstrate the importance of carefully matching control animals to treated animals and the importance of the effect of systemic toxicity, as measured by body weight decreases, on the determination of the magnitude of liver weight gain induced by TCE exposure. These results also show the increased variation in TCE-induced liver weight gain between groups of male mice and an increase incidence of body weight changes due to TCE exposure in comparison to females, regardless of strain.

In terms of strain sensitivity, it is important not only to take into account differing effects on body weight changes due to TCE exposure but also to compare animals of the same age or beginning weight as these, parameters may also affect liver weight gain or toxicity induced by TCE exposure. The authors do not state the age of the animals at the beginning of exposure and report, as stated above, a range of initial body weights between the groups as much as 44% for males and 38% for females. These differences can be due to strain and age. The differences in final body weight between the groups of controls, when all animals would have been 30 days older and more mature, was still as much as 48% for males and 44% for females.

The data for female mice, in which body weight was decreased by TCE exposure only in one group in one strain, suggest that the magnitude of TCE-induced liver weight increase was correlated with body weight of the animals at the beginning of the experiment. For the C57BL and As/n strains, female mice starting weights were averaged 17.5 and 15.5 g, respectively, while the average liver weights were 1.63- and 1.64-fold of control after TCE exposure, respectively. For the B6CBA, wild-type, DBA, and NZB female groups, the starting body weights averaged 22.5, 21.0, 23.0, and 21.0 g, respectively, while the average liver weight increases were 1.70-, 1.78-, 1.88-, and 2.09-fold of control after TCE exposure. Thus, groups of female mice with higher body weights, regardless of strain, generally had higher increases in TCE-induced liver weight increases.

The NMRI group of female mice, did not follow this general pattern and had the highest initial body weight for the single group of 10 mice reported (i.e., 27 g) associated with a

1.66-fold of control value for liver weight. It is probable that the data for these mice had been collected from another study. In fact, the starting weights reported for these groups of 10 mice are identical to the starting weights reported for 26 mice examined in Kjellstrand et al. (1981b). However, while this study reports a 1.66-fold of control value for liver weight after 30 days of TCE exposure, the extrapolated percent liver/body weight given in the 1981 study for 30 days of TCE exposure was 1.74-fold of control in female NMRI mice. In the Kjellstrand et al. (1983a) study, discussed below, 10 female mice were reported to have a 1.66-fold of control value for liver weight after 30 days exposure to 150 ppm TCE with an initial starting weight of 26.7 g. Thus, these data appear to be from that study. Thus, differences in study design, variation between experiments, and strain differences may account for the differences results reported in Kjellstrand et al. (1983b) for NMRI mice and the other strains in regard to the relationship to initial body weight and TCE response of liver weight gain.

These data suggest that initial body weight is a factor in the magnitude of TCE-induced liver weight induction rather than just strain. For male mice, there appeared to be a difference between strains in TCE-induced body weight reduction, which in turn affects liver weight. The DBA and wild-type mice appeared to be the most resistant to this effect (with no groups affected), while the C57BL, B6CBA, and NZB strains appearing to have at least one group affected, and the A/sn strain having both groups of males affected. Only one group of NMRI mice were reported in this study and that group had TCE-induced decreases in body weight.

As stated above, there appeared to be much greater differences between groups of males within the same strain in regard to liver weight increases than for females and that the increases appeared to be affected by concurrent body weight changes. In general, the strains and groups within strains, that had TCE-induced body weight decreases had the smallest increases in liver weight, while those with no TCE-induced changes in body weight in comparison to untreated animals (i.e., wild-type and DBA) or had an actual increase in body weight (one group of NZB mice) had the greatest TCE-induced increase in liver weight. Therefore, only examining liver weight in males rather than percent liver/body weight ratios would not be an accurate predictor of strain sensitivity at this dose due to differences in initial body weight and TCE-induced body weight changes.

E.2.2.6. Kjellstrand et al. (1983a)

This study was conducted in male and female NMRI mice with a similar design as Kjellstrand et al. (1983b). The ages of the mice were not given by the authors. Animals were housed 10 animals per cage and exposed from 30 to 120 days at concentrations ranging from 37 to 3,600 ppm TCE. TCE was stabilized with 0.01% thymol and 0.03% diisopropylene. Animals were exposed continuously with exposure chambers being opened twice a week for change of bedding food and water resulting in a drop in TCE concentration of ~1 hour. A group of mice was exposed intermittently with TCE at night for 16 hours. This paradigm results not

only in inhalation exposure, but also oral exposure from TCE adsorption to food and grooming behavior. The authors state that "the different methodological aspects linked to statistical treatment of body and organ weights have been discussed earlier (Kjellstrand et al., 1981b). The same air-exposed control was used in three cases." The design of the experiment, in terms of measurement of individual organ and body weights and the inability to assign a percent liver/body weight for each animal, and limitations are similar to that of Kjellstrand et al. (1983a).

The exposure design was for groups of male and female mice to be exposed to 37, 75, 150, and 300 ppm TCE continuously for 30 days (n = 10 per gender and group except for the 37 ppm exposure groups) and then for liver weight and body weight to be determined. Additional groups of animals were exposed for 150 ppm continuously for 120 days (n = 10). Intermittent exposure of 4 hours/day for 7 days/week were conducted for 120 days at 900 ppm and examined immediately or 30 days after cessation of exposure (n = 10). Intermittent exposures of 16 hours/day at 255-ppm group (n = 10), 8 hours/day at 450 ppm, 4 hours/day at 900 ppm, 2 hours/day at 1,800 ppm, and 1 hour/day at 3,600 ppm 7 days/week for 30 days were also conducted (n = 10 per group).

As in Kjellstrand et al. (1983b), body weights for individual animals were not recorded in a way that the initial and final body weights could be compared. The approach taken by the authors was to match the control group at the initiation of exposure and compare control and treated average values. At the beginning of the experiment, only one group began the experiment with a statistically significant change in body weight between treated and control animals (female mice exposed 16 hours a day for 30 days). In regard to final body weight, which would indicate systemic TCE toxicity, five groups had significantly decreased body weight (i.e., males exposed to 150 ppm continuously for 30 or 120 days, males and females exposed continuously to 300 ppm for 30 days) and two groups significantly increased body weight (i.e., males exposed to 1,800 ppm for 2 hours/day and 3,600 ppm for 1 hour/day for 30 days) after TCE exposure.

Thus, the accuracy of determining the effect of TCE on liver weight changes, reported by the authors in this study for groups in which body weight were also affected by TCE exposure, would be affected by similar issues as for data presented by Kjellstand et al. (1983b). In addition, comparison in results between the 37 ppm exposure groups and those of the other groups would be affected by difference in number of animals examined (10 vs. 20). As with Kjellstrand et al. (1983b), the ages of the animals in this study are not given by the author. Difference in initial body weight (which can be affected by age and strain) reported by Kjellstrand et al. (1983b) appeared to be correlated with the degree of TCE-induced change in liver weight. Although each exposed group was matched to a control group with a similar average weight, the average initial body weights in this study varied between groups (i.e., as much as 14% in female control, 16% in TCE-exposed female mice, 12% in male control, and 16% in male exposed mice).

For female mice exposed to 37–300 ppm TCE continuously for 30 days, only the 300 pm group experienced a 16% decrease in body weight between control and exposed animals. Thus, liver weight increased reported by this study after TCE exposure were not affected by changes in body weight for exposures <300 ppm in female mice. Initial body weights in the TCE-exposed female mice were similar in each of these groups (i.e., range of 29.2–31.6 g, or 8%), with the exception of the females exposed to 150 ppm TCE for 30 days (i.e., initial body weight of 27.3 g), reducing the effects of differences in initial body weight on TCE-induced liver weight induction. Exposure to TCE continuously for 30 days resulted in a dose-dependent change in liver weight in female mice with 1.06-, 1.27-, 1.66-, and 2.14-fold of control values reported for liver weight at 37, 75, 150, and 300 ppm TCE, respectively. In females, the increase at 300 ppm was accompanied by statistically significant decreased body weight in the TCE exposed groups compared to control (~16%). Thus, the response in liver weight gain at that exposure is in the presence of toxicity. However, the TCE-induced increases in liver weight consistently increased with dose of TCE in a linear fashion.

For male mice exposed to 37–300 ppm TCE continuously for 30 days, both the 150 and 300 ppm groups experienced a 10 and 18% decrease in body weight after TCE exposure, respectively. The 37 and 75 ppm groups did not have decreased body weight due to TCE exposure, but varied by 12% in initial body weight. Thus, there are more factors affecting reported liver weight increases from TCE exposure in the male than female mice, most importantly toxicity. Exposure to TCE continuously for 30 days resulted in liver weights of 1.15-, 1.50-, 1.69-, and 1.90-fold of control for 37, 75, 150, and 300 ppm, respectively. The flattening of the dose-response curve for liver weight in the male mice is consistent with the effects of toxicity at the two highest doses, and thus, the magnitude of response at these doses should be viewed with caution. Consistent with Kjellstrand et al. (1983b) results, male mice in this study appeared to have a higher incidence of TCE-induced body weight changes than female mice.

The effects of extended exposure, lower durations of exposure but at higher concentrations, and of cessation of exposure were examined for ≥150 ppm TCE. Mice exposed to TCE at 150 ppm continuously for 120 days were reported to have increased liver weight (i.e., 1.57-fold of control for females and 1.49-fold of control for males), but in the case of male mice, also to have a significant decrease in body weight of 17% in comparison to control groups. Increasing the exposure concentration to 900-ppm TCE and reducing exposure time to 4 hours/day for 120 days also resulted in increased liver weight (i.e., 1.35-fold of control for females and 1.49-fold of controls for males) but with a significant decrease in body weight in females of 7% in comparison to control groups. For mice that were exposed to 150 ppm TCE for 30 days and then examined 120 days after the cessation of exposure, liver weights were 1.09-fold of control for female mice and the same as controls for male mice.

With the exception of 1,800 and 3,600 ppm TCE groups exposed at 2 and 1 hour, respectively, exposure from 225, 450, and 900 ppm at 16, 8, and 4 hours, respectively, for 30 days did not result in decreased body weight in males or female mice. These exposures did result in increased liver weights in relation to control groups and for female mice the magnitude of increase was similar (i.e., 1.50-, 1.54-, and 1.51-fold of control for liver weight after exposure to 225 ppm TCE 16 hours/day, 450 ppm TCE 8 hours/day, and 900 ppm TCE 4 hours/day, respectively). For these groups, initial body weights varied by 13% in females and 14% in males. Thus, under circumstances without body weight changes due to TCE toxicity, liver weight appeared to have a consistent relationship with the product of duration and concentration of exposure in female mice.

For male mice, the increases in TCE-induced liver weight were more variable (i.e., 1.94-, 1.74-, and 1.61-fold of control for liver weight after exposure to 225 ppm TCE 16 hours/day, 450 ppm TCE 8 hours/day, and 900 ppm TCE 4 hours/day, respectively) with the product of exposure duration and concentration did not result in a consistent response in males (e.g., a lower dose for a longer duration of exposure resulted in a greater response than a larger dose at a shorter duration of exposure).

Kjellstrand et al. (1983a) reported light microscopic findings from this study and report that:

> after 150 ppm exposure for 30 days, the normal trabecular arrangement of the liver cells remained. However, the liver cells were generally larger and often displayed a fine vacuolization of the cytoplasm. The nucleoli varied slightly to moderately in size and shape and had a finer, granular chromatin with a varying basophilic staining intensity. The Kupffer cells of the sinusoid were increased in cellular and nuclear size. The intralobular connective tissue was infiltrated by inflammatory cells. There was no sign of bile stasis. Exposure to TCE in higher or lower concentrations during the 30 days produced a similar morphologic picture. After intermittent exposure for 30 days to a time weighted average concentration of 150 ppm or continuous exposure for 120 days, the trabecular cellular arrangement was less well preserved. The cells had increased in size and the variations in size and shape of the cells were much greater. The nuclei also displayed a greater variation in basophilic staining intensity, and often had one or two enlarged nucleoli. Mitosis was also more frequent in the groups exposed for longer intervals. The vacuolization of the cytoplasm was also much more pronounced. Inflammatory cell infiltration in the interlobular connective tissue was more prominent. After exposure to 150 ppm for 30 days, followed by 120 days of rehabilitation, the morphological picture was similar to that of the air-exposure controls except for changes in cellular and nuclear sizes.

Although not reporting comparisons between changes in male and female mice in the results section of the paper, the authors stated in the discussion section that “However, liver mass

increase and the changes in liver cell morphology were similar in TCE-exposed male and female mice."

The authors do not present any quantitative data on the lesions they describe, especially in terms of dose-response. Most of the qualitative description is for the 150 ppm exposure level, in which there are consistent reports of TCE induced body weight decreases in male mice. The authors suggest that lower concentrations of TCE give a similar pathology as those at the 150 ppm, but did not present data to support that conclusion. Although stating that Kupffer cells were increased in cellular and nuclear size, no differential staining was applied light microscopy sections distinguish Kupffer from endothelial cells lining the hepatic sinusoid in this study. Without differential staining, such a determination is difficult at the light microscopic level. Indeed, Goel et al. (1992) describe proliferation of sinusoidal endothelial cells after 1,000 and 2,000 mg/kg-day TCE exposure for 28 days in male Swiss mice. However, the described inflammatory cell infiltrates in the Kjellstrand et al. (1983a) study are consistent with invasion of macrophages and well as polymorphonuclear cells into the liver, which could activate resident Kupffer cells.

Although not specifically describing the changes as consistent with increased polyploidization of hepatocytes, the changes in cell size and especially the continued change in cell size and nuclear staining characteristics after 120 days of cessation of exposure are consistent with changes in polyploidization induced by TCE. Of note is that in the histological description provided by the authors, although vacuolization is reported and consistent with hepatotoxicity or lipid accumulation, which is lost during routine histological slide preparation, there is no mention of focal necrosis or apoptosis resulting from these exposures to TCE.

E.2.2.7. Buben and O'Flaherty (1985)

This study was conducted with older mice than those generally used in chronic exposure assays (male Swiss-Cox outbred mice between 3 and 5 months of age) with a weight range reported between 34 and 45 g. The mice were administered distilled TCE in corn oil by gavage 5 times/week for 6 weeks at exposure concentrations of either 0, 100, 200, 400, 800, 1,600, 2,400, or 3,200 mg TCE/kg-day. While 12–15 mice were used in most exposure groups, the 100 and 3,200 mg/kg groups contained 4–6 mice and the two control groups consisted of 24 and 26 mice. Liver toxicity was determined by "liver weight increases, decreases in liver glucose-6-phosphate (G6P) activity, increases in liver triglycerides, and increases in serum glutamate-pyruvate transaminase (SGPT) activity." Livers were perfused with cold saline prior to testing for weight and enzyme activity and hepatic DNA was measured.

The authors reported the mice to tolerate the 6-week exposed with TCE with few deaths occurring except at the highest dose and that such deaths were related to CNS depression. Mice in all dose groups were reported to continue to gain weight throughout the 6-week dosing period. However, TCE exposure caused "dose-related increases in liver weight to body weight ratio and

since body weight of mice were generally unaffected by treatment, the increases represent true liver weight increases." Exposure concentrations, as low as 100 mg/kg-day, were reported to be "sufficient to cause statistically significant increase in the liver weight/body weight ratio," and the increases in liver size to be "attributable to hypertrophy of the liver cells, as revealed by histological examination and by a decrease in the DNA concentration in the livers."

Mice in the highest dose group were reported to display liver weight/body weight ratios that were about ~75% greater than those of controls and even at the lowest dose there was a statistically significant increase (i.e., control liver/body weight percent was reported to be 5.22 ± 0.09 vs. 5.85 ± 0.20% in 100 mg/kg-day exposed mice). The percent liver/body ratios were 5.22 ± 0.09, 5.84 ± 0.20, 5.99 ± 0.13, 6.51 ± 0.12, 7.12 ± 0.12, 8.51 ± 0.20, 8.82 ± 0.15, and 9.12 ± 0.15% for control (n = 24), 100 (n = 5), 200 (n = 12), 400 (n = 12), 800 (n = 12), 1,600 (n = 12), 2,400 (n = 12), and 3,200 (n = 4) mg/kg-day TCE. This represents 1.12-, 1.15-, 1.25-, 1.36-, 1.63-, 1.69-, and 1.75-fold of control for these doses. All dose groups of TCE induced a statistically significant increase in liver/body weight ratios. For the 200–1,600 mg/kg-day exposure levels, the magnitudes of the increases in TCE exposure concentrations were similar to the magnitudes of TCE-induced increases in percent liver/body weight ratios (i.e., an approximately twofold increase in TCE dose resulted in ~1.7-fold increase change in percent liver/body weight).

TCE exposure was reported to induce a dose-related trend towards increased triglycerides (i.e., control values of 3.08 ± 0.29 vs. 6.89 ± 1.40 at 2,400 mg/kg TCE) with variation of response increased with TCE exposure. For liver triglycerides, the reported values in mg/g liver were 3.08 ± 0.29 (n = 24), 3.12 ± 0.49 (n = 5), 4.41 ± 0.76 (n = 12), 4.53 ± 1.05 (n = 12), 5.76 ± 0.85 (n = 12), 5.82 ± 0.93 (n = 12), 6.89 ± 1.40 (n = 12), and 7.02 ± 0.69 (n = 4) for control, 100, 200, 400, 800, 1,600, 2,400, and 3,200 mg/kg-day dose groups, respectively.

For G6P, the values in μg phosphate/mg protein/20 minutes were 125.5 ± 3.2 (n = 12), 117.8 ± 6.0 (n = 5), 116.4 ± 2.8 (n = 9), 117.3 ± 4.6 (n = 9), 111.7 ± 3.3 (n = 9), 89.9 ± 1.7 (n = 9), 83.8 ± 2.1 (n = 8), and 83.0 ± 7.0 (n = 3) for the same dose groups. Only the 2,400 mg/kg-day group was reported to be statistically significantly increased for triglycerides after TCE exposure although there appeared to be a dose-response. For decreases in G6P, doses ≥800 mg/kg-day were statistically significant.

The numbers of animals varied between groups in this study but, in particular, only a subset of the animals were tested for G6P with the authors providing no rationale for the selection of animals for this assay. The differences in the number of animals per group and small number of animals per group affected the ability to determine a statistically significant change in these parameters but the changes in liver weights were robust enough and the variation was small enough between groups that all TCE-induced changes were described as statistically significant. The livers of TCE treated mice, although enlarged, were reported to appear normal.

A dose-related decrease in G6P activity was reported with similar small decreases (~10%) observed in the TCE exposed groups that did not reach statistical significance until the dose reached 800 mg/kg TCE exposure. SGPT activity was not observed to be increased in TCE-treated mice except at the two highest doses and even at the 2,400 mg/kg-day dose half of the mice had normal values. The large variability in SGPT activity was indicative of heterogeneity of this response between mice at the higher exposure levels for this indicator of liver toxicity. However, the results of this study also demonstrate that hepatomegaly was a robust response that was observed at the lowest dose tested, was dose-related, and was not accompanied by toxicity.

Liver histopathology and DNA content were determined only in control, 400, and 1,600 mg/kg-day TCE exposure groups. DNA content was reported to be significantly decreased from 2.83 ± 0.17 mg/g liver in controls to 2.57 ± 0.14 in 400 mg/kg-day TCE treated group, and to 2.15 ± 0.08 mg/kg-day liver in the 1,600 mg/kg-day exposed group. This result was consistent with a decreased number of nuclei/g of liver and hepatocellular hypertrophy.

Liver degeneration was reported as swollen hepatocytes and to be common with treatment. "Cells had indistinct borders; their cytoplasm was clumped and a vesicular pattern was apparent. The swelling was not simply due to edema, as wet weight/dry weight ratios did not increase." Karyorrhexis (the disintegration of the nucleus) was reported to be present in nearly all specimens and suggestive of impending cell death. A qualitative scale of negative, 1, 2, 3, or 4 was given by the authors to rate their findings without further definition or criterion given for the ratings. "No karyorrhexis, necrosis, or polyploidy was reported in controls, but a score of 1 for karyorrhexis was given for 400 mg/kg TCE and 2 for 1,600 mg/kg TCE." Central lobular necrosis reported to be present only at the 1,600 mg/kg-day TCE exposure level and as a score of 1. "Polyploidy was also characteristic in the central lobular region" with a score of 1 for both 400 and 1,600 mg/kg TCE. The authors reported that "hepatic cells had two or more nuclei or had enlarged nuclei containing increased amounts of chromatin, suggesting that a regenerative process was ongoing" and that there were no fine lipid droplets in TCE-exposed animals.

The finding of "no polyploidy" in control mouse liver is unexpected given that binucleate and polyploid hepatocytes are a common finding in the mature mouse liver. It is possible that the authors were referring to unusually high instances of "polyploidy" in comparison to what would be expected for the mature mouse. The score given by the authors for polyploidy did not indicate a difference between the two TCE exposure treatments and that it was of the lowest level of severity or occurrence.

No score was given for centrolobular hypertrophy although the DNA content and liver weight changes suggested a dose response. The "karyorrhexis" described in this study could have been a sign of cell death associated with increased liver cell number or dying of maturing hepatocytes associated with the increased ploidy, and suggests that TCE treatment was inducing polyploidization. Consistent with enzyme analyses, centrilobular necrosis was only seen at the

highest dose and with the lowest qualitative score, indicating that even at the highest dose there was little toxicity.

Thus, the results of this study of TCE exposure for 6 weeks are consistent with acute studies and show that the region of the liver affected by TCE is the centrilobular region, that hepatocellular hypertrophy is observed in that region, and that increased liver weight is induced at the lowest exposure level tested and much lower than those inducing overt toxicity. These authors suggest that polyploidization is occurring as a result of TCE exposure, although a quantitative dose-response cannot be determined from these data.

E.2.2.8. Channel et al. (1998)

This study was performed in male hybrid B6C3F_1/CrlBR mice (13 weeks old, 25–30 g) and focused on indicators of oxidative stress. TCE was administered by gavage 5 days/week in corn oil for up to 55 days for some groups. Although the study design indicated that water controls, corn oil controls, and exposure levels of 400, 800, and 1,200 mg/kg-day TCE in corn oil, results were not presented for water controls for some parameters measured. Initial body weights and those recorded during the course of the study were not reported for individual treatment groups. Liver samples were collected on study days 2, 3, 6, 10, 14, 21, 28, 35, 42, 49, and 56. Histopathology was studied from a single section taken from the median lobe. Thiobarbituric acid-reactive substances (TBARS) were determined from whole-liver homogenates. Nuclei were isolated from whole-liver homogenates and DNA assayed for 8-hydroxy-2' deoxyguanosine (8-OHdG). There was no indication that parenchymal cell and nonparenchymal cells were distinguished in the assay. Free radical electron paramagnetic resonance (EPR) for total radicals was analyzed in whole-liver homogenates. For peroxisome detection and analysis, livers from three mice from the 1,200 mg/kg-day TCE and control (oil and water) groups were analyzed via electron microscopy. Only centrilobular regions, the area stated by the authors to be the primary site of peroxisome proliferation, were examined. For each animal, 7 micrographs of randomly chosen hepatocytes immediately adjacent to the central vein were examined with peroxisomal area to cytoplasmic area, the number of peroxisomes per unit area of cytoplasm, and average peroxisomal size quantified. Proliferation cell nuclear antigen (PCNA), described as a marker of cell cycle except G0, was examined in histological sections for a minimum of 18 fields per liver section. The authors did not indicate what areas of the liver lobule were examined for PCNA. Apoptosis was detected on liver sections using a apoptosis kit using a single liver section from the median lobe and based on the number of positively labeled cells per 10 mm^2 in combination with the morphological criteria for apoptosis of Columbano et al. (1985). However, the authors did not indicate what areas of the liver lobule were specifically examined.

The authors reported that body weight gain was not adversely affected by TCE dosing of the time course of the study but did not show the data. No gross lesions were reported to be

observed in any group. For TBARS, no water control data were reported by the authors. Data were presented for six animals per group for the corn oil control group and the 1,200 mg/kg-day group (error bars representing the SE). No data were presented without corn oil so that the effects of corn oil on the first day of the study (day 2 of dosing) could not be determined.

After 2 and 3 days of dosing, the corn oil and 1,200 mg/kg-day TCE groups appeared to have similar levels of TBAR detected in whole liver as nmol TBARS/mg protein. However, by day 6, the corn oil treated control had a decrease in TBAR that continued until day 15 where the level was ~50% of that reported on days 2 and 3. The variation between animals as measured by SE was reported to be large on day 10. By day 20, there was a slight increase in variation that declined by day 35 and stayed the same through day 55. For the TCE-exposed group, the TBARS remained relatively consistent and began to decline by about day 20 to a level that similar to the corn oil declines by day 35. Therefore, corn oil alone had a significant effect on TBAR detection inducing a decline by 6 days of administration that persisted thought 55 days. TCE administration at the 1,200 mg/kg-day dose in corn oil appeared to have a delayed decline in TBARS. The authors interpreted this pattern to show that lipid peroxidation was elevated in the 1,200 mg/kg-day TCE group at day 6 over corn oil. However, corn oil alone induced a decrease in TBARs. At no time was TBARS in the TCE treatment groups reported to be greater than the initial levels at days 2 and 3, a time in which TCE and corn oil treatment groups had similar levels. Rather than inducing increasing TBARS over the time course of the study, TCE, at the 1,200 mg/kg-day dose, appeared to delay the corn oil induced suppression of TBARS detection. Because the authors did not present data for aqueous control animals, the time course of TBARS detection in the absence of corn oil cannot be established.

For the 800 and 400 mg/kg-day TCE data, the authors presented a figure, without SE information, for up to 35 days that shows little difference between 400 mg/kg TCE treatment and corn oil suppression of TBAR induction. There was little difference between the patterns of TBAR detection for 800 and 400 mg/kg-day TCE, indicating that both delayed TBARS suppression by corn oil to a similar extent and did not induce greater TBARS than corn oil alone.

For 8-OHdG levels, the authors reported that elevations were modest with the greatest increase noted in the 1,200 mg/kg-day TCE treatment group of 196% of oil controls on day 56. Levels fluctuated throughout the study with most of the time points that were elevated showing 129% of control for the 1,200 mg/kg-day group. Statistically significant elevations were noted on days 2, 10, 28, 49, and 56 with depression on day 3. On all other days (i.e., days 6, 14, 21, 35, and 42), the 8-OHdG values were similar to those of corn oil controls. No statistically significant effects were reported to be observed at lower doses.

The figure presented by the authors shows the percent of controls by TCE treatment at 1,200 mg/kg-day but not the control values themselves. The pattern by corn oil is not shown and neither is the SE of the data. As a percent of control values, the variations were very large for many of the data points and largest for the data given at day 55 in which the authors report the

largest difference between control and TCE treatment. There was no apparent pattern of elevation in 8-OHdG when the data were presented in this manner. Because the data for the corn oil control was not given, as well as no data given for aqueous controls, the effects of corn oil alone cannot be discerned.

Given that for TBARS corn oil had a significant effect and showed a pattern of decline after 6 days, with TCE showing a delayed decline, it is especially important to discern the effects of corn oil and to see the pattern of the data. At time points when TBARS levels were reported to be the same between corn oil and TCE (days 42, 49 and 56), the pattern of 8-OHdG was quite different with a lower level at day 42, a slightly increased level at day 49, and the highest difference reported at day 56 between corn oil control and TCE treated animals. The authors reported that the pattern of "lipid peroxidation" was similar between the 1,200 and 800 mg/kg-day doses of TCE, but that there was no significant difference between 800 mg/kg-day TCE and corn oil controls. Thus, the pattern of TBARS as a measure of lipid peroxidation and 8-OHdG level in nuclear DNA did not match.

In regard to total free radical levels as measured by EPR, results were reported for the 1,200 mg/kg TCE as a signal that was subtracted from control values with the authors stating that only this dose level induced an elevation significantly different from controls. Again, aqueous control values were not presented to discern the effects of corn oil or the pattern that may have arisen with time of corn oil administration.

The pattern of total free radical level appeared to differ from that of lipid peroxidation and for that of 8-OHdG DNA levels, with no changes at days 2, 3, a peak level at day 6, a rapid drop at day 10, mild elevation at day 20, and a significant decrease at day 49. The percentage differences between control and treated values reported at days 6 and 20 by the authors was not proportional to the fold-difference in signal indicating that there was not a consistent level for control values over the time course of the experiment. While differences in lipid peroxidation detection between 1,200 mg/kg-day TCE and corn oil control were greatest at day 14, total free radicals showed their biggest change between corn oil controls and TCE exposure on day 6, time points in which 8-OHdG levels were similar between TCE treatment and corn oil controls. Again, there was no reported difference between corn oil control and the 800 mg/kg-day TCE exposed group in total free radical formation, but for lipid peroxidation, the 800 mg/kg-day TCE exposed group had a similar pattern as that of 1,200 mg/kg-day TCE.

Only the 1,200 mg/kg-day group was evaluated for peroxisomal proliferation at days 6, 10, and 14. Thus, correlations with peroxisome proliferation and other parameters in the report at differing times and TCE exposure concentrations could not be made. The authors reported that there was a treatment and time effect for percent peroxisomal area, a "treatment only" effect for number of peroxisome and no effect for peroxisomal size. They also reported that hepatocytes examined from corn oil control rats were no different than those from water control rats for all peroxisomal parameter, thus discounting a vehicle effect.

However, there was an effect on peroxisomal size between corn oil control and water with corn oil decreasing the peroxisomal size in comparison to water on all days tested. The highest TCE-induced percent peroxisomal area and number occurred on day 10 of the three time points measured for this dose and the fold increase was ~4.5- and ~3.1-fold increase, respectively. The day-10 peak in peroxisomal area and number did not correlate with the reported pattern of free radical or 8-OHdG generation.

For cell proliferation and apoptosis, data were given for days 2, 6, 10, 14, and 21 in a figure. PCNA cells, a measure of cells that have undergone DNA synthesis, was elevated only on day 10 and only in the 1,200 mg/kg-day TCE exposed group with a mean of ~60 positive nuclei per 1,000 nuclei for six mice (~6%). Given that there was little difference in PCNA positive cells at the other TCE doses or time points studied, the small number of affected cells in the liver could not account for the increase in liver size reported in other experimental paradigms at these doses.

The PCNA positive cells as well as "mitotic figures" were reported to be present in centrilobular, midzonal, and periportal regions with no observed predilection for a particular lobular distribution. No data were shown regarding any quantitative estimates of mitotic figures and whether they correlated with PCNA results. Thus, whether the DNA synthesis phases of the cell cycle indicated by PCNA staining were indentifying polyploidization or increased cell number cannot be determined. The authors reported that there was no cytotoxicity manifested as hepatocellular necrosis in any dose group and that there was no significant difference in apoptosis between treatment and control groups with data not shown. The extent of apoptosis in any of the treatment groups, or which groups and timepoints were studied for this effect cannot be determined. No liver weight or body weight data were provided in this study.

These results confirm that as a vehicle corn oil is not neutral in its affects in the liver. The TBARS results indicate a reduction in detection of TBARS in the liver with increasing time of exposure to corn oil alone. Although control animals "treated with water" gavage were studied, only the results for peroxisome proliferation were presented by the study, so that the effects of corn oil gavage were not easy to discern. In addition, the data were presented in such a way for 8-OHdG and total free radical changes that the pattern of corn oil administration was obscured. It is not apparent from this study that TCE exposure induces oxidative damage.

E.2.2.9. Dorfmueller et al. (1979)

The focus of this study was the evaluation of "teratogenicity and behavioral toxicity with inhalation exposure of maternal rats" to TCE. Female Long-Evans hooded rats (n = 12) of ~210 g weight were treated with 1,800 ± 200 ppm TCE for 6 hours/day, 5 days/week, for 22 ± 6 days (until pregnancy confirmation) continuing through GD 20. Control animals were exposed 22 ± 3 days before pregnancy confirmation. The TCE used in this study contained 0.2% epichlorohydrin. Body weights were monitored as well as maternal liver weight at the end of

exposure. Other than organ weight, no other observations regarding the liver were reported in this study. The initial weights of the dams were 212 ± 39 g (mean ± SD) and 204 ± 35 g for treated and control groups, respectively. The final weights were 362 ± 32 g and 337 ± 48 g for treated and control groups, respectively. There was no indication of maternal toxicity by body weight determinations as a result of TCE exposure in this experiment and there was also no significant difference in absolute or relative percent liver/body weight between control and treated female rats in this study.

E.2.2.10. Kumar et al. (2001a)

In this study, adult male Wistar rats (130 ± 10 g body weight) were exposed to 376 ± 1.76 ppm TCE ("AnalaR grade") for 8, 12, and 24 weeks for 4 hours/day 5 days/week. The ages of the rats were not given by the authors. Each group contained six rats. The animals were exposed in whole-body chambers and thus, additional oral exposure was probable. Along with histopathology of light microscopic sections, enzymatic activities of ALP and acid phosphatase, glutamic oxoacetate transaminase, glutamic pyruvate transaminase, reduced GSH, and "total sulphydryl" were assayed in whole-liver homogenates as well as total protein. The authors stated that "the size and weight of the liver were significantly increased after 8, 12, and 24 weeks of TCE exposure." However, the authors did not report the final body weight of the rats after treatment nor did they give quantitative data of liver weight changes. In regard to histopathology, the authors stated:

> After 8 weeks of exposure enlarged hepatocytes, with uniform presence of fat vacuoles were found in all of the hepatocytes affecting the periportal, midzonal, and centrilobular areas, and fat vacuoles pushing the pyknosed nuclei to one side of hepatocytes. Moreover congestion was not significant. After exposure of 12 and 24 weeks, the fatty changes became more progressive with marked necrosis, uniformly distributed in the entire organ.

No other description of pathology was provided in this report. In regard to the description of fatty change, the authors only did conventional H&E staining of sections with no precautions to preserve or stain lipids in their sections. The authors provided a table with histological scoring of simply + or – for minimal, mild, or moderate effects and do not define the criteria for that scoring. There was also no quantitative information given as to the extent, nature, or location of hepatocellular necrosis. The authors reported "no change was observed in GOT and GPT levels of liver in all the three groups. The GSH level was significantly decreased while TSH level was significantly increased during 8, 12, and 24 weeks of TCE exposure. The acid and ALPs were significantly increased during 8, 12, and 24 weeks of TCE exposure." The authors presented a series of figures that are poor in quality to demonstrate histopathological

TCE-induced changes. No mortality was observed from TCE exposure in any group despite the presence of liver necrosis.

E.2.2.11. Kawamoto et al. (1988b)

The focus of this study was the long-term effects of TCE treatment on induction of metabolic enzymes in male adult Wistar rats. The authors reported that eight rats weighing 200 g were treated with 2 g/kg TCE in olive oil administered subcutaneously twice a week for 15 weeks with seven rats serving as olive oil controls. In a separate experiment, five rats were injected with 1 g/kg TCE in olive oil i.p. once a day for 5 continuous days. For comparative purposes, groups of five rats each were administered 3-methylcholanthrene (20 mg/kg in olive oil i.p.), phenobarbital (80 mg/kg in saline i.p.) for 4 days as well as ethanol administered in drinking water containing 10% ethanol for 14 days. Microsomes were prepared 1 week after the last exposure from rats administered TCE for 15 weeks and 24 hours after the last exposure for the other treatments.

Body weights were reported to be slightly less for the TCE treated group than for controls with the initial weights, shown in a figure, to be similar for the first weeks of exposure. At 15 weeks, there appeared to be ~7.5% difference in mean body weights between control and TCE treated rats, which the authors reported to not be significantly different. Organ weights at the termination of the experiment were reported to only be different for the liver with a 1.21-fold of control value reported as a percentage of body weight with TCE treatment. The authors reported their increase in liver weights in male rats from subcutaneous exposure to TCE in olive oil (2.0 g/kg) to be consistent with the range of liver weight gain in rats reported by Kjellstrand et al. (1981b) for 150 ppm TCE inhalation exposure (see comments on that study above). The 5-day i.p. treatment with TCE was also reported to only produce increased liver weight but the data were not shown and the magnitude of the percentage increase was not given by the authors. No liver pathology results were studied or reported.

Along with an increase in liver weight, 15-week treatment with TCE was reported to cause a significant increase of microsomal protein/g liver of ~20% (10.64 ± 0.88 vs. 12.58 ± 0.71 mg/g liver for olive oil controls and TCE treatment, respectively). Microsomal CYP content was reported to show a mild increase that was not statistically significant of 1.08-fold (1.342 ± 0.205 vs. 1.456 ± 0.159 nmol/mg protein for olive oil controls and TCE treatment, respectively) of control. However, CYP content showed 1.28-fold of control value (14.28 ± 2.41 vs. 18.34 ± 2.31 nmol/g liver for olive oil controls and TCE treatment, respectively) in terms of g/liver. Chronic treatment of TCE was also reported to cause a significant increase in cytochrome b-5 level (~1.35-fold of control) and NADPH-cytochrome c reductase activity (~1.50-fold of control) in g/liver.

The 5-day TCE treatment via the i.p. route of administration was reported to cause a significant increase in microsomal protein (~20%) and induce CYP (~50% increase g/liver and

22% increase in microsomal protein), but to also increase cytochrome b-5 and NADPH-cytochrome c reductase activity by 50 and 70% in g/liver, respectively. Although weaker, 5-day i.p. treatment with TCE induced an enzyme pattern more similar to that of phenobarbital and ethanol rather methylcholanthrene (i.e., increased CYP but not microsomal protein and NADPH-cytochrome c reductase). Direct quantitative comparisons of vehicle effects and potential impact on response to TCE treatments for 15 weeks subcutaneous exposure and 5-day i.p. exposure could not be made as baseline levels of all enzyme and protein levels changed as a function of age.

Of note is that, in the discussion section of the paper, the authors disclosed that injection of TCE 2 or 3 g/kg i.p. for 5 days resulted in paralytic ileus from TCE exposure as unpublished observations. They noted that the rationale for injecting TCE subcutaneously was that it not only did not require an inhalation chamber, but also guarded against peritonitis that sometimes occurs following repeated i.p. injection. In terms of comparison with inhalation or oral results, the authors noted that the subcutaneous treatment paradigm will result in TCE not immediately being metabolized but retained in the fatty tissue and that after cessation of exposure, TCE metabolites continued to be excreted into the urine for >2 weeks.

E.2.2.12. NTP (1990)

E.2.2.12.1. 13-Week studies

The NTP conducted a 13-week study of 7-week-old F344/N rats (10 rats per group) that received doses of 125–2,000 mg/kg (males [0, 125, 250, 500, 1,000, or 2,000 mg/kg]) and 62.5 to 1,000 mg/kg (females [0, 62.5, 125, 250, 500, or 1,000 mg/kg] TCE via corn oil gavage 5 days/week (see Table E-1). For 7-week-old B6C3F$_1$mice (n = 10 per group), the dose levels were reported to be 375–6,000 mg/kg TCE (0, 375, 750, 1,500, 3,000, or 6,000 mg/kg). Animals were exposed via corn oil gavage to TCE that was epichlorhydrin-free.

Table E-1. Mice data for 13 weeks: mean body and liver weights

Dose (mg/kg TCE)	Survival	Body weight (mean in g)		Liver weight (mean final in g)	% liver weight/body weight (fold change vs. control)
		Initial	Final		
Male					
0	10/10	21	36	2.1	5.8
375	10/10	20	35	1.74	5.0 (0.86)
750	10/10	21	32	2.14	6.8 (1.17)
1,500	8/10	19	29	2.27	7.6 (1.31)
3,000	3/10	20	30	2.78	8.5 (1.46)
6,000	0/10	22	–	–	–
Female					
0	10/10	18	26	1.4	5.5
375	10/10	17	26	1.31	5.0 (0.91)
750	9/10	17	26	1.55	5.8 (1.05)

1,500	9/10	17	26	1.8	6.5 (1.18)
3,000	9/10	15	26	2.06	7.8 (1.42)
6,000	1/10	15	27	2.67	9.5 (1.73)

All rats were reported to survive the 13-week study, but males receiving 2,000 mg/kg exhibited a 24% difference in final body weight. However, there was great variation in initial weights between the dose groups with mean initial weights at the beginning of the study reported to be 87, 88, 92, 95, 101, and 83 g for the control, 125, 250, 500, 1,000, and 2,000 mg/kg dose groups in male rats, respectively. This represents a 22% difference between the highest and lowest initial weights between groups. Thus, changes in final body weight after TCE treatment also reflect differences in starting weights between the groups that, in the case of the 500 and 1,000 mg/kg groups, would result in a lower-than-expected change in weight due to TCE exposure.

For female rats, the mean initial starting weights were reported to be 81, 72, 74, 75, 73, and 76 g, respectively for the control, 62.5, 125, 250, 500, and 1,000 mg/kg dose groups. This represents a ~13% difference between initial weights. In the case of female rats, the larger mean initial weight in the control group would tend to exaggerate the effects of TCE exposure on final body weight. The authors did not report the variation in initial or final body weights within the dose groups. At the lowest doses for male and female rats, body mean weights were reported to be decreased by 6 and 7% in male and female rats, respectively. Organ weight changes were not reported for rats.

For male mice, mean initial body weights ranged from 19 to 22 g (~16% difference) and for female mice ranged between 18 and 15 g (20% difference), and thus, similar to rats, the final body weights in the groups dose with TCE reflect not only the effects of the compound but also differences in initial weights. For male mice, the mean final body weights were reported to be 3–17% less than controls for the 375–3,000 mg/kg doses. For female mice, the percent difference in final body weight was reported to be the same except for the 6,000 mg/kg dose group, but this lack of difference between controls and treated female mice reflected no change in mice that started at differing weights.

Male mice started to exhibit mortality at 1,500 mg/kg with 8/10 surviving the 1,500 mg/kg dose, 3/10 surviving the 3,000 mg/kg dose, and none surviving the 6,000 mg/kg dose of TCE until the end of the study. For females, 1 animal out of 10 died in the 750, 1,500, and 3,000 mg/kg dose groups and 1/10 survived the 6,000 mg/kg group.

In general, the magnitude of increase in TCE exposure concentration was similar to the magnitude of increase in percent liver/body weight for the 750 and 1,500 mg/kg TCE exposure groups in male B6C3F$_1$ mice and for the 750–3,000 mg/kg TCE exposure groups in female mice (i.e., a twofold increase in TCE exposure resulted in an approximate twofold increase in percent liver/body weight).

The descriptions of pathology in rats and mice given by this study were not very detailed. For rats, only control and high-dose rats were examined histologically. For mice, only controls and the two highest dose groups were examined histologically. Only mean liver weights were reported with no statistical analyses provided to ascertain quantitative differences between study groups.

Pathological results were reported to reveal that 6/10 males and 6/10 female rats had pulmonary vasculitis at the highest concentration of TCE. This change was also reported to have occurred in 1/10 control male and female rats. Most of those animals were also reported to have had mild interstitial pneumonitis. The authors report that viral titers were positive during this study for Sendai virus.

In mice, liver weights (both absolute and as a percent of body weight) were reported to increase with TCE-exposure level. Liver weights were reported to have increased by >10% relative to controls for males receiving ≥750 mg/kg and for females receiving ≥1,500 mg/kg. The most prominent hepatic lesions detected in the mice were reported to be centrilobular necrosis, observed in 6/10 males and 1/10 females administered 6,000 mg/kg.

> Although centrilobular necrosis was not seen in either males or females administered 3000 mg/kg, 2/10 males had multifocal areas of calcifications scattered throughout their livers. These areas of calcification were considered to be evidence of earlier hepatocellular necrosis. Multifocal calcification was also seen in the liver of a single female mouse that survived the 6000 mg/kg dosage regime. One female mouse administered 3000 mg/kg also had a hepatocellular adenoma, an extremely rare lesion in female mice of this age (20 weeks).

There appeared to be consistent decrease in liver weight at the lowest dose in both female and male mice after 13 weeks of TCE exposure. Liver weight was increased at exposure concentrations in which there was not increased mortality due to TCE exposure at 13 weeks of TCE exposure.

E.2.2.12.2. 2-Year Studies

In the 2-year phase of the NTP study, TCE was administered by corn oil gavage to groups of 50 male and 50 female F344/N rats, and B6C3F_1 mice. Dosage levels were 500 and 1,000 mg/kg for rats and 1,000 mg/kg for mice. TCE was administered 5 times/week for 103 weeks and surviving animals were killed between weeks 103 and 107. The same number of animals receiving corn oil gavage served as controls. The animals were 8 weeks old at the beginning of exposure. The focus of this study was to determine if there was a carcinogenic response due to TCE exposure so there was little reporting of non-neoplastic pathology or toxicity. There was no report of liver weight at termination of the study, only body weight.

The authors reported that there was no increase in necrosis in the liver from TCE exposure in comparison to control mice. In control male mice, the incidence of HCC (tumors with markedly abnormal cytology and architecture) was reported to be 8/48 in controls, and 31/50 in TCE-exposed male mice. For female control mice, HCCs were reported in 2/48 of controls and 13/49 of TCE-exposed female mice. Specifically, the authors described liver pathology in mice as follows:

> Microscopically the hepatocellular adenomas were circumscribed areas of distinctive hepatic parenchymal cells with a perimeter of normal appearing parenchyma in which there were areas that appeared to be undergoing compression from expansion of the tumor. Mitotic figures were sparse or absent but the tumors lacked typical lobular organization. The hepatocellular carcinomas had markedly abnormal cytology and architecture. Abnormalities in cytology included increased cell size, decreased cell size, cytoplasmic eosinophilia, cytoplasmic basophilia, cytoplasmic vacuolization, cytoplasmic hyaline bodies, and variations in nuclear appearance. In many instance, several or all of the abnormalities were present in different areas of the tumor. There were also variations in architecture with some of the hepatocellular carcinomas having areas of trabecular organization. Mitosis was variable in amount and location.

The authors reported that the non-neoplastic lesion in male mice differing from controls was focal necrosis in four vs. one animal in the dosed group (8 vs. 2%). There was no fatty metamorphosis in treated male mice vs. two animals in control. In female mice, there was focal inflammation in 29 vs. 19% of animals (dosed vs. control) and no other changes. Therefore, the reported pathological results of this study did not show that the liver was showing signs of toxicity after 2 years of TCE exposure except for neoplasia.

For hepatocellular adenomas, the incidence was reported to be "7/48 control vs. 14/50 dosed in males and 4/48 in control vs. 16/49 dosed female mice." The administration of TCE to mice was reported to cause increased incidences of HCCs in males (control, 8/48; dosed, 31/50: $p = 0.001$) and in females (control 2/48; dosed 13/49; $p < 0.005$). HCCs were reported to metastasize to the lungs in five dosed male mice and one control male mouse, while none were observed in females. The incidences of hepatocellular adenomas were reported to be increased in male mice (control 7/48; dosed 14/50) and in female mice (control 4/48; dosed 16/49; $p < 0.05$).

The survival of both low- and high-dose male rats and dosed male mice was reported to be less than that of vehicle controls with body weight decreases dose dependent. Female mice body weights were comparable to controls. The authors report adjusted rates of 20.6% for control vs. 53.1% for dosed males for adenoma, 22.1% control, and 92.9% for carcinoma in males, and liver carcinoma or adenoma adjusted rates of 100%. For female mice, the adjusted rates were reported to be 12.5% adenoma for control vs. 55.6% for dosed, and 6.2% control carcinoma vs. 43.9% dosed, with liver carcinoma or adenoma adjusted rates of 18.7% for control

vs. 69.7% for dosed. All of the liver results for male and female mice were reported to be statistically significant. The administration of TCE was reported to cause earlier expression of tumors as the first animals with carcinomas were 57 weeks for TCE-exposed animals and 75 weeks for control male mice.

In male rats, there was no reported treatment-related non-neoplastic liver lesions. In female rats, a decrease in basophilic cytological change was reported to be of note in TCE treated rats (~50% in controls but ~5% in TCE treatment groups). However, the authors reported that "the results in male F344/N rats were considered equivocal for detecting a carcinogenic response because both groups receiving TCE showed significantly reduced survival compared to vehicle controls (35/70, 70%; 20/50, 40%; 16/50, 32%) and because 20% of the animals in the high-dose group were killed accidently by gavage error." Specifically 1 male control, 3 low-dose males, 10 high-dose males, 2 female controls, 5 low-dose females, and 5 high-dose female rats were killed by gavage error.

E.2.2.13. NTP (1988)

The studies described in the NTP (1988) TCE report were conducted "to compare the sensitivities of four strains of rats to diisopropylamine-stabilized TCE." However, the authors concluded:

> that because of chemically induced toxicity, reduced survival, and incomplete documentation of experimental data, the studies are considered inadequate for either comparing or assessing TCE-induced carcinogenesis in these strains of rats. TCE (more than 99% pure, stabilized with 8ppm diisopropylamine) was administered via corn oil gavage at exposure concentrations of 0, 500 or 1000 mg/kg per day, 5 days per week, for 103 weeks to 50 male and female rats of each strain. The survival of "high-dose male Marshal rats was reduced by a large number of accidental deaths (25 animals were accidentally killed).

However, the report stated that survival was decreased at both exposure levels of TCE because of mortality that occurred during the administration of the chemical. The number of animals accidently killed were reported to be: 11 male ACI rats at 500 mg/kg, 18 male ACI rats at 1,000 mg/kg, 2 vehicle control female ACI rats, 14 female ACI rats at 500 mg/kg, 12 male ACI rats at 1,000 mg/kg, 6 vehicle control male August rats, 12 male August rats at 500 mg/kg, 11 male August rats at 1,000 mg/kg, 1 vehicle control female August rats, 6 female August rats at 500 mg/kg, 13 male August rats at 1,000 mg/kg, 2 vehicle control male Marshal rats, 12 male Marshal rats at 500 mg/kg, 25 male Marshal rats at 1,000 mg/kg, 3 vehicle control female Marshal rats, 14 female Marshal rats at 500 mg/kg, 18 female Marshal rats at 1,000 mg/kg, 1 vehicle control male Osborne-Mendel rat, 6 male Osborne-Mendel rats at 500 mg/kg, 7 male Osborne-Mendel rats at 1,000 mg/kg, 8 vehicle control female Osborne-Mendel rats, 6 female Osborne-Mendel rats at 500 mg/kg, and 6 female Osborne-Mendel rats at 1,000 mg/kg. The ages

of the rats "when placed on the study" were reported to differ and were for ACI rats (6.5 weeks), August rats (8 weeks), Marshal rats (7 weeks), and Osborne-Mendel rats (8 weeks). The ages of sacrifice also varied and were 17–18 weeks for the ACI and August rats and 110–111 weeks for the Marshal rats.

Results from a 13-week study were briefly mentioned in the report. For the 13-week duration of exposure, groups of 10 male ACI and August rats were administered 0,125, 250, 500, 1,000, or 2,000 mg/kg TCE in corn oil gavage. Groups of 10 female ACI and August rats were administered 0, 62.5, 125, 250, 500, or 1,000 mg/kg TCE. Groups of 10 male Marshal rats received 0, 268, 308, 495, 932, or 1,834 mg/kg and groups of female Marshal rats were given 0, 134, 153, 248, 466, or 918 mg/kg TCE. With the exception of three male August rats receiving 2,000 mg/kg TCE, all animals survived to the end of the 13-week experimental period. "The administration of the chemical for 13 weeks was not associated with histopathological changes."

In the 2-year study the report noted that there:

> was no evidence of liver toxicity described as non-neoplastic changes in male ACI rats due to TCE exposure with 4% or less incidence of any lesion in control or treated animals. For female ACI rats, the incidence of fatty metamorphosis was 6% in control vehicle, 9% in low dose TCE, and 13% in high dose TCE groups. There was also a 2%, 11%, and 8% incidence of clear cell change, respectively. A 6% incidence of hepatocytomegaly was reported in vehicle control and 15% incidence in the high dose group.

All other descriptors had reported incidences of <4%.

For August rats, there was also little evidence of liver toxicity. In male August rats, there was a reported incidence of 8, 4, and 10% focal necrosis in vehicle control, low dose, and high dose, respectively. Fatty metamorphosis was reported to be 8% in control, and 2 and 4% in low and high dose. All other descriptors were reported to be <4%. In female August rats, all descriptors of pathology were reported to have a <4% incidence except for hepatomegaly, which was 10% for vehicle control, 6% for the low dose, and 2% for high dose TCE.

For male Marshal rats, there was a reported 63% incidence of inflammation, NOS in vehicle control, 12% in low dose, and values not recorded at the high dose. There was a reported 6 and 14% incidence of fatty metamorphosis in control and low-dose male rats. Clear cell change was 8% in vehicle with all other values ≤4%. For female Marshal rats, all values were ≤4% except for fatty metamorphosis in 6% of vehicle controls.

For male Osborne-Mendel rats, there was a reported 4, 10, and 4% incidence of focal necrosis in vehicle control, low, and high dose, respectively. For "cytoplasmic change/NOS," there were reported incidences of 26, 32, and 27% in vehicle, low-dose, and high-dose animals, respectively. All other descriptors were reported to be ≤4%. In female Osborne-Mendel rats,

there was a reported incidence of 10% of focal necrosis at the low dose with all other descriptors reported at ≤4%.

Obviously, the negative results in this bioassay are confounded by the killing of a large portion of the animals accidently by experimental error. Still, these large exposure concentrations of TCE did not seem to be causing overt liver toxicity in the rat. Organ weights were not reported in this study, which would have been hard to interpret if they had been reported because of the mortality.

E.2.2.14. Fukuda et al. (1983)

In this 104-week bioassay designed primarily to determine a carcinogenic response, female noninbred Crj:CD-1 (ICR) mice and female Crj:CD (Sprague-Dawley) rats 7 weeks of age were exposed to "reagent grade" TCE at 0, 50, 150, and 450 ppm for 7 hours/day, 5 days/week. During the 2-year duration of the experiment, inhalation concentrations were reported to be within 2% of target values. The numbers of animals per group were reported to be 49–50 mice and 49–51 rats at the beginning of the experiment. The impurities in the TCE were reported to be 0.128% carbon tetrachloride benzene, 0.019% epichlorohydrin, and 0.019% 1,1,2-trichloroethane. After 107 weeks from commencement of the exposure, surviving animals were reported to be killed and completely necropsied. "Tumors and abnormal organs as well as other major organs were excised and prepared for examination in H&E sections." No other details of the methodologies used for pathological examination of tissues were given including what areas of the liver and number of sections examined by light microscopy.

Body weights were not given, but the authors reported that "body weight changes of the mice and rats were normal with a normal range of standard deviation." It was also reported that there were no significant differences in average body weight of animals at specified times during the experiments and no significant difference in mortality between the groups of mice. The report included a figure showing, that for the first 60 weeks of the experiment, there was a difference in cumulative mortality at the 450 ppm dose in ICR mice and the other groups. The authors reported that significantly increased mortalities in the control group of rats compared to the other dosed groups were observed at 85 weeks and after 100 weeks, reflecting many deaths during the 81–85-week and 96–100-week periods for control rats. No significant comparable clinical observations were reported to be noted in each group but that major symptoms such as bloody nasal discharge (in rats), local alopecia (in mice and rats), hunching appearance (in mice), and respiratory disorders (in mice and rats) were observed in some animals mostly after 1 year.

The authors reported that "the numbers of different types of tumors were counted and only malignant tumors were counted when both malignant and benign tumors were observed within one organ." They also reported that "all animals were included in the effective numbers except for a few that were killed accidently, severely autolyzed or cannibalized, and died before the first appearance of tumors among the groups."

In mice, the first tumors were observed at 286 days as thymic lymphoma and most of the malignant tumors appearing later were described as lymphomas or lymphatic leukemias. The incidences of mice with tumors were 37, 36, 54, and 52% in the control, 50, 150 and 450 ppm groups, respectively, by the end of the experiment. "Tumors of the ovary, uterus, subcutaneous tissue, stomach, and liver were observed in the dose groups at low incidences (2–7%) but not in the controls." For the liver, the control, 50 and 150 ppm groups were all reported to have no liver tumors with one animal (2%) having an adenoma at the 450 ppm dose.

For rats, the first tumor was reported to be observed at 410 days and the incidences of animals with tumors were 64, 78, 66, and 63% for control, 50, 150, and 450 ppm TCE, respectively, by the end of the experiment. Most tumors were distributed in the pituitary gland and mammary gland with other tumors reported at a low incidence of 2–4% with none in the controls. For the liver, there were no liver tumors in the control or 150 ppm groups, but one animal (2%) had a cystic cholangioma in 50 ppm group and one animal (2%) had a HCC in the 450 ppm group of rats. No details concerning the pathology of the liver or organ weight changes were given by the authors, including any incidences of hepatomegaly or preneoplastic foci. Of note is that there were no background liver tumors in either strain, indicative of the relative insensitivity of these strains to hepatocarcinogenicity. However, the carcinogenic potential of TCE was reflected by a number of other tumor sites in this paradigm.

E.2.2.15. Henschler et al. (1980)

This report focused on the potential carcinogenic response of TCE in mice (NMRI random bred), rats (WIST random bred), and hamsters (Syrian random bred) exposed to 0, 100, and 500 ppm TCE for 6 hours/day 5 days/week for 18 months. The TCE used in the experiment was reported to be pure with the exception of trace amounts of chlorinated hydrocarbons, epoxides, and triethanolamines (<0.000025% w/w) and stabilized with 0.0015% triethanolamine. The number of animals in each group was 30 and the ages and initial and final body weights of the animals were not provided in the report. For the period of exposure (8 am–2 pm), animals were deprived of food and water. The exposure period was for 18 months with mice and hamsters sacrificed after 30 months and rats after 36 months. "Deceased animals" were reported to be autopsied; spleen, liver, kidneys, lungs, and heart were weighed; and these organs, as well as stomach, CNS, and tumorous tissues, were examined in H&E sections.

Body weight gain was reported to be normal in all species with no noticeable differences between control and exposed groups but data were not shown. However, a "clearly dose-dependent decrease in the survival rate for both male and female mice" was reported to be statistically significant in both sexes and concentrations of TCE with no other significant differences reported in other species. The increase in mortality was more pronounced in male mice, especially after 50 weeks of exposure. Hence, the opportunity for tumor development was diminished due to decreased survival in TCE treated groups.

No organ weights were provided for the study due to the design, in which a considerable period of time occurred between the cessation of exposure and the sacrifice of the animals. Liver weights changes due to TCE may have been diminished with time.

For the 30 autopsied male mice in the control group, one hepatocellular adenoma and one HCC was reported. Whether they occurred in the same animal cannot be determined from the data presentation. In the 29 animals in the 100 ppm TCE exposure group, two hepatocellular adenomas and one mesenchymal liver tumor were reported but no HCCs also without a determination as whether they occurred in the same animal or not. In the 30 animals autopsied in the 500 ppm exposure group, no liver tumors were reported. In female mice, of the 29 animals autopsied in the control group, 30 animals autopsied in the 100 ppm group, and the 28 animals autopsied in the 500 ppm group, there were also no liver tumors reported.

In both the 100 and 500 ppm exposure groups, of male mice especially, low numbers of animals studied, abbreviated TCE exposure duration, and lower numbers of animals surviving to the end of the experiment limit the power of this study to determine a treatment-related difference in liver carcinogenicity. As discussed in Section E.2.3.2 below, the use of an abbreviated exposure regime or study duration and low numbers of animals examined limits the power of a study to detect a treatment-related response. The lack of any observed background liver tumors in the female mice and a very low background level of two tumors in the male mice are indicative of a low sensitivity to detect liver tumors in this paradigm, which may have occurred either through its design, or a low sensitivity of mouse strain used for this endpoint. However, the carcinogenic potential of TCE in mice was reflected by a number of other tumor sites in this paradigm.

For rats and hamsters the authors reported "no dose-related accumulation of any kind of tumor in either sex of these species." For male rats, there was only one hepatocellular adenoma reported at 100 ppm in the 30 animals autopsied and no carcinomas. For female rats, there were no liver tumors reported in control animals but, more significantly, at 100 ppm, there was one adenoma and one cholangiocarcinoma reported at 100 ppm, and at 500 ppm, there were two cholangioadenomas. Although not statistically significant, the occurrence of this relatively rare biliary tumor was observed in both TCE dose groups in female rats. The difference in survival, as reported in mice, did not affect the power to detect a response in rats, but the low numbers of animals studied, abbreviated exposure duration, and apparent low sensitivity to detect a hepatocarcinogenic response suggest a study of low power. Nevertheless, the occurrence of cholangioadenomas and one cholangiocarcinoma in female rats after TCE treatments is of concern, especially given the relationship in origin and proximity of the bile and liver cells and the low incidence of this tumor. For hamsters, the low background rate of tumors of any kind suggests that in this paradigm, the sensitivity for detection of this tumor is relatively low.

E.2.2.16. Maltoni et al. (1986)

The report by Maltoni et al. (1986) included a series of "systematic and integrated experiments (BT 301, 302, 303, 304, 304bis, 305, 306 bis) started in sequence, testing TCE by inhalation and by ingestion." The first experiment (BT 301) was begun in 1976 and the last in 1983, with this report representing the completed summary of the findings and results of project. The focus of the study was detection of a neoplastic response with only a generalized description of tumor pathology phenotype given and no reporting of liver weight changes induced by TCE exposure.

In experiment BT 301, TCE was administered in male and female Sprague-Dawley rats (13 weeks at start of experiment) via olive oil gavage at control, 50, or 250 mg/kg exposure levels for 52 weeks (4–5 days weekly). The animals (30 male, 30 female for each dose group) were examined during their lifetime. In experiment BT 302, male and female Sprague-Dawley rats (13 weeks old at start of the experiment) were exposed to TCE via inhalation at 0, 100, and 600 ppm, 7 hours/day, 5 days/week, for 8 weeks. The animals (90 animals in each control group, 60 animals in each 100 ppm group, and 72 animals in each 600 ppm group) were examined during their lifetime. In experiment BT 304, male and female Sprague-Dawley rats (12 weeks old at start of the experiment) were exposed TCE via inhalation at 0, 100, 300, and 600 ppm 7 hours/day, 5 days/week, for 104 weeks. The animals (95 male, 100 female rats control groups, 90 animals in each 100 ppm group, 90 animals in each 300 ppm group, and 90 animals in each 600 ppm group) were examined during their lifetime. In experiment BT304bis, male and female Sprague-Dawley rats (12 weeks old at start of the experiment) were exposed to TCE via inhalation at 0, 100, 300, and 600 ppm for 7 hours/day, 5 days/week, for 104 weeks. The animals (40 male, 40 female rats control groups, 40 animals in each 100 ppm group, 40 animals in each 300 ppm group, and 40 animals in each 600 ppm group) were examined during their lifetime.

In experiment BT 303, Swiss mice (11 weeks old at the start of the experiment) were exposed to TCE via inhalation in for 8 weeks using the same exposure concentrations as for experiment BT 302. The animals (100 animals in each control group, 60 animals in the 100 ppm exposed group, and 72 animals in each 600 ppm group) were examined during their lifetime. In experiment BT 305, Swiss mice (11 weeks old at the start of the experiment) were exposed to TCE via inhalation in for 78 weeks, 7 hours/day, 5 days/week. The animals (90 animals in each control group, 90 animals in the 100 ppm exposed group, 90 animals in the 300 ppm group, and 90 animals in each 600 ppm group) were examined during their lifetime. In experiment BT 306, $B6C3F_1$ mice (from NCI source) (12 weeks old at the start of the experiment) were exposed to TCE via inhalation in for 78 weeks, 7 hours a day, 5 days a week. The animals (90 animals in each control group, 90 animals in the 100-ppm-exposed group, 90 animals in the 300-ppm group, and 90 animals in each 600-ppm group) were examined during their lifetime. In experiment BT 306bis, $B6C3F_1$ mice (from Charles River Laboratory as source) (12 weeks old at the start of

the experiment) were exposed to TCE via inhalation for 78 weeks, 7 hours/day, 5 days/week. The animals (90 animals in each control group, 90 animals in the 100 ppm exposed group, 90 animals in the 300 ppm group, and 90 animals in each 600 ppm group) were examined during their lifetime.

In all experiments, TCE was supplied, tested, and reported by the authors of the study to be was highly purified and epoxide free with butyl-hydroxy-toluene at 20 ppm used as a stabilizer. Extra virgin olive oil was used as the carrier for ingestion experiments and was reported to be free of pesticides. The authors described the treatment of the animals and running of the facility in detail and reported that:

> Animal rooms were cleaned every day and room temperature varied from 19 degrees to 22 degrees and was checked 3 times daily. Bedding was changed every two days and cages changes and washed once weekly. The animals were handled very gently and, therefore, were neither aggressive nor nervous. Concentrations of TCE were checked by continuous gas-chromatographic monitoring. Treatment was performed by the same team. In particular, the same person carried out the gavage of the same animals. This is important, since animals become accustomed to the same operators. The inhalation chambers were maintained at 23 ± 2 degrees C and 50 ± 10% relative humidity. Ingestion from Monday to Friday was usually performed early in the morning. The status and behavior of the animals were examined at least three times daily and recorded. Every two weeks the animals were submitted to an examination for the detection of the gross changes, which were registered in the experimental records. The animals which were found moribund at the periodical daily inspection were isolated in order to avoid cannibalism. The animals were weight every two weeks during treatment and then every eight weeks. Animals were kept under observation until spontaneous death. A complete necropsy was performed. Histological specimens were fixed in 70% ethyl alcohol. A higher number of samples was taken when particular pathological lesions were seen. All slides were screened by a junior pathologist and then reviewed by a senior pathologist. The senior pathologist was the same throughout the entire project. Analysis of variance was used for statistical evaluation of body weights. Results are expressed as means and standard deviations. Survival time is evaluated using the Kruskal-Wallis test. For different survival rates between groups, the incidence of lesions is evaluated by using the Log rank test. Non-neoplastic, preneoplastic, and neoplastic lesions were evaluated using the Chi-square of Fisher's exact test. The effect of different doses was evaluated using the Cochran-Armitage test for linear trends in proportions and frequencies.

The authors stated that: "Although the BT project on TCE was started in 1976 and most of the experiments were performed from the beginning of 1979, the methodological protocol adopted substantially met the requirements of the Good Laboratory Practices Act." Finally, it was reported that "the experiments ran smoothly with no accidents in relation to the conduct of the experiment and the health of the animals, apart from an excess in mortality in the male

$B6C3F_1$ mice of the experiment BT 306, due to aggressiveness and fighting among the animals." This is in contrast to the description of the gavage studies conducted by NTP (1990, 1988) in which gavage error resulted in significant loss of experimental animals.

Questions have been raised about the findings, experimental conditions, and experimental paradigm of the European Ramazzini Foundation (ERF) from which the Maltoni et al. (1986) experiments were conducted (EFSA, 2006). However, these concerns were addressed by Caldwell et al. (2008a), who concluded that the ERF bioassay program produced credible results that were generally consistent with those of NTP

In regards to effects of TCE exposure on survival:

> a nonsignificant excess in mortality correlated to TCE treatment was observed only in female rats (treated by ingestion with the compound) and in male $B6C3F_1$ mice. In $B6C3F_1$ mice of the experiment BT 306 bis, the excess in mortality in treated animals was higher ($p < 0.05$ after 40 weeks) but was not dose correlated. No excess in mortality was observed in the other experiments.

The authors reported that "no definite effect of TCE on body weight was observed in any of the experiments, apart from experiment BT 306 bis, in which a slight nondose correlated decrease was found in exposed animals."

In mice, "hepatoma" was the term used by the authors of these studies to describe all malignant tumors of hepatic cells, of different subhistotypes, and of various degrees of malignancy. The authors reported that the hepatomas induced by exposure to TCE:

> may be unique or multiple, and have different sizes (usually detected grossly at necropsy). Under microscopic examination these tumors proved to be of the usual type observed in Swiss and $B6C3F_1$ mice, as well as in other mouse strains, either untreated or treated with hepatocarcinogens. They frequently have medullary (solid), trabecular, and pleomorphic (usually anaplastic) patterns. The hepatomas may produce distant metastases, more frequently in the lungs.

In regard to the induction of "hepatomas" by TCE exposure, the authors report that in Swiss mice exposed to TCE by inhalation for 8 weeks (BT303), the percentage of animals with hepatomas was 1.0% in male mice and 1.0% in female mice in the control group (n = 100 for each gender). For animals exposed to 100 ppm TCE, the percentage in female mice was 1.7% and male mice 5.0% (n = 60 for each gender). For animals exposed to 600 ppm TCE, the percentage in female mice was 0% and in male mice 5.5% (n = 72 for each gender).

The relatively larger number of animals used in this bioassay, in comparison to NTP standard assays, allows for a greater power to detect a response. It is also apparent from these results that Swiss mice in this experimental paradigm are a "less sensitive" strain in regard to spontaneous liver cancer induction over the lifetime of the animals. These results suggest that

8 weeks of TCE exposure via inhalation at 100 or 600 ppm may have been associated with a small increase in liver tumors in male mice in comparison to concurrent controls.

In Swiss mice exposed to TCE via inhalation for 78 weeks (BT 305), the percentage of animals with hepatomas was reported to be 4.4% in male mice and 0% in female mice in the control group (n = 90 for each gender). For animals exposed to 100 ppm TCE, the percentage in female mice was reported to be 0% and male mice 2.2% (n = 90 for each gender). For animals exposed to 300 ppm TCE, the percentage in female mice was reported to be 0% and in male mice 8.9% (n = 90 for each gender). For animals exposed to 600 ppm TCE, the percentage in female mice was reported to be 1.1% and in male mice 14.4%. As with experiment BT303, there is a consistency in the relatively low background level of hepatomas reported for Swiss mice in this paradigm. After 78 weeks of exposure, there appears to be a dose-related increase in hepatomas in male but not female Swiss mice via inhalation exposure.

In $B6C3F_1$ mice exposed to TCE by inhalation for 78 weeks (BT306), the percentage of animals with hepatomas was reported to be 1.1% in male mice and 3.3% in female mice in the control group (n = 90 for each gender). For animals exposed to 100 ppm TCE, the percentage in female mice was reported to be 4.4% and in male mice 1.1% (n = 90 for each gender). For animals exposed to 300 ppm TCE, the percentage in female mice was reported to be 3.3% and in male mice 4.4% (n = 90 for each gender). For animals exposed to 600 ppm TCE, the percentage in female mice was reported to be 10.0% and in male mice 6.7%. This was the experimental group with excess mortality in the male group due to fighting. The excess mortality could have affected the results. The authors reported that there was a difference in the percentage of males bearing benign and malignant tumors that was due to early mortality among males in experiment BT306. It is unexpected for the liver cancer incidence to be less in male mice than female mice and not consistent with the results reported for the Swiss mice.

In $B6C3F_1$ male mice exposed to TCE via inhalation (BT 306 bis), the percentage of animals with hepatomas was reported to be 18.9% in male mice in the control group (n = 90). For animals exposed to 100 ppm TCE, the percentage in male mice was reported to be 21.1% (n = 90). For animals exposed to 300 ppm TCE, the percentage in male mice was reported to be 30.0% (n = 90). For animals exposed to 600 ppm TCE, the percentage in male mice was reported to be 23.3%. This experiment did not examine female mice. The authors reported a decrease in survival in mice from this experiment that could have affected results. It is apparent from the BT 306 and BT 306 bis experiments that the background level of liver cancer was significantly different in male mice, although they were supposed to be of the same strain. The finding of differences in response in animals of the same strain but from differing sources has also been reported in other studies for other endpoints (see Section E.3.1.2).

The authors reported four liver angiosarcomas: one in an untreated male rat (BT 304); one in a male and one in a female rat exposed to 600 ppm TCE for 8 weeks (experiment BT302);

and one in a female rat exposed to 600 ppm TCE for 104 weeks (BT 304). The authors concluded that:

> the tumors observed in the treated animals cannot be considered to be correlated to TCE treatment, but are spontaneously arising. These findings are underlined because of the extreme rarity of this tumor in control Sprague-Dawley rats, untreated or treated with vehicle materials. The morphology of these tumors is of the liver angiosarcoma type produced by vinyl chloride in this strain of rats.

In rats treated for 104 weeks, TCE was reported to not affect the percentages of animals bearing benign and malignant tumor and of animals bearing malignant tumors. Moreover, it did not affect the number of total malignant tumors per 100 animals. This study did not report a treatment-related increase in liver cancer in rats. The report only explicitly described positive findings so it is assumed that there were no increases in "hepatomas" in rat liver associated with TCE treatment. The authors concluded that "under the tested experimental conditions, the evidence of TCE (without epoxide stabilizer) carcinogenicity, gives the result of TCE treatment-related hepatomas in male Swiss and $B6C3F_1$ mice. A borderline increased frequency of hepatomas was also seen after 8 weeks of exposure in male Swiss mice." Thus, the increase in liver tumors in both strains of mice exposed to TCE via inhalation reported in this study is consistent with the gavage results from the NTP (1990) study in $B6C3F_1$ mice, where male mice had a higher background level and greater response from TCE exposure than females.

E.2.2.17. Maltoni et al. (1988)

This report was an abbreviated description of an earlier study (Maltoni et al., 1986) focusing on the identification of a carcinogenic response in rats and mice by chronic TCE exposure.

E.2.2.18. Van Duuren et al. (1979)

This study exposed male and female noninbred HA:ICR Swiss mice at 6–8 weeks of age to distilled TCE with no further descriptions of purity. Gavage feeding of TCE was once weekly in 0.1 mL trioctanoin. Neither initial nor final body weights were reported by the authors. The authors reported that, at the termination of the experiments or at death, animals were completely autopsied with specimens of all abnormal-appearing tissues and organs excised for histopathologic diagnosis. Tissues from the stomachs, livers, and kidneys were reported to be taken routinely for the intragastric feeding experiments. Tissues were reported to be stained for H&E for pathologic examination, but no further description of the lobe(s) of the liver examined or the sections examined was provided by the authors.

Results were only reported for the no of mice with forestomach tumors exposed to 0.5 mg/mouse of TCE treatment given once a week in 0.1 mL trioctanoin. Mouse body weights

were not given, so the dose in mg/kg for the mice cannot be ascertained. The protocol used in this experiment kept the mg/mouse constant with a 1-week dosing schedule so that as the mice increased weight with age, the dose as a function of body weight was decreased. The days on test were reported to be 622 for 30 male and female mice.

Two male and one female mice were reported as having forestomach tumors. For 30 mice treated with trioctanoin alone, the number of forestomach tumors was reported to be zero. For mice with no TCE treatment, 5 of 100 male mice were reported to have forestomach tumors and of 8 of 60 female mice were reported to have forestomach tumors for 636 and 649 days on test. No results for liver were presented by the authors by the intragastric route of administration including background rates of the incidences of liver tumors or treatment results. The authors noted that except for repeated skin applications of certain chemicals, no significant difference between the incidence of distant tumors in treated animals compared with no treatment and vehicle control groups was noted. Given the uncertainties in regard to dose, the once-a-week dosing regime, the low number of animals tested with resulting low power, and the lack of reporting of experimental results, the ability to use the results from this experiment in regard to TCE carcinogenicity is very limited.

E.2.2.19. NCI (1976)

This bioassay was "initiated in 1972 according to the methods used and widely accepted at that time" with the design of carcinogenesis bioassays having "evolved since then in some respects and several improvements" having been developed. The most notable changes reported in the foreword of the report are changes "pertaining to preliminary toxicity studies, numbers of controls used, and extent of pathological examination." Industrial-grade TCE was tested (99% TCE, 0.19% 1,2,-epoxybutane, 0.04% ethyl acetate, 0.09% epichlorhydrin, 0.02% *N*-methyl pyrrole, and 0.03% diisobutylene) with rats and mice exposed via gavage in corn oil 5 times/week for 78 weeks using 50 animals per group at two doses with both sexes of Osborne-Mendel rats and B6C3F_1 mice. However, for control groups, only 20 of each sex and species were used. Rats were killed after 110 weeks and mice after 90 weeks. Rats and mice were initially 48 and 35 days of age, respectively, at the start of the experiment with control and treated animals born within 6 days of each other. Initial weight ranges were reported for treated and control animals to be 168–229 g for male rats, 130–170 g for female rats, 11–22 g for male mice, and 11–18 g for female mice. Animals were reported to be "randomly assigned to treatment groups so that initially the average weight in each group was approximately the same." Mice treated with TCE were reported to be:

> maintained in a room housing other mice being treated with one of the following 17 compounds: 1,1,2-2-tetrachloroethane, chloroform, 3-chloropropene, chloropicrin, 1,2-dibromochloropropane, 1,2, dibromoethane, ethylene dichloride, 1,1-diochloroethane, 3-sulfolene, idoform, methyl chloroform, 1,1,2-trichloro-

ethane, tetrachloroethylene, hexachloroethane, carbon disulfide, trichlorofluoromethane, and carbon tetrachloride. Nine groups of vehicle controls and 9 groups of untreated controls were also housed in this same room.

The authors noted that:

TCE-treated rats and their controls were maintained in a room housing other rats being treated with one of the following compounds: dibromochloropropane, ethylene dichloride, 1,1-dichloroethane, and carbon disulfide. Four groups of vehicle-treated controls were in the same room." Thus, there was the potential of co-exposure to a number of other chemicals, especially for the mice, resulting from exhalation in treated animals housed in the same room, including the control groups, as noted by the authors. The authors also noted that "samples of ambient air were not tested for presence of volatile materials" but state that "although the room arrangement is not desirable as is stated in the Guidelines for Carcinogen Bioassay in Small Rodents, there is no evidence the results would have been different with a single compound in a room.

The initial doses of TCE for rats were reported to be 1,300 and 650 mg/kg. However, these levels were changed based on survival and body weight data "so that the TWA doses were 549 and 1,097 mg/kg for both male and female rats." For mice, the initial doses were reported to be 1,000 and 2,000 mg/kg for males and 700 and 1,400 mg/kg for females. The "doses were increased so that the time weighted average doses were 1,169 and 2,339 mg/kg for male mice and 869 and 1,739 mg/kg for female mice."

The authors reported that signs of toxicity, including reduction in weight, were evident in treated rats, which, along with increased mortality, "necessitated a reduction in doses during the test." In contrast "very little evidence of toxicity was seen in mice, so doses were increased slightly during the study." Doses were "changed for the rats after 7 and 16 weeks of treatment, and for the mice after 12 weeks." At 7 weeks of age, male and female rats were dosed with 650 mg/kg TCE, at 14 weeks they were dosed with 750 mg/kg TCE, and at 23 weeks of age 500 mg/kg TCE. For the high exposure level, the exposure concentrations were 1,300, 1,500, and 1,000 mg/kg TCE, respectively, for the same changes in dosing concentration. For rats the percentage of TCE in corn oil remained constant at 60%. For female mice, the TCE exposure at the beginning of dosing was 700 mg/kg TCE (10% in corn oil) at 5 weeks of age for the "lower dose" level. The dose was increased to 900 mg/kg-day (18% in corn oil) at 17 weeks of age and maintained until 83 weeks of age. For male mice, the TCE exposure at the beginning of dosing was 1,000 mg/kg TCE (15% in corn oil) at 5 weeks of age for the "lower dose" level. At 11 weeks, the level of TCE remained the same but the percentage of TCE in corn oil was reduced to 10%. The dose was increased to 1,200 mg/kg-day at 17 weeks of age (24% in corn oil) and maintained until 83 weeks of age. For the "higher dose," the TCE exposure at the beginning of dosing was 1,400 mg/kg TCE (10% in corn oil) at 5 weeks of age in female mice.

At 11 weeks of age, the exposure level of TCE was kept the same but the percentage of TCE in corn oil increased to 20%. By 17 weeks of age, the exposure concentration of TCE in corn oil was increased to 1,800 mg/kg (18% in corn oil) in female mice. For the "higher dose" in male mice, the TCE exposure at the beginning of dosing was 2,000 mg/kg (15% in corn oil) which was maintained at 11 weeks in regard to TCE administered but the percent of TCE corn oil was increased to 20%. For male mice, the exposure concentration was increased to 2,400 mg/kg (24% in corn oil). For all of the mice, treatment continued on a 5 days/week schedule of gavage dosing throughout the timecourse of treatment (78 weeks of treatment). Thus, not only did the total dose administered to the animals change, but the volumes of vehicle in which TCE was administered changed throughout the experiment.

The authors stated that at 37 weeks of age, "To help assure survival until planned termination the dosing schedule was changed for rats to a cycle of 1 week of no treatment followed by 4 weeks of treatment." for male and female rats. Thus, the duration of exposure in rats was also changed. All lobes of the liver were reported to be taken including the free margin of each lobe with any nodule or mass represented in a block 10 × 5 × 3 mm cut from the liver and fixed in a marked capsule.

Body weights (mean ± SD) were reported to be 193 ± 15.0 g (n = 20), 193 ± 15.8 g (n = 50), and 195 ± 16.7 g (n = 50) for control, low-, and high-dose male rats at initiation of the experiment. By 1 year of exposure (50 weeks), 20/20 control male rats were still alive to be weighed, 42/50 of the low dose rats were alive and 34/50 of high dose rats were still alive. The body weights of those remaining were decreased by 6.2 and 17% in the low- and high-dose animals in comparison with the controls. For female rats, the mean body weights were reported to be 146 ± 11.4 g (n = 20), 144 ± 11.0 g (n = 50), and 144 ± 9.5 g (n = 50) for control, low-, and high-dose female rats at initiation of the experiment. By 1 year of exposure (50 weeks), 17/20 control female rats were still alive; 28/50 low-dose and 39/50 high-dose rats were alive. The body weights of those remaining were decreased by 25 and 30% in the low- and high-dose animals in comparison with the controls.

For male mice, the initial body weights were 17 ± 0.5 g (n = 20), 17 ± 2.0 g (n = 50), and 17 ± 1.1 g (n = 50) for control, low, and high doses. By 1 year of exposure (50 weeks), 18/20 control male mice were still alive; 47/50 or the low-dose and 34/50 high-dose mice were still alive. The body weights of those remaining were unchanged in comparison to controls. For female mice, the initial body weights were 14 ± 0.0 g (n = 20), 14 ± 0.6 g (n = 50), and 14 ± 0.7 g (n = 50) for control, low, and high doses. By 1 year of exposure (50 weeks), 18/20 control male mice were still alive; 45/50 of the low dose and 41/50 of the high-dose groups were still alive. The body weights of those remaining were unchanged in comparison to controls.

A high proportion of rats were reported to die during the experiment with 17/20 control, 42/50 low-dose, and 47/50 high-dose animals dying prior to scheduled termination. For female rats, 12/20 control, 35/48 low-dose, and 37/50 high-dose animals were reported to die before

scheduled termination with two low-dose females reported to be missing and not counted in the denominator for that group. The authors reported that earlier death was associated with higher TCE dose. A decrease in the percentage of tumor-bearing animals was reported to be lower in treated animals and attributed by the authors to be likely related to the decrease in their survival.

A high percentage of respiratory disease was reported to be observed among the rats without any apparent difference in the type, severity, or morbidity as to sex or group. The authors reported that "no significant toxic hepatic changes were observed" but no other details regarding results in the liver of rats were provided.

Carbon tetrachloride was administered to rats as a positive control. A low incidence of both HCC and neoplastic nodule was reported to be found in both colony controls (1/99 HCC and 0/99 neoplastic nodule in male rats and 0/98 HCC and 2/98 neoplastic nodules in female rats) and carbon-tetrachloride-treated rats. Hepatic adenomas were included in the description of neoplastic nodules in this study with the diagnosis of HCC to be "based on the presence of less organized architecture and more variability in the cells comprising the neoplasms."

The authors reported that "increased mortality in treated male mice appears to be related to the presence of liver tumors." For both male and female mice, the incidences of HCC were reported to be high from TCE treatment with 1/20 in age matched controls, 26/50 in low-dose, and 31/48 in high-dose males. Colony controls for male mice were reported to be 5/77 for vehicle and 5/70 for untreated mice. For female mice HCCs were reported to be observed in 0/20 age-matched controls, 4/50 low-dose, and 11/47 high-dose mice. Colony controls for female mice were reported to be 1/80 for vehicle and 2/75 for untreated mice. In male mice, HCCs were reported to be observed early in the study with the first seen at 27 weeks. HCCs were not observed so early in low-dose male or female mice.

The diagnosis of HCC was reported to be based on histologic appearance and the presence of metastasis especially to the lung with no other lesions significantly elevated in treated mice. The tumors were reported to be:

> varied from those composed of well differentiated hepatocytes in a relatively uniform trabecular arrangement to rather anaplastic lesions in which mitotic figures occurred in cells which varied greatly in size and tinctorial characteristics. Many of the tumors were characterized by the formation of relatively discrete areas of highly anaplastic cells within the tumor proper which were, in turn, surrounded by relatively well differentiated neoplastic cells. In general, various arrangements of the hepatocellular carcinoma occurred, as described in the literature, including those with an orderly cord-like arrangement of neoplastic cells, those with a pseudoglandular pattern resembling adenocarcinoma, and those composed of sheets of highly anaplastic cells with minimal cord or gland-like arrangement. Multiple metaplastic lesions were observed in the lung, including several neoplasms which were differentiated and relative benign in appearance."

The authors noted that almost all mice treated with carbon tetrachloride exhibited

liver tumors and that the "neoplasms occurring in treated [sic carbon tetrachloride treated] mice were similar in appearance to those noted in the TCE-treated mice.

Thus, phenotypically this study reported that the liver tumors induced in mice by TCE were heterogeneous and typical of those arising after carbon tetrachloride administration. The descriptions of liver tumors in this study and the tendency of metastasis to the lung are similar to the descriptions provided by Maltoni et al. (1986) for TCE-induced liver tumors in mice via inhalation.

In terms of noncancer pathology of the liver, one control male rat was reported to display fatty metamorphosis of the liver at 102 weeks. However, for the low dose, three male rats were reported to display fatty metamorphosis (90, 110, and 110 weeks), two rats to display cystic inflammation (76, 110 weeks), and one rat to display general inflammation (110 weeks). At the high dose, six rats were reported to display fatty metamorphosis (12, 35, 49, 52, 52, and 58 weeks), one rat to display cytomegaly (42 weeks), two rats to display centrilobular degeneration (53 and 58 weeks), one rat to display diffuse inflammation (62 weeks), 1 rat to display congestion (Week 12), and five rats to display angiectasis or abnormally enlarged blood vessels, which can be manifested by hyperproliferation of endothelial cells and dilatation of sinusoidal spaces (35, 42, 52, 54, and 65 weeks). One control female rat was reported o display fatty metamorphosis of the liver at 110 weeks, and one control female rats to display "inflammation" of the liver at 110 weeks. Of the TCE dosed female rats, only one high-dose female rat displayed fatty metamorphosis at week 96.

Thus, for male rats, there was liver pathology present in some rats due to TCE exposure examined from 12 weeks to a year at their time of their premature death. For mice, the liver pathology was dominated by the presence of HCC with additional hyperplasia noted in two mice of the high-dose male and female groups and one or less mouse exhibiting hyperplasia in the control or low-dose groups.

The authors noted that "while the absence of a similar effect in rats appears most likely attributable to a difference in sensitivity between the Osborne-Mendel rat and $B6C3F_1$ mouse, the early mortality of rats due to toxicity must also be considered." They concluded that "the test in rats is inconclusive: large numbers of rats died prior to planned termination; in addition, the response of this rat strain to the hepatocarcinogenicity of the positive control compound, carbon tetrachloride, appeared relatively low." Finally, the authors noted that "while the results obtained in the present bioassay could possibly have been influenced by an impurity in the TCE used, the extremely low amounts of impurities found make this improbable."

E.2.2.20. Herren-Freund et al. (1987)

This study gave results primarily in initiated male $B6C3F_1$ mice that were also exposed to TCE metabolites in drinking water for 61 weeks. However, in Table 1 of the report, results were

given for mice that received no initiator but were given 40 mg/L TCE or 2 g/L sodium chloride as control. The mice were reported to be 28 days of age when placed on drinking water containing TCE. The authors reported that concentrations of TCE fell by about half at the 40 mg/L dose of TCE during the twice a week change in drinking water solution. For control animals (n = 22), body weight at termination was reported to be 32.93 ± 0.54 g, liver weight 1.80 ± 0.05 g, and percent liver/body weight 5.47% ± 0.16%. For TCE treated animals (n = 32), body weight at termination was reported to be 35.23 ± 0.66 g, liver weight was 1.97 ± 0.10 g, and percent liver/body weight was 5.57% ± 0.24%. Thus, hepatomegaly was not reported for this paradigm at this time of exposure. The study reported that for 22 control animals the prevalence of adenomas was 2/22 animals (or 9%), with the mean number of adenomas per animal to be 0.09 ± 0.06 (SEM). The prevalence of carcinomas in the control group was reported to be 0/22. For 32 animals exposed to 40 mg/L TCE, the prevalence of adenomas was 3/32 animals (or 9%), with the mean number of adenomas per animal to be 0.19 ± 0.12 (SEM). The prevalence of animals with HCCs was 3/32 animals (or 9%) with the mean number of HCCs to be 0.10 ± 0.05 (SEM).

Thus, similar to the acute study of Tucker et al. (1982), significant loss of TCE is a limitation for trying to evaluate TCE hazard in drinking water. However, despite difficulties in establishing accurately the dose received, an increase in adenomas per animal and an increase in the number of animals with HCCs were reported to be associated with TCE exposure after 61 weeks of exposure. Also of note is that the increase in tumors was reported without significant increases in hepatomegaly at the end of exposure. The authors did not report these increases in tumors as being significant but did not do a statistical test between TCE exposed animals without initiation and control animals without initiation. The low numbers of animal tested limits the statistical power to make such a determination. However, for carcinomas, there was none reported in controls but 9% of TCE-treated mice had HCCs.

E.2.2.21. Anna et al. (1994)

This report focused on presenting incidence of cancer induction after exposure to TCE or its metabolites and included a description of results for male B6C3F$_1$ mice (8 weeks old at the beginning of treatment) receiving 800 mg/kg-day TCE via gavage in corn oil, 5 days/week for 76 weeks. There was very limited reporting of results other than tumor incidence. There was no reporting of liver weights at termination of the experiment. Although the methods section of the report gives 800 mg/kg-day as the exposure level, Table 1 in the results section reports that TCE was administered at 1,700 mg/kg-day. This could be a typographical error in the table as a transposition with the dose of "perc" administered to other animals in the same study. The methods section of the report states that the authors based their dose in mice that used in the 1990 (NTP) study. The NTP study only used a 1,000 mg/kg-day in mice, suggesting that the table is mislabeled and that the actual dose is 800 mg/kg-day in the Anna et al. (1994) study.

All treated mice were reported to be alive after 76 weeks of treatment. For control animals, 10 animals exposed to corn oil and 10 untreated controls were killed in a 9-day period. The remaining controls were killed at 96, 103, and 134 weeks of treatment. Therefore, the control group (all) contains a heterogeneous group of animals that were sacrificed from 76 to 134 weeks and were not comparable to the animals sacrificed at 76 weeks.

At 76 weeks, 3 of 10 the untreated and two of the 10 corn oil treated controls were reported to have one small hepatocellular adenoma. None of the controls examined at 76 weeks were reported to have any observed HCCs. The authors reported no cytotoxicity for TCE, corn oil, and untreated control group. At 76 weeks, 75 mice treated with 800 mg/kg-day TCE were reported to have a prevalence of 50/75 animals having adenomas with the mean number of adenomas per animal to be 1.27 ± 0.14 (SEM). The prevalence of carcinomas in these same animals was reported to be 30/70 with the mean number of HCCs per animal to be 0.57 ± 0.10 (SEM).

Although not comparable in terms of time until tumor observation, corn oil control animals examined at much later time points did not have as great a tumor response as did those exposed to TCE. At 76–134 weeks, 32 mice treated with corn oil were reported to have a prevalence of 4/32 animals having adenomas with the mean number of adenomas per animal to be 0.13 ± 0.06 (SEM). The prevalence of carcinomas in these same animals was reported to be 4/32 with the mean number of HCCs per animal to be 0.12 ± 0.06 (SEM). Despite only examining one exposure level of TCE and the limited reporting of findings other than incidence data, this study also reported that TCE exposure in male $B6C3F_1$ mice to be associated with increased induction of adenomas and HCC, without concurrent cytotoxicity.

In terms of liver tumor phenotype, Anna et al. (1994) reported the percent of H-ras codon 61 mutations in tumors from concurrent control animals (water and corn oil treatment groups combined) examined in their study, historical controls in $B6C3F_1$ mice, and in tumors from TCE or DCA (0.5% in drinking water) treated animals. From their concurrent controls, they reported H-ras codon 61 mutations in 17% (n = 6) of adenomas and 100% (n = 5) of carcinomas. For historical controls (published and unpublished), they reported mutations in 73% (n = 33) of adenomas and in 70% (n = 30) of carcinomas. For tumors from TCE-treated animals, they reported mutations in 35% (n = 40) of adenomas and 69% (n = 36) of carcinomas, while for DCA-treated animals, they reported mutations in 54% (n = 24) of adenomas and in 68% (n = 40) of carcinomas. The authors reported that "in this study, the H-ras codon 61 mutation frequency was not statistically different in liver tumors from DCA and TCE-treated mice and combined controls (62, 51 and 69%, respectively)." In regard to mutation spectra in H-ras oncogenes detected $B6C3F_1$ mouse liver "tumors," the authors reported combined results for concurrent and historical controls of 58% AAA, 27% CGA, and 14% CTA substitutions for CAA at codon 61 out of 58 mutations. For TCE "tumors" the substitution pattern was reported to be 29% AAA,

24% CGA, and 40% CTA substitutions for CAA at codon 61 out of 39 mutations and for DCA 28% AAA, 35% CGA, and 38% CTA substitutions for CAA at codon 61 out of 40 mutations.

E.2.2.22. Bull et al. (2002)

This study primarily presented results from exposures to TCE, DCA, TCA, and combinations of DCA and TCA after 52 weeks of exposure with some animals examined at 87 weeks. It only examined and described results for liver. In a third experiment, 1,000 mg/kg TCE was administered once daily 7 days/week for 79 weeks in 5% alkamuls in distilled water to 40 $B6C3F_1$ male mice (6 weeks old at the beginning of the experiment). At the time of euthanasia, the livers were removed, tumors were identified, and the tissues section was examined by a pathologist and immunostaining. Liver weights were not reported. For the TCE gavage experiment, there were 6 gavage-associated deaths during the course of this experiment among a total of 10 animals that died with TCE treatment. No animals were lost in the control group.

The limitations of this experiment were discussed in Caldwell et al. (2008b). Specifically, for the DCA- and TCA-exposed animals, the experiment was limited by low statistical power, a relatively short duration of exposure, and uncertainty in reports of lesion prevalence and multiplicity due to inappropriate lesions grouping (i.e., grouping of hyperplastic nodules, adenomas, and carcinomas together as "tumors"), and incomplete histopathology determinations (i.e., random selection of gross lesions for histopathology examination).

For the TCE results, Bull et al. (2002) reported a high prevalence (23/36 $B6C3F_1$ male mice) of adenomas and HCC (7/36) and gave results of an examination of approximately half of the lesions induced by TCE exposure. Tumor incidence data were provided for only 15 control mice and reported as 2/15 (13%) having adenomas and 1/15 (7%) carcinomas. Thus, this study presents results that are consistent with other studies of chronic exposure that show TCE induction of HCC in male $B6C3F_1$ mice.

For determinations of immunoreactivity to c-Jun as a marker of differences in "tumor" phenotype, Bull et al. (2002) did include all lesions in most of their treatment groups, decreasing the uncertainty of his findings. The exceptions were the absence of control lesions and inclusion of only 16/27 and 38/72 lesions for 0.5 g/L DCA + 0.05 g/L TCA and 1 g/kg-day TCE exposure groups, respectively. Immunoreactivity results were reported for the group of hyperplastic nodules, adenomas, and carcinomas. Thus, changes in c-Jun expression between the differing types of lesions were not determined.

Bull et al. (2002) reported lesion reactivity to c-Jun antibody to be dependent on the proportion of the DCA and TCA administered after 52 weeks of exposure. Given alone, DCA produced lesions in mouse liver for which approximately half displayed a diffuse immunoreactivity to a c-Jun antibody, half did not, and none exhibited a mixture of the two. After TCA exposure alone, no lesions were reported to be stained with this antibody. When

given in various combinations, DCA and TCA co-exposure induced a few lesions that were only c-Jun+, many that were only c-Jun–, and a number with a mixed phenotype whose frequency increased with the dose of DCA. For TCE exposure of 79 weeks, TCE-induced lesions also had a mixture of phenotypes (42% c-Jun+, 34% c-Jun–, and 24% mixed) and were most consistent with those resulting from DCA and TCA co-exposure but not either metabolite alone.

Mutation frequency spectra for the H-ras codon 61 in mouse liver "tumors" induced by TCE (n = 37 tumors examined) were reported to be significantly different than that for TCA (n = 41 tumors examined), with DCA-treated mice tumors giving an intermediate result (n = 64 tumors examined). In this experiment, TCA-induced "tumors" were reported to have more mutations in codon 61(44%) than those from TCE (21%) and DCA (33%). This frequency of mutation in the H-ras codon 61 for TCA is the opposite pattern as that observed for a number of peroxisome proliferators in which the mutation spectra in tumors has been reported to be much lower than spontaneously arising tumors (see Section E.3.4.1.5).

Bull et al. (2002) noted that the mutation frequency for all TCE-, TCA-, or DCA-induced tumors was lower in this experiment than for spontaneous tumors reported in other studies (they had too few spontaneous tumors to analyze in this study), but that this study utilized lower doses and was of shorter duration than that of Ferreira-Gonzalez et al. (1995). These are additional concerns along with the effects of inappropriate lesion grouping, in which a lower stage of progression is grouped with more advanced stages. In a limited subset of tumor that were both sequenced and characterized histologically, only 8 of 34 (24%) TCE-induced adenomas but 9/15 (60%) of TCE-induced carcinomas had mutated H-ras at codon 61, which the authors suggest is evidence that this mutation is a late event.

The issues involving identification of mode of action through tumor phenotype analysis are discussed in detail below for the more general case of liver cancer as well as for specific hypothesized modes of action (see Sections E.3.1.4, E.3.1.8, E.3.2.1, and E.3.4.1.5). In an earlier paper, Bull (2000) suggested that "the report by Anna et al. (1994) indicated that TCE-induced tumors possessed a different mutation spectra in codon 61 of the H-ras oncogene than those observed in spontaneous tumors of control mice." Bull (2000) stated that "results of this type have been interpreted as suggesting that a chemical is acting by a mutagenic mechanism" but went on to suggest that it is not possible to a priori rule out a role for selection in this process and that differences in mutation frequency and spectra in this gene provide some insight into the relative contribution of different metabolites to TCE-induced liver tumors. Bull (2000) noted that data from Anna et al. (1994), Ferreira-Gonzalez et al. (1995), and Maronpot et al. (1995a) indicated that mutation frequency in DCA-induced tumors did not differ significantly from that observed in spontaneous tumors, that the mutation spectra found in DCA-induced tumors has a striking similarity to that observed in TCE-induced tumors, and that DCA-induced tumors were significantly different than that of TCA-induced liver tumors.

What is clear from these observations is that the phenotype of TCE-induced tumors appears to be more like DCA-induced tumors (which are consistent with spontaneous tumors), or those resulting from a co-exposure to both DCA and TCA, than from those induced by TCA. More importantly, these data suggest that using measures other than dysplasticity and tincture indicate that mouse liver tumors induced by TCE are heterogeneous in phenotype. The descriptions of tumors in mice reported by the NTP (1990) and Maltoni et al. (1986) studies are also consistent with phenotypic heterogeneity as well as consistency with spontaneous tumor morphology.

E.2.3. Mode of Action: Relative Contribution of TCE Metabolites

Several metabolites of TCE have also been shown to induce liver cancer in rodents with DCA and TCA having been the focus of study as potential active agent(s) of TCE liver toxicity and/or carcinogenesis and both able to induce peroxisome proliferation (Caldwell and Keshava, 2006). A variety of DCA effects from exposure have been noted that are consistent with conditions that increase risk of liver cancer (e.g., effects on the cytosolic enzyme GST-zeta, diabetes, and glycogen storage disease), with the pathological changes induced by DCA on whole liver consistent with changes observed in preneoplastic foci from a variety of agents (Caldwell and Keshava, 2006). CH is one of the first metabolites from oxidative metabolism of TCE with a large fraction of TCE metabolism appearing to go through CH and then subsequent metabolism to TCA and TCOH (Chiu et al., 2006b). Similarities in toxicity may indicate that common downstream metabolites may be toxicologically important, and differences may indicate the importance of other metabolic pathways.

Although both induce liver tumors, DCA and TCA have distinctly different actions (Caldwell and Keshava, 2006) and apparently differ in induced tumor phenotype (see discussions above in Section E.2.2. and many studies have been conducted to try to elucidate the nature of those differences (Caldwell et al., 2008b). Limitations of all of the available chronic studies of TCA and most of the studies of DCA include less-than-lifetime exposures, varying and small numbers of animals examined, and few exposure concentrations that were relatively high.

E.2.3.1. Acute studies of DCA/TCA

The studies in this section focus on studies of DCA and TCA that examine, to the extent possible, similar endpoints using similar experimental designs as those of TCE examined above and that give insight into proposed modes of action for all three. Of note for any experiment involving TCA is whether exposure solutions were neutralized. Unbuffered TCA is commonly used as a reagent to precipitate proteins so that any result from studies using unbuffered TCA could potentially be confounded by the effects on pH.

E.2.3.1.1. Sanchez and Bull (1990)

In this report TCA and DCA were administered to male B6C3F$_1$ mice (9 weeks of age) and male and female Swiss-Webster mice (9 weeks of age) for up to 14 days. At 2, 4, or 14 days, mice were injected with tritiated thymidine. Experiments were replicated at least once but results were pooled so that variation between experiments could not be determined. B6C3F$_1$ male mice were given DCA or TCA at 0, 0.3, 1.0, or 2.0 g/L in drinking water (n = 4 for each group for 2 and 5 days, but n = 15 for control and n = 12 for treatment groups at day 14). Swiss-Webster mice (n = 4) at were exposed to DCA only on day 14 at 0, 1.0, or 2.0 g/L. Mice were injected with tritiated thymidine 2 hours prior to sacrifice. The pH of the drinking water was adjusted to 6.8–7.2 with sodium hydroxide. Concentrations of TCA and DCA were reported to be stable for a minimum of 3 weeks.

Hepatocyte diameters were reported to be determined by randomly selecting five different high power fields (400×) in five different sections per animals (total of 25 fields/animal with "cells in and around areas of necrosis, close to the edges of the section, or displaying mitotic figures were not included in the cell diameter measurements." PAS staining was reported to be done for glycogen and lipofuscin determined by autofluorescence. Tritiated thymidine was reported to be given to the animals 2 hours prior to sacrifice. In two of three replications of the 14-day experiment, a portion of the liver was reported to be set aside for DNA extraction with the remaining group examined autoradiographically for tritiated thymidine incorporation into individual hepatocytes. Autoradiographs were also reported to be examined in the highest dose of either DCA or TCA for the 2- and 5-day treatment groups. Autoradiographs were reported to be analyzed in randomly selected fields (5 sections per animal in 10 different fields) for a total of 50 fields/animal and reported as percentage of cells in the fields that were labeled. There was no indication by the authors that they characterized differing zones of the liver for preferential labeling. DNA thymidine incorporation results were not examined in the same animals as those for individual hepatocyte incorporation and also not examined at 2- or 5-day time periods. The only analyses reported for the Swiss-Webster mice were of hepatic weight change and histopathology. Variations in results were reported as SE of the mean.

Liver weights were reported but not body weights, so the relationship of liver/body weight ratio could not be determined for the B6C3F$_1$ mice. For liver weight, the numbers of animals examined varied greatly between and within treatment groups. The number of control animals examined were reported to be n = 4 on day 2, n = 8 on day 5, and n = 15 on day 14. There was also a large variation between control groups in regard to liver weight. Control liver weights for day 2 were reported to be 1.3 ± 0.1, day 5 to be 1.5 ± 0.05, and for day 14 to be 1.3 ± 0.04 g. Liver weights in Day 5 control animals were much greater than those for day 2 and day 14 animals and thus, the means varied by as much as 15%.

For DCA, there was no reported change in liver weights compared to controls values at any exposure level of DCA after 2 days of exposure. After 5 days of exposure, there was no

difference in liver weight between controls and 0.3 g/L exposed animals. However, the animals exposed at 1.0 or 2.0 g/L DCA had identical increases in liver weight of 1.7 ± 0.13 and 1.7 ± 0.8 g, respectively. Due to the low power of the experiment, only the 2.0 g/L DCA result was identified by the authors as significantly different from the control value. For TCA, there was a slight decrease reported between control values and the 0.3 g/L treatment group (1.2 ± 0.1 g vs. 1.3 ± 0.1 g), but the 1.0 and 2.0 g/L treatment groups had similar slight increases over control (for 1.0 g/L liver weight was 1.5 ± 0.1 and for 2.0 g/L liver weight was 1.4 ± 0.1 g). The same pattern was apparent for the 5-day treatment groups for TCA as for the 2-day treatment groups.

For 14-day exposure periods, the number of animals studied was increased to12 for the TCA and DCA treatment groups. After 14 days of DCA treatment, there was a reported dose-related increase in liver weight that was statistically significant at the two highest doses (i.e., at 0.3 g/L DCA liver weight was 1.4 ± 0.04, at 1.0 g/L DCA liver weight was 1.7 ± 0.07 g, and at 2.0 g/L DCA liver weight was 2.1 ± 0.08 g). This was 1.08-, 1.31-, and 1.62-fold of controls, respectively. After 14 days of TCA exposure, there was a dose-related increase in liver weight that the authors reported to be statistically significant at all exposure levels (i.e., at 0.3 g/L liver weight was 1.5 ± 0.06, at 1.0 g/L liver weight was 1.6 ± 0.07 g, and at 2.0 g/L liver weight was 1.8 ± 0.10 g). This represents 1.15-, 1.23-, and 1.38-fold of control.

The authors note that at 14 days, that DCA-associated increases in hepatic liver weight were greater than that of TCA. What is apparent from these data are that while the magnitude of difference between the exposures was ~6.7-fold between the lowest and highest dose, the differences between TCA exposure groups for change in liver weight was ~2.5. For DCA, the slope of the dose-response curve for liver weight increases appeared to be closer to the magnitude of difference in exposure concentrations between the groups (i.e., a difference of 7.7-fold between the highest and lowest dose for liver weight induction). Given that the control animal weights varied as much as 15%, the small number of animals examined, and that body weights were also not reported, there are limitations for making quantitative comparisons between TCA and DCA treatments. However, after 14 days of treatment, it is apparent that there was a dose-related increase in liver weight after either DCA or TCA exposure at these exposure levels. For male and female Swiss-Webster mice, 1 and 2 g/L DCA treatment (n = 4) was reported to also induce an increase in percent liver/body weight that was similar to the magnitude of exposure difference (see below).

Grossly, livers of $B6C3F_1$ mice treated with DCA for 1 or 2 g/L were reported to have "pale streaks running on the surface" and occasionally, discrete, white, round areas were also observed on the surface of these livers. Such areas were not observed in TCA-treated or control $B6C3F_1$ mice. Pale streaks on the surface of the liver were not observed in Swiss-Webster mice. Again there was no significant effect on total body or renal weights (data not shown).

Swiss-Webster mice were reported to have dose-related increases in hepatic weight and hepatic/body weight ratios were observed. DCA-associated increases in relative hepatic weights

in both sexes were comparable to those in $B6C3F_1$ mice. The authors report liver weights for the Swiss-Webster male mice (n = 4 for each group) to be 2.1 ± 0.1 g for controls, 2.1 ± 0.1 g for 1.0 g/L DCA, and 2.4 ± 0.2 g for 2.0 g/L DCA 14-day treatment groups. The percent liver/body weights for these same groups were reported to be 6.4 ± 0.4, 6.9 ± 0.2, and 8.1 ± 0.3%, respectively. For female Swiss-Webster mice (n = 4 for each group), the liver weights were reported to be 1.1 ± 0.1 g for controls, 1.5 ± 0.1 g for 1.0 g/L DCA, and 1.7 ± 0.2 g for 2.0 g/L DCA 14-day treatment groups. The percent liver/body weights for these same groups of Swiss mice were reported to be 4.8 ± 0.2, 6.0 ± 0.2, and 6.8 ± 0.4%, respectively.

Thus, while there was no significant difference in "liver weight" between the control and the 1.0 g/L DCA treatment group for male or female Swiss-Webster mice, there was a statistically significant difference in liver/body weight ratio reported by the authors. These data illustrate the importance of reporting both measures and the limitations of using small numbers of animals (n = 4 for the Swiss Webster vs. n = 12–14 for $B6C3F_1$ 14-day experiments).

Relative liver weights were reported by the authors for male $B6C3F_1$ mice only for the 14-day groups, as a function of calculated mean water consumption, as pooled data from the three experiments, and as a figure that was not comparable to the data reported for Swiss-Webster mice. The liver weight data indicate that male mice of the same age appeared to differ in liver weight between the two strains without treatment (i.e., male $B6C3F_1$ mice had control liver weights at 14 days of 1.3 ± 0.04 g for 15 mice, while Swiss-Webster mice had control values of 2.1 ± 0.1 for 4 mice). While the authors report that results were "comparable" between the $B6C3F_1$ mice in regard to DCA-induced changes in liver weight, the increase in percent liver/body weight ratios were 1.27-fold of control for Swiss-Webster male mice (n = 4) and 1.42-fold of control for females while the increase in liver weight for $B6C3F_1$ male mice (n = 12–14) was 1.62-fold of controls after 14 days of exposure to 2 g/L DCA.

The concentration of DNA in the liver was reported as mg hepatic DNA/g of liver. This measurement can be associated with hepatocellular hypertrophy when decreased, or increased cellularity (of any cell type), increased DNA synthesis, and/or increased hepatocellular ploidy in the liver when increased. The number of animals examined for this parameter varied. For control animals, there were four animals reported to be examined at 2 days, eight animals examined at 5 days, and at 14 days eight animals were examined.

The mean DNA content in control livers were not reported to vary greatly, however, and the variation between animals was relatively low in the 5- and 14-day control groups (i.e., 1.67 ± 0.27, 1.70 ± 0.05, and 1.69 mg DNA/g, for 2-, 5-, or 14-day control animals, respectively). For treatment groups, the number of animals reported to be examined appeared to be the same as the control animals.

For DCA treatment, there did not appear to be a dose-response in hepatic DNA content with the 1 g/L exposure level having the same reported value as control but the 0.3 and 2.0 g/L values reported to be lower (mean values of 1.49 and 1.32 mg DNA/g, respectively). After

5 days of exposure, all treatment groups were reported to have a lower DNA content that the control value (i.e., 1.44 ± 0.06, 1.47 ± , and 1.30 ± 0.14 mg DNA/g, for 0.3, 1.0, and 2.0 g/L exposure levels of DCA, respectively). After 14 days of exposure, there was a reported increase in hepatic DNA at the 0.3 g/L exposure level, but significant decreases at the 1.0 and 2.0 g/L exposure levels (i.e., 1.94 ± 0.20, 1.44 ± 0.14, and 1.19 ± 0.16 mg DNA/g for the 0.3, 1.0, and 2.0 g/L exposure levels of DCA, respectively).

Changes in DNA concentration in the liver were not correlated with the pattern of liver weight increases after DCA treatment. For example, while there was a clear dose-related increase in liver weight after 14 days of DCA treatment, the 0.3 g/L DCA exposed group was reported to have a higher rather than lower level of hepatic DNA than controls. After 2 or 5 days of DCA treatment, liver weights were reported to be the same between the 1.0 and 2.0 g/L treatment groups but hepatic DNA was reported to be decreased.

For TCA, there appeared to be a dose-related decrease in reported hepatic DNA after 2 days of treatment (i.e., 1.63 ± 0.07, 1.53 ± 0.08, and 1.43 ± 0.04 mg DNA/g for the 0.3, 1.0, and 2.0 g/L exposure levels of TCA, respectively). After 5 days of TCA exposure, there was a reported decrease in hepatic DNA for all treatment groups that was similar at the 1.0 and 2.0 g/L exposure groups (i.e., 1.45 ± 0.17, 1.29 ± 0.18, and 1.26 ± 0.22 mg DNA/g for the 0.3, 1.0, and 2.0 g/L exposure levels of TCA, respectively). After 14 days of TCA treatment, there was a reported decrease in all treatment groups in hepatic DNA content that did not appear to be dose-related (i.e., 1.31 ± 0.17, 1.21 ± 0.17, and 1.33 ± 0.18 mg DNA/g for the 0.3, 1.0, and 2.0 g/L exposure levels of TCA, respectively).

Thus, similar to the results reported for DCA, the patterns of liver weight gain did not match those of hepatic DNA decrease for TCA-treated animals. For example, although there appeared to be a dose-related increase in liver weight gain after 14 days of TCA exposure, there was a treatment- but not dose-related decrease in hepatic DNA content.

In regard to the ability to detect changes, the low number of animals examined after 2 days of exposure (n = 4) limited the ability to detect a significant change in liver weight and hepatic DNA concentration. For hepatic DNA determinations, the larger number of animals examined at 5- and 14-day time points and the similarity of values with relatively smaller SE of the mean reported in the control animals made quantitative differences in this parameter easier to determine. However, animals varied in their response to treatment and this variability exceeded that of the control groups. For DCA, results reported at 14 days and those for TCA reported at 5 and 14 days, the SEs for treated animals showed a much greater variability than those of the control animals (range of 0.04–0.05 mg DNA/g for control groups, but ranges of 0.17–0.22 mg DNA/g for TCA at 5 days and 0.14–0.20 mg DNA/g for DCA or TCA at 14 days). The authors stated that:

> the increases in hepatic weights were generally accompanied by decreases in the concentration of DNA. However, the only clear changes were in animals treated with DCA for 5 or 14 days where the ANOVAs were clearly significant (P<0.020 and 0.005, respectively). While changes of similar magnitude were observed in other groups, the much greater variation observed in the treated groups resulted in not significant differences by ANOVA (p = 0.41, 0.66. 0.26, 0.15 for DCA – 2 days, and TCA for 2,5, and 14 days, respectively).

The size of hepatocytes is heterogeneous and correlated with its ploidy, zone, and age of the animal (see Section E.1.1). The authors did not indicate if there was predominance in zone or ploidy for hepatocytes included in their analysis of average hepatocyte diameter in the random selection of 25 fields per animal (n = 3–7 animals). There appeared to be a dose-related increase in cell diameter associated with DCA exposure and a treatment but not dose-related increase with TCA treatment after 14 days of treatment. For control B6C3F_1 male mice (n = 7), the hepatocyte diameter was reported to be 20.6 ± 0.4 microns. For mice exposed to DCA, hepatocyte diameter was reported to be 22.2 ± 0.2, 25.2 ± 0.6, and 26.0 ± 1.0 microns for 0.3, 1.0, and 2.0 g/L treated mice (n = 4 for each group), respectively. For mice exposed to TCA hepatocyte diameter was reported to be 22.2 ± 0.2, 22.4 ± 0.6, and 23.2 ± 0.4 microns for 0.3, 1.0, and 2.0 g/L treated mice (n = 4 for the 0.3 and 1.0 g/L groups and n = 3 for the 2.0 g/L group), respectively.

The small number of animals examined limited the power of the experiment to determine statistically significant differences with the authors reporting that only the 1.0 g/L DCA and 2.0 g/L DCA- and TCA-treated groups statistically significant from control values. The dose-related increases in reported cell diameter were consistent with the dose-related increases in liver weight reported for DCA after 14 days of exposure. However, the pattern for hepatic DNA content did not. For TCA, the dose-related increases in cell diameter were also consistent with the dose-related increases in liver weight after 14 days of exposure. Similar to DCA results, the changes in hepatic DNA content did not correlate with changes in cell size. In regard to the magnitude of increases over control values, the 68 vs. 38% increase in liver weight for DCA vs. TCA at 2.0 g/L, was less than the 26 and 13% increases in cell diameter for the same groups, respectively. Therefore, for both DCA and TCA exposure, there appeared to be dose-related hepatomegaly and increased cell size after 14-days of exposure.

The authors reported PAS staining for glycogen content as an attempt to examine the nature of increased cell size by DCA and TCA. However, they did not present any quantitative data and only provided a brief discussion. The authors reported that:

> hepatic sections of DCA-treated B6C3F_1 mice (1 and 2 g/L) contained very large amounts of perilobular PAS-positive material within hepatocytes. PAS stained hepatic sections from animals receiving the highest concentration of TCA displayed a much less intense staining that was confined to periportal areas. Amylase digesting confirmed the majority of the PAS-positive material to by glycogen. Thus, increased hepatocellular size in groups receiving DCA appears

to be related to increased glycogen deposition. Similar increases in glycogen deposition were observed in Swiss-Webster mice.

There is no way to discern whether DCA-induced glycogen deposition was dose-related and therefore correlated with increased liver weight and cell diameter. While the authors suggest that Swiss-Webster mice displayed "similar increased in glycogen deposition," the authors did not report a similar increase in liver weight gain after DCA exposure at 14 days (1.27-fold of control percent liver/body weight ratio in Swiss male mice and 1.42-fold in female Swiss-Webster mice vs. 1.62-fold of control in $B6C3F_1$ mice after 14 days of exposure to 2 g/L DCA). Thus, the contribution of glycogen deposition to DCA-induced hepatomegaly and the nature of increased cell size induced by acute TCA exposure cannot be determined by this study. However, this study does show that DCA and TCA differ in respect to their effects on glycogen deposition after short-term exposure.

The authors report that:

> localized areas of coagulative necrosis were observed histologically in both $B6C3F_1$ and Swiss-Webster mice treated with DCA at concentrations of 1 and 2 g/L for 14 days. The necrotic areas corresponded to the pale streaked areas seen grossly. These areas varied in size, shape and location within sections and occupied up to several mm^2. An acute inflammatory response characterized by thin rims of neutrophils was associated with the necrosis, along with multiple mitotic figures. No such areas of necrosis were observed in animals treated at lower concentrations of DCA, or in animals receiving the chemical for 2 or 5 days. Mice treated with 2 g/L TCA for 14 days have some necrotic areas, but at such low frequency that it was not possible to determine if it was treatment-related (2 lesions in a total of 20 sections examined). No necrosis was observed in animals treated at the lower concentrations of TCA or at earlier time points.

Again there were no quantitative estimates given of the size of necrotic areas, variation between animals, variation between strain, or dose-response of necrosis reported for DCA exposure by the authors. The lack of necrosis after 2 and 5 days of exposure at all treatment levels and at the lower exposure level at 14 days of exposure is not correlated with the increases in liver weight reported for these treatment groups.

Autoradiographs of randomly chosen high powered fields (400×) (50 fields/animal) were reported as the percentage of cells in the fields that were labeled. There was significant variation in the number of animals examined and in the reported mean percent of labeled cells between control groups. The number of control animals was not given for the 2-day group but for the 5- and 14-day groups were reported to be n = 4 and n = 11, respectively. The mean percent of labeling in control animals was reported at 0.11 ± 0.03, 0.12 ± 0.04, and 0.46 ± 0.07% of hepatocytes for 2-, 5-, and 14-day control groups, respectively. Only the 2.0 g/L exposures of

DCA and TCA were examined at all three times of exposure, while all groups were examined at 14 days. However, the number of animals examined in all treatment groups appeared to be only four animals in each group.

There was not an increase over controls reported in the 2.0 g/L DCA or TCA 2- and 5-day exposure groups in hepatocyte labeling with tritiated thymidine. After 14 days of exposure, there was a statistically significant but very small dose-related increase over the control value after DCA exposure (i.e., 0.46 ± 0.07, 0.64 ± 0.15, 0.75 ± 0.22, and 0.94 ± 0.05% labeling of hepatocytes in control, 0.3, 1.0, and 2.0 g/L DCA treatment groups, respectively). For TCA, there was no change in hepatocyte labeling except for a 50% decrease from control values at after 14 days of exposure to 2.0 g/L TCA (i.e., 0.46 ± 0.07, 0.50 ± 0.14, 0.52 ± 0.26, and 0.26 ± 0.14% labeling of hepatocytes in control, 0.3, 1.0, and 2.0 g/L TCA treatment groups, respectively). The authors report that:

> labeled cells were localized around necrotic areas in these [sic DCA treated] groups. Since counts were made randomly, the local increased in DCA-treated animals at concentrations of 1 and 2 g/L are in fact much higher than indicated by the data. Labeling indices in these areas of proliferation were as high as 30%. Labeled hepatocytes in TCA-treated and the control animals were distributed uniformly throughout the sections. There was an apparent decrease in the percentage of labeled cells in the group of animals treated with the highest dose of TCA. This is because no labeled cells were found in any of the fields examined for one animal.

The data for control mice in this experiment are consistent with others showing that the liver is quiescent in regard to hepatocellular proliferation with few cells undergoing mitosis (see Section E.1.1). For up to 14 days of exposure with either DCA or TCA, there was little increase in hepatocellular proliferation except in instances and in close proximity to areas of proliferation. The increases in liver weight reported for this study were not correlated with and cannot be a result of hepatocellular proliferation as only a very small population of hepatocytes is undergoing DNA synthesis. For TCA, there was no increase in DNA synthesis in hepatocytes, even at the highest dose, as shown by autoradiographic data of tritiated thymidine incorporation in random fields.

Whole-liver sections were examined for tritiated thymidine incorporation from DNA extracts. The number of animals examined varied (i.e., n = 4 for the 2-day exposure groups and n = 8 for 5- and 14-day exposure groups), but the number of control animals examined was the same as the treated groups for this analysis. The levels of tritiated thymidine incorporation in hepatic DNA (dpm/mg DNA expressed as mean × 10^3 ± SE of the number of animals) were reported to be similar across control groups (i.e., 56 ± 11, 56 ± 6, and 56 ± 7 dpm/mg DNA, for 2-, 5-, and 14-day treatment groups, respectively).

After 2 days of DCA exposure, there appeared to be a slight treatment-related, but not dose-related, increase in reported tritiated thymidine incorporation into hepatic DNA (i.e., 72 ± 23, 80 ± 6, and 68 ± 7 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L DCA, respectively). After 5 days of DCA exposure, there appeared to be a dose-related increase in reported tritiated thymidine incorporation into hepatic DNA (i.e., 68 ± 18, 110 ± 20, and 130 ± 7 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L DCA, respectively). However, after 14 days of DCA exposure, levels of tritiated thymidine incorporation were less than those reported at 5 days and the level for the 0.3 g/L exposure group was less than the control value (i.e., 33 ± 11, 77 ± 9, and 81 ± 12 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L DCA, respectively).

After 2 days of TCA exposure, there did not appear to be a treatment-related increase in tritiated thymidine incorporation into hepatic DNA (i.e., 82 ± 16, 52 ± 7, and 54 ± 7 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L TCA, respectively). Similar to the reported results for DCA, after 5 days of TCA exposure, there appeared to be a dose-related increase in reported tritiated thymidine incorporation into hepatic DNA (i.e., 79 ± 23, 86 ± 17, and 158 ± 33 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L TCA, respectively). After 14 days of TCA exposure, there were treatment-related increases, but not a dose-related increase, in reported tritiated thymidine incorporation into hepatic DNA (i.e., 71 ± 10, 73 ± 14, and 103 ± 14 dpm/mg DNA for 0.3, 1.0, or 2.0 g/L TCA, respectively).

It would appear that for both TCA and DCA, the increase in tritiated thymidine incorporation into hepatic DNA was dose related and peaked after 5 days of exposure. The authors report that the decrease in incorporation into hepatic DNA observed after 14 days of DCA treatment at 0.3 g/L to be statistically significant as well as the increases after 5 and 14 days of TCA exposure at the 2.0 g/L level. The small numbers of animals examined, the varying number of animals examined, and the degree of variation in treatment-related effects limit the statistical power of this experiment to detect quantitative changes.

Given the limitations of this experiment, determination of an accurate measure of the quantitative differences in tritiated thymidine incorporation into whole-liver DNA or that observed in hepatocytes are hard to determine. In general, the results for tritiated thymidine incorporation into hepatic DNA were consistent with those for tritiated thymidine incorporation into hepatocytes in that they show that there were, at most, a small population of hepatocytes undergoing DNA synthesis after up to 14 days of exposure at relative high levels of exposure to DCA and TCA (i.e., the largest percentage of hepatocytes undergoing DNA synthesis for any treatment group was <1% of hepatocytes). The highest increases over control levels for hepatic DNA incorporation for the whole liver were reported at the highest exposure level of TCA treatment after 5 days of treatment (threefold of control) and after 14 days of TCA treatment (twofold of control).

Although the authors report small areas of focal necrosis with concurrent localized increases in hepatocyte proliferation in DCA-treated animals exposed to1.0 g/L and 2.0 g/L

DCA, the levels of whole-liver tritiated thymidine incorporation were only slightly elevated over controls at these concentrations, and were decreased at the 0.3 g/L exposure concentration for which no focal necrosis was reported. The whole-liver DNA incorporation of tritiated thymidine was not consistent with the pattern of tritiated thymidine incorporation observed in individual hepatocytes. The authors state that "at present, the mechanisms for increased tritiated thymidine uptake in the absence of increased rates of cell replication with increasing doses of TCA cannot be determined." The authors do not discuss the possibility that the difference in hepatocyte labeling and whole-liver DNA tritiated thymidine incorporation could have been due to the labeling representing increased polyploidization rather than cell proliferation, as well as increased numbers of proliferating nonparenchymal and inflammatory cells. The increased cell size due from TCA exposure without concurrent increased glycogen deposition could have been indicative of increased polyploidization. Finally, although both TCA- and DCA-induced increases in liver weight were generally consistent with cell size increases, they were not correlated with patterns of change in hepatic DNA content, incorporation of tritiated thymidine in DNA extracts from whole liver, or incorporation of tritiated thymidine in hepatocytes. In regard to cell size, although increased glycogen deposition with DCA exposure was noted by the authors of this study, lack of quantitative analyses of that accumulation precludes comparison with DCA-induced liver weight gain.

E.2.3.1.2. Nelson et al. (1989) and Nelson and Bull (1988)

Nelson and Bull (1988) administered TCE (0, 3.9, 11.4, 22.9, and 30.4 mmol/kg) in Tween 80® via gavage to male Sprague-Dawley rats and male B6C3F$_1$ mice, sacrificed them 4 hours after treatment (n = 4–7), and measured the rate of DNA unwinding under alkaline conditions. They assumed that this assay represented increases in SSBs. For rats, there was little change from controls up to 11.4 mmol/kg (1.5 g/kg TCE) but a significantly increased rate of unwinding at 22.9 and 30.4 mmol/kg TCE (approximately twofold greater at 30.4 mmol). For mice, there was a significantly increased level of DNA unwinding at 11.4 and 22.9 mmol. Concentrations >22.9 mmol/kg were reported to be lethal to the mice. In this same study, TCE metabolites were administered in unbuffered solution using the same assay. DCA was reported to be most potent in this assay with TCA being the lowest, while CH closely approximated the dose-response curve of TCE in the rat. In the mouse, the most potent metabolite in the assay was reported to be TCA followed by DCA with CH considerably less potent.

The focus of the Nelson et al. (1989) study was to examine whether reported SSBs in hepatic DNA induced by DCA and TCA (Nelson and Bull, 1988) were secondary to peroxisome proliferation also reported to be induced by both. Male B6C3F$_1$ mice (25–30 g but no age reported) were given DCA (10 mg/kg or 500 mg/kg) or TCA (500 mg/kg) via gavage in 1% aqueous Tween 80® with no pH adjustment. The animals were reported to be sacrificed 1, 2, 4, or 8 hours after administration, and livers were examined for SSBs as a whole-liver homogenate.

In a separate experiment (experiment #2), treatment was parallel to the first (500 mg/kg treatment of DCA or TCA), but levels of PCO activity were measured as an indication of peroxisome proliferation and expressed as μmol/minute/g liver. In a separate experiment (experiment #3), mice were administered 500 mg/kg DCA or TCA for 10 days with Clofibrate administered at a dose of 250 mg/kg as a positive control. Twenty-four hours after the last dose, animals were killed, and liver was examined by light microscopy and PCO activity. Finally, in an experiment parallel in design to experiment #3, SSBs were measured in total hepatic DNA after 500 mg/kg exposure to TCA (experiment #4). Electron microscopy was performed on two animals/group for vehicle, DCA, or TCA treatment, with six randomly chosen micrographic fields utilized for peroxisome profiles. These micrographs were analyzed without identification as to what area of the liver lobules they were being taken from. Hence, there is a question as to whether the areas that are known to be peroxisome rich were assayed or not.

The data from all control groups were reported as pooled data in figures, but statistical comparisons were made between concurrent control and treated groups. The results for DNA SSBs were reported for "13 control animals" and each experimental time point "as at least 6 animals."

DNA strand breaks were reported to be significantly increased over concurrent control by a single exposure to 10 or 500 mg/kg DCA or 500 mg/kg TCA for 1, 2, or 4 hours after administration but not at 8 or 24 hours. There did not appear to be a difference in the magnitude of response between the three treatments (the fraction of unwound DNA was ~2.5 times that of control). PCO activity was reported to be not increased over control within 24 hours of either DCA or TCA treatment (n = 6 animals per group). The fraction of alkaline unwinding rates as an indicator of SSBs were reported to not be significantly different from controls and TCA-treated animals after 10 days of exposure (n = 5).

Relative to controls, body weights were reported to not be affected by exposures to DCA or TCA for 10 days at 500 mg/kg (data were not shown.) (n = 6 per group). However, both DCA and TCA were reported to significantly increase liver weight and liver/body weight ratios (i.e., liver weights were 1.3 ± 0.05, 2.1 ± 0.10, and 1.7 ± 0.09 g for control, 500 mg/kg DCA, and 500 mg/kg TCA treatment groups, respectively while percent liver/body weights were 4.9 ± 0.14, 7.5 ± 0.18, and 5.7 ± 0.14% for control, 500 mg/kg DCA, and 500 mg/kg TCA treatment groups, respectively).

PCO activity (μmol/minute/g liver) was reported to be significantly increased by DCA (500 mg/kg), TCA (500 mg/kg), and Clofibrate (250 mg/kg) treatment (i.e., levels of oxidation were 0.63 ± 0.07, 1.03 ± 0.09, 1.70 ± 0.08, and 3.26 ± 0.05 for control, 500 mg/kg DCA, 500 mg/kg TCA, and 250 mg/kg Clofibrate treatment groups, respectively). Thus, the increases were ~1.63-, 2.7-, and 5-fold of control for DCA, TCA, and Clofibrate treatments.

Results from randomly selected electron photomicrographs from two animals (six per animal) were reported for DCA and TCA treatment and to show an increase in peroxisomes per

unit area that was reported to be statistically significant (i.e., 9.8 ± 1.2, 25.4 ± 2.9, and 23.6 ± 1.8 for control, 500 mg/kg DCA, and 500 mg/kg TCA, respectively). The 2.5- and 2.4-fold of control values for DCA and TCA gave a different pattern than that of PCO activity. The small number of animals examined limited the power of the experiment to quantitatively determine the magnitude of peroxisome proliferation via electron microscopy. The enzyme analyses suggested that both DCA and TCA were weaker inducers of peroxisome proliferation than Clofibrate.

The authors reported that there was no evidence of gross hepatotoxicity in vehicle or TCA-treated mice. Light microscopic sections from mice exposed to TCA or DCA for 10 days were stained with H&E and PAS for glycogen. For TCA treatment, PAS staining "produced approximately the same intensity of staining and amylase digesting revealed that the vast majority of PAS-positive staining was glycogen." Hepatocytes were reported to be "slightly larger in TCA-treated mice than hepatocytes from control animals throughout the liver section with the architecture and tissue pattern of the liver intact." The histopathology after DCA treatment was reported to be "markedly different than that observed with either vehicle or TCA treatments" with the "most pronounced change in the size of hepatocytes." DCA was reported to:

> produce marked cellular hypertrophy uniformly throughout the liver. The hepatocytes were approximately 1.4 times larger in diameter than control liver cells. This hypertrophy was accompanied by an increase in PAS staining; indicating greater glycogen deposition than in TCA-treated and control liver tissue. Multiple white streaks were grossly visible on the surface of the liver of DCA-treated mice. The white areas corresponded with subcapsular foci of coagulative necrosis. These localized necrotic areas were not encapsulated and varied in size. The largest necrotic foci occupied the area of a single lobule. These necrotic areas showed a change in staining characteristics. Often this change consisted of increased eosinophilia. A slight inflammatory response, characterized by neutrophil infiltration, was present. These changed were evident in all DCA-treated mice.

The results from this experiment cannot inform as to dose-response relationships for the parameters tested with the exception of DNA SSBs where two concentrations of DCA were examined (10 and 500 mg/kg). For this parameter, the 10 mg/kg exposure of DCA was as effective as the 500 mg/kg dose where toxicity was observed. This effect on DNA was observed before evidence of induction of peroxisome proliferation. The authors did not examine Clofibrate for effects on DNA so whether it too, would have produced this effect is unclear. The results from this study are consistent with those of Sanchez and Bull (1990) for induction of hepatomegaly by DCA and TCA, the lack of hepatotoxicity at this dose by TCA, and the difference in glycogen deposition between DCA and TCA.

E.2.3.1.3. Styles et al. (1991)

In this report, a similar paradigm is used as Nelson et al. (1989) for the intention of repeating that work on SSBs and to study DNA synthesis and peroxisome proliferation. In regard to the findings of SSBs, Styles et al. (1991) reported for a similar paradigm of 500 mg/kg neutralized TCA administered to male B6C3F_1 mice (7–8 weeks of age) and examined at 1, 4, 8, and 24 hours after dosing. They reported no increased unwinding of DNA 1 or 24 hours after TCA administration. In a separate experiment, tritiated thymidine was administered to mice 1 hour before sacrifice at 24, 36, 48, 72, and 96 hours after the first dose of 500 mg/kg TCA for 3 days via gavage (n = 5 animals per group).

The hepatic DNA uptake of tritiated thymidine was reported to be similar to control levels up to 36 hours after the first dose and then to increase to a level approximately sixfold greater than controls by 72 hours after the first dose of TCA. By 96 hours, the level of tritiated thymidine incorporation had fallen to approximately fourfold greater than controls. The variation, reported by SD, was very large in treated animals (e.g., SD was equal to approximately ±1.3-fold of control for 48 hour time point). Individual hepatocytes were examined with the number of labeled hepatocytes/1,000 cells reported for each animal.

The control level was reported to be ~1 with a SD of similar magnitude. The number of labeled hepatocytes was reported to decrease between 24 and 36 hours and then to rise slowly back to control levels at 48 hours and then to be significantly increased 72 hours after the first dose of TCA (~9 cells/1,000 with a SD of 3.5) and then to decrease to a level of ~5 cells/1,000. Thus, it appears that increases in hepatic DNA tritiated thymidine uptake preceded those of increased labeled hepatocytes and did not capture the decrease in hepatocyte labeling at 36 hours. By either measure, the population of cells undergoing DNA synthesis was small, with the peak level being <1% of the hepatocyte population.

The authors go on to report the zonal distribution of mean number of hepatocytes incorporating tritiated thymidine but no variations between animals were reported. The decrease in hepatocyte labeling at 36 hours was apparent at all zones. By 48 hours, there appeared to be slightly more periportal than midzonal cells undergoing DNA synthesis with centrilobular cells still below control levels. By 72 hours, all zones of the liver were reported to have a similar number of labeled cells. By 96 hours, the midzonal and centrilobular regions have returned almost to control levels while the periportal areas were still elevated. These results are consistent with all hepatocytes showing a decrease in DNA synthesis by 36 hours and then a wave of DNA synthesis occurring starting at the periportal zone and progressing through to the pericentral zone until 72 hours and then the midzonal and pericentral hepatocytes completing their DNA synthesis activity.

Peroxisome proliferation was assessed via electron photomicrographs taken in mice (four controls and four treated animals) given 10 daily doses of 500 mg/kg TCA and killed 14 hours after the last dose. No details were given by the authors as to methodology for peroxisome

volume estimate (e.g., how many photos per animals were examined and whether they were randomly chosen). The mean percent cell volume occupied by peroxisome was reported to be 2.1 ± 0.386 and 3.9 ± 0.551% for control and 500 mg/kg TCA, respectively. Given that there were no time points examined before 10 days for peroxisome proliferation, correlations with DNA synthesis activity induced by TCA cannot be made from this experiment. However, it is clear from this study that a wave of DNA synthesis occurs throughout the liver after treatment of TCA at this exposure concentration and that it has peaked by 72 hours even with continuous exposure to 96 hours. Whether the DNA synthesis represents polyploidization or cell proliferation cannot be determined from these data; neither can a dose-response be determined.

E.2.3.1.4. Carter et al. (1995)

The aim of this study was to "use correlative biochemical, pathologic and morphometric techniques to characterize and quantify the acute, short-term responses of hepatocytes in the male B6C3F_1 mouse to drinking water containing DCA." This report used tritiated thymidine incorporation, DNA concentration, hepatocyte number per field (cellularity), nuclear size, and binuclearity (polyploidy) parameters to study 0, 0.5, and 5 g/L neutralized DCA exposures up to 30 days. Male B6C3F_1 mice were started on treatment at 28 days of age. Tritiated thymidine was administered by miniosmotic pump 5 days prior to sacrifice.

The experiment was conducted in two phases, which consisted of 5–15 days of treatment (Phase I) and 20–30 days of treatment (Phase II) with five animals per group in groups sacrificed at 5-day intervals. Liver sections were stained for H&E, PAS (for glycogen) or methyl green pyronin stain (for RNA). DNA was extracted from liver homogenates and the amount of tritiated thymidine determined as dpm/µg DNA. Autoradiography was performed with the number of hepatocyte nuclei scored in 1,000 hepatocytes selected randomly to provide a labeling index of "number of labeled cells/1000 X 100%." Changes in cellularity, nuclear size and number of multinucleate cells were quantified in H&E sections at 40 × power. Hepatocyte cellularity was determined by counting the number of nuclei in 50 microscopic fields with multinucleate cells being counted as one cell and nonparenchymal cells not counted. Nuclear size was also measured in 200 nuclei with the mean area plus 2 SD was considered to be the largest possible single nucleus. Therefore, polyploid diploid cells were identified by the authors but not cells that had undergone polyploidy with increased DNA content in a single nucleus.

Mean body weights at the beginning of the experiment varied between 18.7 and 19.6 g in the first three exposure groups of Phase I of the study. Through 15 days of exposure, there did not appear to be a change in body weight in the 0.5 g/L exposure groups but in the 5 g/L exposure group body weight was reduced at 5, 10, and 15 days with that reduction statistically significant at 5 and 15 days. Liver weights did not appear to be increased at day 5 but were increased at days 10 and 15 in both treatment groups (i.e., means ± SEM. for day 10, 1.36 ± 0.03, 1.46 ± 0.03, and 1.59 ± 0.08 g for control, 0.5, and 5 g/L DCA, respectively; and for day 15,

1.51 ± 0.06, 1.72 ± 0.05, and 2.08 ± 0.11 g for control, 0.5, and 5 g/L DCA, respectively). The percent liver/body weight followed a similar pattern with the exception that at day 5, the 5 g/L exposure group had a statistically significant increase over control (i.e., for day 10, 6.00 ± 0.10, 6.72 ± 0.17, and 8.21 ± 0.10% for control, 0.5, and 5 g/L DCA, respectively; and for day 15, 6.22 ± 0.08, 6.99 ± 0.15, and 10.37 ± 0.27% g for control, 0.5, and 5 g/L DCA, respectively).

In Phase II of the study, control body weights were smaller than Phase I and varied between 16.6 and 16.9 g in the first three exposure groups. Liver weights of controls were also smaller making it difficult to quantitatively compare the two groups in terms of absolute liver weights. However, the pattern of DCA-induced increases in liver weight and percent liver/body weight remained. The patterns of body weight reduction only in the 5 g/L treatment groups and increased liver weight with DCA treatment at both concentrations continued from 20 to 30 days of exposure.

For liver weight, there was a slight but statistically significant increase in liver weight for the 0.5 g/L treatment groups over controls (i.e., for day 20, 1.02 ± 0.02, 1.18 ± 0.05, and 1.98 ± 0.05 g for control, 0.5, and 5 g/L DCA, respectively; for day 25, 1.15 ± 0.03, 1.34 ± 0.04, and 2.06 ± 0.12 g for control, 0.5, and 5 g/L DCA, respectively, for day 30, 1.15 ± 0.03, 1.39 ± 0.08, and 1.90 ± 0.12 g for control, 0.5, and 5 g/L DCA, respectively). For percent liver/body weight, there was a small increase at 0.5 g/L that was not statistically significant but all other treatments induced increases in percent liver/body weight that were statistically significant (i.e., for day 20, 4.82 ± 0.07, 5.05 ± 0.09, and 9.71 ± 0.11% for control, 0.5, and 5 g/L DCA, respectively; for day 25, 5.08 ± 0.04, 5.91% ± 0.09, and 10.38 ± 0.58% for control, 0.5, and 5 g/L DCA, respectively; for day 30, 5.17 ± 0.09, 6.01 ± 0.08, and 10.28 ± 0.28% for control, 0.5, and 5 g/L DCA, respectively).

Of note is the dramatic decrease in water consumption in the 5 g/L treatment groups that were consistently reduced by 64% in Phase I and 46% in Phase II. The 0.5 g/L treatment groups had no difference from controls in water consumption at any time in the study. The effects of such water consumption decreases would affect body weight as well as dose received. Given the differences in the size of the animals at the beginning of the study and the concurrent differences in liver weights and percent liver/body weight in control animals between the two phases, the changes in these parameters through time from DCA treatments cannot be accurately determined (e.g., control liver/body weights averaged 6.32% in Phase I but 5.02% in Phase II). However, percent liver/body weight increase were reported to be consistently increased within and between both phases of the study for the 0.5 g/L DCA treatment from 5 to 30 days of treatment (i.e., for Phase I, the average increase was 9.5% and for Phase II, the average increased was 12.5% for 0.5 g/L DCA treated groups). Although increased at 5 days, the nonsignificance of the change may be resultant from the small number of animals examined. The difference in magnitude of dose and percent liver/body weight increase is difficult to determine given that the 5 g/L dose of DCA reduced body weight and significantly reduced water consumption by ~50% in both phases

of the study. Of note is that the differences in DCA-induced percent liver/body weight were ~6-fold for the 15, 25, and 30-day data between the 0.5 and 5 g/L DCA exposures rather than the 10-fold difference in exposure concentration in the drinking water.

The incorporation of tritiated thymidine into total hepatic DNA control treatment groups was reported to be 73.34 ± 11.74 dpm/μg DNA at 5 days, 34 ± 4.12 dpm/μg DNA at 15 days, and 28.48 ± 3.24 dpm/μg DNA at 20 days but was not reported for other treatments. The results for 0.5 g/L treatments were not reported quantitatively but the authors stated that the results "showed similar trends of initial inhibition followed by enhancement of labeling, the changes relative to controls were not statistically significant." For 5 g/L treatment groups, the 5-day treated groups DNA tritiated thymidine incorporation was reported to be 42.8% of controls and followed by a transient increase at 15 and 20 days (i.e., 2.65- and 2.45-fold of controls, respectively) but after 25 and 30 days, was not significantly different from controls (data not shown).

Labeling indices of hepatocytes were reported as means, but variations as either SEM or SD were not reported. Control means were reported as 5.5, 4, 2, 2, 3.2, and 3.5% of randomly selected hepatocytes for 5, 10, 15, 20, 25, and 30 days, respectively, for four to five animals per group. In contrast to the DNA incorporation results, no increase in labeling of hepatocytes was reported to be observed in comparison to controls for any DCA treatment group from 5 to 30 days of DCA exposure. The 5 g/L treatment group showed an immediate decrease in hepatocyte labeling from day 5 onwards that gradually increased approximately half of control levels by day 30 of exposure (i.e., <0.5% labeling index at day 5, ~1% labeling index at day 10, ~0.6% labeling index at day 20, 1% labeling index at day 25, and 2% labeling index at day 30). For the 0.5 g/L treatment, the labeling index was reported to not differ from controls from days 5 though 15, but to be significantly decreased between days 20 and 30 to levels similar to those observed for the 5 g/L exposures. The relatively higher number of hepatocytes incorporating label reported in this study than others can be a reflection of the longer times of exposure to tritiated thymidine. Here, incorporation was shown for 1 weeks worth of exposure and reflects the percent of cell undergoing synthesis during that time period. Also, the higher labeling index in control animals at the 5- and 10-day exposure periods is probably a reflection of the age of the animals at the time of study.

From the data reported by the authors, there was a correlation between the patterns of total DNA incorporation of label and hepatocyte labeling indices in control groups (i.e., higher level of labeling at 5 days than at 15 and 20 days). However, the patterns of decreased thymidine labeling reported for hepatocytes were not correlated with a transient increase in total DNA thymidine incorporation reported with DCA treatment, especially at the 5 g/L exposure level with a large decrease reported for the number of labeled hepatocytes at the same time an increase in total DNA thymidine incorporation was reported.

Although reported to be transiently increased, the total hepatic DNA labeling still represented at most a 2.5-fold increase over control liver, which represents a small population of cells. Given that the study examined hepatocyte labeling in random fields and did not report quantitative zonal differences in proliferation, a more accurate determination of what hepatocytes were undergoing proliferation cannot be made from the labeling index results. Also, although the authors report signs of inflammatory cells for 5-day treatment there is no reference to any inflammatory changes that may have been observed at later time periods when cellular degeneration and loss of nuclei were apparent. Such an increase inflammatory infiltrates can increase the DNA synthesis measurements in the liver. The difference in labeling index and total DNA synthesis could reflect differences in nonparenchymal cell proliferation or ploidy changes vs. mitoses in hepatocytes. Clearly, the increases in liver weight that were reported as early as 5 days of exposure could not have resulted from increased hepatocyte proliferation.

The H&E sections were reported to have been fixed in an aqueous solution that reduced glycogen content. However, residual PAS positive material (assumed to be glycogen) was reported to be present indicating that not all of the glycogen had been dissolved. The authors report changes in pathology between 5 and 30 days in control animals that included straightening of hepatocyte cording, decreased mitoses, less clarity and more fine granularity of pericentral hepatocellular cytoplasm, increased numbers of larger nuclei that were not labeled, and reported differences between animals in the amount of glycogen present (i.e., two or three animals out of the five had less glycogen than other members of the group with less glycogen in the central and midzonal areas). These changes are consistent with increased polyploidization expected for maturing mice (see Sections E.1.1 and E.1.2).

After 5 days of treatment, 0.5 g/L exposed animals were reported to have livers with fewer mitoses and tritiated thymidine hepatocyte labeling, but by 10 days, there was an increase in nuclear size. Labeling was reported to be predominantly in small nuclei. Animals given 0.5 g/L DCA for 15, 20, and 25 days were reported to have "focal cells in the middle zone with less detectable or no cell membranes and loss of the coarse granularity of the cytoplasm" with some cells not having nuclei or cells having a loss of nuclear membrane and apparent karyolysis. "Cells without nuclei because the plane of the section did not pass through the nuclei had the same type of nuclei. Cells without nuclei not related to plane of section had a condensed cytoplasm." Livers from 20-day and later sacrifice groups treated with 0.5 g/L DCA were reported to have normal architecture. After 25 days of treatment, apoptotic bodies were reported to be observed with fewer nuclei around the central veins nuclei that were larger in central and midzonal areas.

In animals treated with 5 g/L DCA, the authors report similar features as for 0.5 g/L but in a zonal pattern. Inflammatory cells were reported to not be observed, and after 5 and 10 days, a marked decrease in labeled nuclei. After 5 days of 5 g/L DCA, nuclear depletion in the central and mid-zonal areas was reported. In methyl green pyronin-stained slides a marked loss of

cellular membranes was reported at 5 days with a loss of nuclei and formation of "lakes of liver cell debris." After 15 days of treatment, there was a reported increase in labeling in comparison to animals sacrificed after 5 or 10 days. The cells nearest to the triads were reported to have clearing of their cytoplasms and an increase in PAS positivity. Hepatocytes of both 0.5 and 5 g/L DCA treatment groups were reported to have "enlarged, presumably polyploidy nuclei." Some of the nuclei were reported to be "labeled, usually in hepatocytes in the mid-zonal area."

The morphometric analyses of liver sections were reported to reveal statistically significant changes in cellularity, nuclear size (as measured by either nuclear area or mean diameter of the nuclear area equivalent circle), and multinucleated cells during 30 days of exposure to DCA. The authors reported that the concentration of total DNA in the liver, reported as total μg nuclear DNA/g liver, ranged between 278.17 ± 16.88 and 707.00 ± 25.03 in the control groups (i.e., two- to fivefold range). No 0.5 g/L DCA treatment groups differed from their control group in terms of liver DNA concentration. However, for 10–30 days of exposure, hepatic DNA concentrations were reported to be decreased in the 5 g/L treatment groups (at 5 days, there appeared to be ~30% increase over control). The number of cells per field was reported to range between 24.28 ± 1.94 and 43.81 ± 1.93 in control livers (i.e., 1.8-fold range). From 5 to 15 days, the number of cells/field decreased with 0.5 g/L DCA treatment, although only at day 15 was the change statistically significant. From 20 to 30 days of treatment, only the 30-day treatment showed a slight decrease in cells/field and that change was statistically significant. After 5 days of treatment, the number of cells/field was 1.6-fold of control, by 15 days, it was reduced by ~20%, and for 20–30 days, it continued to be reduced by as much as 40%.

Although the authors reported that the changes in cellularity and DNA concentration to be closely correlated, the patterns in the number of cells/field varied in their consistency with those of DNA concentration (i.e., for days 5, 20, and 25 the direction of change with dose was similar between the two parameters but not for days 10, 15, and 30). If changes in liver weight were due to hepatocellular hypertrophy, the increased liver size would be matched by a decrease in liver DNA concentration and by the number of cells/field. The large increases in liver/body weight induced by 5 g/L DCA were matched by decreases in liver DNA concentration except for the 5-day exposure group. In general, the small increases in liver/body weight consistently induced by 0.5 g/L treatment from days 5 through 30 were not correlated with DNA concentrations or cells/field.

The small number of animal examined for these parameters (i.e., n = 4–5) and the highly variable control values limit the power to accurately detect changes. The apparent dehydration in the animals treated at 5 g/L DCA was cited by the authors for the transient increase in cellularity and DNA concentration in the 5-day exposure group. However, drinking water consumption was reported to be similarly reduced at all treatment periods for 5 g/L DCA-treated animals so that all groups would experience the same degree of dehydration.

The percentage of mononucleated cells was reported as percent of mononucleated hepatocytes with results given as means, but with no reports of variation within groups. The mean control values were reported to range between 60 and 75% for Phase I and between 58 and 71% for Phase II of the experiment (n = 4–5 animals per group). The percent of mononucleated hepatocytes was reported to be similar between control and DCA treatment groups at 5- and 10-day exposures. At 15 days, both DCA treatments were reported to give a similar increase in mononucleated hepatocytes (~80 vs. 60% in control) with only the 5 g/L DCA group statistically significant. The increase in mononucleated cells reported for DCA treatment is similar in size to the variation between control values. For Phase II of the study, DCA treatment was reported to increase the number of mononucleated cells in at all concentrations and exposure time periods in comparison to control values. However, only the increases for the 5 g/L treatments at days 20 and 25, and the 0.5 g/L treatment at day 30 were reported to be statistically significant. Again, small numbers of animals limit the ability to accurately determine a change. However, the consistent reporting of an increasing number of mononucleated cells between 15 and 30 days could be associated with clearance of mature hepatocytes as suggested by the report of DCA-induced loss of cell nuclei.

Mean nuclear area was reported to range between 45 and 54 μ^2 in Phase I and between 41 and 48 μ^2 in Phase II of the experiment with no variation in measurements given by the authors. The only statistically significant differences reported between control and treated groups in Phase I was a decrease from 54 to ~42 μ^2 in the 0.5 g/L DCA 10-day treatment group and a small increase from 50 to ~52 μ^2 in the 15-day treatment group. Clearly, the changes reported by the authors as statistically significant did not show a dose-related pattern and were within the range of variation reported between control groups. For Phase II of the experiment, both DCA treatment concentrations were reported to induce a statistically significant increase the nuclear area that was dose-related, with the exception of day 30 in which the nuclear area was similar between the 0.5 and 5 g/L treatment groups. The largest increase in nuclear area was reported at 20 days for the 5 g/L treatment group (~72 vs. 41 μ^2 for control).

The patterns of increases in nuclear area were correlated with those of increased percentage of mononucleated cells in Phase II of the study (20–30 days of treatment) as well as the small changes seen in Phase I of the experiment. An increase in nuclear cell area is consistent with increase polyploidization without mitosis, as cells are induced towards polyploidization. A decrease in the numbers of binucleated cells in favor of mononucleated cells is consistent with clearance of mature binucleated hepatocyte as well induction of further polyploidization of diploid or tetraploid binucleated cell to tetraploid or octoploid mononucleated cells. The authors suggested that the "large hyperchromatic mononucleated hepatocytes are tetraploid" and suggest that such increases in tetraploid cells have also been observed with nongenotoxic carcinogens and with di(2-ethylhexyl) phthalate (DEHP).

In terms of increased cellular granularity observed by the authors with DCA treatment, this result is also consistent with a more differentiated phenotype (Sigal et al., 1999). Thus, these results for DCA are consistent with a DCA-induced change in polyploidization of the cells without cell proliferation.

The pattern of consistent increase in percent liver/body weight induced by 0.5 g/L DCA treatment from days 5 though 30 was not consistent with the increased numbers of mononucleated cells and increase nuclear area reported from day 20 onward. The large differences in liver weight induction between the 0.5 and 5 g/L treatment groups at all times studied also did not correlate with changes in nuclear size and percent of mononucleated cells. Thus, increased liver weight was not a function of cellular proliferation, but probably included both aspects of hypertrophy associated with polyploidization and increased glycogen deposition induced by DCA. The similar changes reported after short-term exposure for both the 0.5 and 5 g/L exposure concentration were suggested by the authors to indicate that the carcinogenic mechanism at both concentrations would be similar. Furthermore, they suggest that although there is evidence of cytotoxicity (e.g., loss of cell membranes and apparent apoptosis), DeAngelo et al. (1999) suggested that the present study does not support that the mechanism of DCA-induced hepatocellular carcinogenesis is one of regenerative hyperplasia following massive cell death nor peroxisome proliferation as the 0.5 g/L exposure concentration has been shown to increase hepatocellular lesions after 100 weeks of treatment without concurrent peroxisome proliferation or cytotoxicity.

E.2.3.1.5. DeAngelo et al. (1989)

Various strains of rats and mice were exposed to TCA (12 and 31 mM) or DCA (16 and 39 mM) for 14 days with Sprague-Dawley rats and $B6C3F_1$ mice exposed to an additional concentration of 6 mM TCA and 8 mM DCA. Although noting that in a previous study, with high concentrations of chloracids, there was decreased water consumption, the authors did not measure drinking water consumption in this study.

This study exposed several strains of male rats and mice to TCA at two concentrations in drinking water (12 and 31 mM neutralized TCA) for 14 days. The conversion of mmol/L or mM TCA is 5, 2, and 1 g/L TCA for 31, 12, and 6 mM TCA, respectively. The conversion of mmol/L of mM DCA is 5, 2, and 1 g/L DCA for 39, 16, and 8 mM DCA, respectively. The strains of mice tested were Swiss-Webster, $B6C3F_1$, C57BL/6, and C3H and for rats were Sprague-Dawley, Osborne-Mendel, and F344. For the F344 rat and $B6C3F_1$ mice, data from two separate experiments were reported for each. The number of animals in each group was reported to be six for most experiments with the exception of the Sprague-Dawley rats (n = 3 at the highest dose of TCA and n = 4 or 5 for the control and the lower TCA dose), one study in $B6C3F_1$ mice (n = 4 or 5 for all groups), and one study in F344 rats (n = 4 for all groups).

The body weight of the controls was reported to range from 269 to 341 g in the differing strains of rats (1.27-fold) and 21–28 g in the differing strains of mice (1.33-fold, age not reported). For percent liver/body weight ratios, the range was 4.4–5.6% in control rats (1.27-fold) and 5.1–6.8% in control mice (1.33-fold).

As discussed in other studies, the determination of PCO activity appears to be highly variable. This enzyme activity is often used as a proxy for peroxisome proliferation. For PCO activity, the range of activity in controls was much greater than for either body weight or percent liver/body weight. For rats, there was a 2.8-fold difference in PCO control activity, and in mice, there was a 4.6-fold difference in PCO activity. Between the two studies performed in the same strain of rat (F344), there was a 2.83-fold difference in PCO activity between controls, and for the two studies in the same strain of mouse ($B6C3F_1$) there was a 3.14-fold difference in PCO activity between controls. Not only were there differences between strains and experiments in the same strain, but also differences in control values between species with a wider range of values in the mice. The lowest level of PCO activity in control rats, expressed as nanomoles NAD reduced/minute/mg/protein, was 3.34, and for control mice, was 1.40. The highest level reported in control in rats was 9.46, and for control mice, was 6.40.

These groups of rats and mice were exposed to 2 g/L sodium chloride, or 2 or 5 g/L TCA in drinking water for 14 days and their PCO activity was assayed. These doses of TCA did not affect body weight except for the Sprague-Dawley rats, which lost ~16% of their body weight. This was also the same group in which only three rats survived treatment. The Osborne-Mendel and F344 strains did not exhibit loss of body weight or mortality due to TCA exposure.

There was a large variation in response to TCA exposure between the differing strains of rats and mice with a much larger difference between the strains of mice. For the three rat strains tested, there was a range between 0% change and 2.38-fold of control for PCO activity at the 5 g/L TCA exposure. For the 2 g/L TCA exposure, there was a range of 0% change to 1.54-fold of control for PCO activity. The Osborne-Mendel rats had 1.54-fold of control value for PCO activity at 2 g/L TCA and 2.38-fold of control value for PCO activity reported at 5 g/L, exhibiting the most consistent increase in PCO with increased dose of TCA. Two experiments were reported for F344 rats with one reporting a 1.63-fold of control and the other a 1.79-fold of control value for 5 g/L TCA. Only one of the F334 experiments also exposed rats to 2 g/L TCA and reported no change from control values.

For the four strains of mice tested, there was a range of 7.44–22.13-fold of control values reported at the 5 g/L TCA exposures and 3.76–25.92-fold of control values at the 2 g/L TCA exposures for PCO activity. For the C57BL/6 strain of mice, there was little difference between the 5 and 2 g/L TCA exposures and a generally threefold higher induction of PCO activity by TCA at the 5 g/L TCA exposure level than for the other mouse strains. Although there was a 2.5-fold difference between the 5 and 2 g/L TCA exposure dose, the difference in magnitude of PCO activity between these doses ranged from 0.85- to 2.23-fold for all strains of mice. For the

$B6C3F_1$ mice, there was a difference between reported increases of PCO activity in the text (i.e., reported as 9.59-fold of control) for one of the experiments and that presented graphically in Figure 2 (i.e., 8.70-fold of control). Nevertheless in the two studies of $B6C3F_1$ mice, 5 g/L TCA was reported to induce 7.78-fold of control and 8.70-fold of control for PCO activity, and 2 g/L TCA was reported to induce 5.56-fold of control and 4.70-fold of control for PCO activity.

For the two F344 rat studies in which ~200 mg/kg or 5 g/L TCA was administered for 10 or 14 days, there was 1.63-fold of control and 1.79-fold of control values reported for PCO activity. Thus, for experiments in which the same strain and dose of TCA were administered, there was not as large a difference in PCO response than between strains and species.

Whether increases in percent liver/body weight ratios were similar in magnitude to increased PCO activity can be assessed by examination of the differences in magnitude of increase over control for the 5 and 2 g/L TCA treatments in the varying rat and mouse strains. The relationship in exposure concentration was a 2.5:1 ratio for the 5 and 2 g/L doses. For rats treatment of 5 g/L TCA to Sprague-Dawley rats resulted in a significant decrease in body weight, and therefore, affected the magnitude of increase in percent liver/body weight ratio for this group. However, for the rest of the rat and mouse data, this dose was not reported to affect body weight so that there is more confidence in the dose-response relationship.

For the Sprague-Dawley rat, there was no change in the percent liver/body weight ratio at 2 g/L but a 10% decrease at 5 g/L TCA exposure with no change in PCO activity for either. However, for the Osborne-Mendel rats, there was no change in percent liver/body weight ratios for either exposure concentration of TCA, but PCO activity was reported to be 1.54-fold of control at 2 g/L and 2.38-fold of control at 5 g/L TCA. Thus, there was a ratio of 2.5-fold increase in PCO activity between the 5 and 2 g/L treatment groups. For the F344 rats, there was a 2-fold difference in liver weight increases (i.e., 12 vs. 6% increase over control) between the two exposure concentrations but 1.6-fold of control value for PCO activity at the 5 g/L TCA exposure concentration and no increase in PCO activity at the 2 g/L level. Thus, for the three strains of rats, there did not appear to be a consistent correlation between liver weight induction by TCA and PCO activity.

For differing strains of mice, similar concentrations of TCA were reported to vary in the induction of liver weight increases. The range of liver weight induction was 1.26–1.66-fold of control values between the four strains of mice at 5 g/L TCA and 1.16–1.63-fold at 2 g/L TCA. In general, for mice the magnitudes of the difference in the increase in dose between the 5 g/L and 2 g/L TCA exposure concentration (2.5-fold) was generally higher than the increase percent liver/body weight ratios at these doses. The differences in liver weight induction between the 2 and 5 g/L doses were ~40% for the Swiss-Webster, C3H, and for one of the $B6C3F_1$ mouse experiments. For the C57BL/6 mouse, there was no difference in liver weight induction between the 2 and 5 g/L TCA exposure groups. For the other $B6C3F_1$ mouse experiments, there was a

2.5-fold greater induction of liver weight increase for the 5 g/L TCA group than for the 2 g/L exposure group (1.39- vs. 1.16-fold of control for percent liver/body weight, respectively).

For PCO activity, the Swiss-Webster, C3H, and one of the $B6C3F_1$ mouse experiments were reported to have approximately twofold difference in the increase in PCO activity between the two doses. For the other $B6C3F_1$ mouse experiment, there was only about a 50% increase and for the C57BL/6 mouse data, there was 15% less PCO activity induction reported at the 5 g/L TCA dose that at the 2 g/L dose. None of the difference in increases in liver weight or PCO activity in mice from the 2 or 5 g/L TCA exposures were of the same magnitude as the difference in TCA exposure concentration (i.e., 2.5-fold) except for liver weight from the one experiment in $B6C3F_1$ mice. These are also the data used for comparisons with the Sprague-Dawley rat discussed below.

In regard to strain differences for TCA response in mice, there did not appear to be correlations of the magnitude of 5 g/L TCA-induced changes in percent liver/body weight ratio or PCO activity with the body weights reported for control mice for each strain. The control weights between the four strains of mice varied from 21 to 28 g. The strain with the greatest response (C57Bl/6) for TCA-induced changes in percent liver/body weight ratio (i.e., 1.66-fold of control) and PCO activity (22.13-fold of control) had a mean body weight reported to be 26 g for controls. At this dose, the range of percent liver/body weight for the other strains was reported to be 1.26–1.39-fold of control and the range of PCO activity reported to be of 7.48–8.71-fold of control.

Of note is that in the literature, this study has been cited as providing evidence of differences between rats and mice for peroxisomal response to TCA and DCA. Generally, the PCO data from the Sprague-Dawley rats and $B6C3F_1$ mice at the highest dose of TCA and DCA have been cited. However, the Sprague-Dawley strain was reported to have greater mortality from TCA at this exposure than the other strains tested (i.e., only three rats survived and provided PCO levels) and a lower PCO response (no change in PCO activity over control) that the other two strains tested in this study (i.e., Osborne-Mendel rats was reported to have had 2.38-fold of control and the F344- had a 1.63–1.79-fold of control for PCO activity after exposure to 5 g/L TCA with no mortality). The $B6C3F_1$ mouse was reported to have a 7.78- or 8.71-fold of control for PCO activity from 5 g/L TCA exposure. Certainly, the male mouse is more responsive to TCA induction of PCO activity. However, as discussed above, there are large variations in control levels of PCO activity and in the magnitude and dose-response of TCA-induction of PCO activity between rat and mouse strains and between species. It is not correct to state that the rat is refractory to TCA-induction of peroxisome activity.

Unfortunately, the authors chose the Sprague-Dawley rat (i.e., the most unresponsive strain for PCO activity and most sensitive to toxicity) for studies for comparative studies between DCA and TCA effects. The authors also tested for carnitine acetyl CoA transferase (CAT) activity as a marker of peroxisomal enzyme response and took morphometric analysis of

peroxisome number and cytoplasmic volume for one liver section for each of two $B6C3F_1$ mice or Sprague-Dawley rats from the 5 g/L TCA and 5 g/L DCA treatment groups. Only six electron micrograph fields were analyzed from each section (12 fields total) were analyzed without identification as to what area of the liver lobules they were being taken from. Hence, there is a question as to whether the areas that are known to be peroxisome rich were assayed of not. Also as noted above, previous studies have indicate that such high concentration of DCA and TCA inhibit drinking water consumption and therefore, raising issues not only about toxicity, but also the dose that rats and mice received.

The number of peroxisomes per 100 μm^3 and cytoplasmic volume of peroxisomes was reported to be 6.60 and 1.94%, respectively, for control rats, and 6.89 and 0.61% for control mice, respectively. For 5 g/L TCA and 5 g/L DCA, the numbers of peroxisomes were reported to be increased to 7.14 and 16.75, respectively, in treated Sprague-Dawley rats. Thus, there was 2.5- and 1.08-fold of control reported in peroxisome numbers for 5 g/L DCA and TCA, respectively. The cytoplasmic volume of peroxisomes was reported to be 2.80 and 0.89% for 5 g/L DCA and 5 g/L TCA, respectively (i.e., a 1.44-fold of control and ~60% reduction for 5 g/L DCA and 5 g/L TCA, respectively). Thus, 5 g/L TCA was reported to slightly increase the number of peroxisomes, but decrease the percent of the cytoplasmic volume occupied by peroxisome by half. For DCA, the reported pattern was for both to increase. PCO activity was reported to increase by a similar magnitude as peroxisome numbers but not volume in the 5 g/L TCA treated Sprague-Dawley rats. However, although peroxisomal volume was reported to be cut nearly in half and for peroxisome number to be similar, 5 g/L TCA treatment was not reported to change PCO activity in the Sprague-Dawley rat.

For comparisons between DCA and TCA, $B6C3F_1$ mice were examined at 1, 2, and 5 g/L concentrations. DCA was reported to induce a higher percent liver/body weight ratio that did TCA at every concentration (i.e., 1.55-, 1.27-, and 1.21-fold of control for DCA and 1.39-, 1.16-, and 1.08-fold of control for TCA at 1, 2, and 5 g/L concentrations, respectively). As noted above, for other strains of mice tested and a second experiment with $B6C3F_1$ mice, there was $\leq$40% difference in percent liver/body weight ratio between the 2 and 5 g/L exposures to TCA, but for this experiment, there was a 2.5-fold difference. Thus, at 5 g/L, there was ~40% greater induction of liver weight for DCA than TCA.

In the $B6C3F_1$ mice, 5 g/L TCA was reported to increase peroxisome number to 30.75 and cytoplasmic volume to 4.92% (i.e., 4.4- and 8.1-fold of control, respectively). For 5 g/L DCA treatment, the peroxisome number was reported to be 30.77 and 3.75% (i.e., 4.5- and 6.1-fold of control, respectively). While there was no difference in peroxisome number and ~40% difference in cytoplasmic volume at the 5 g/L exposures of DCA and TCA, there was a greater difference in the magnitude of PCO activity increase. The 5 g/L TCA exposure was reported to induce 4.3-fold of control for PCO activity, while 5 g/L DCA induced as 9.6-fold of control PCO activity (although a figure in the report shows 8.7-fold of control), which is a

~2.5-fold difference between DCA and TCA at this exposure concentration. Thus, for one of the $B6C3F_1$ mouse studies, 5 g/L DCA and TCA treatments were reported to give a similar increase peroxisome number, TCA to induce a 40% greater increase in peroxisomal cytoplasmic volume than DCA and a 2.5-fold greater increase in PCO activity, but DCA to induce ~40% greater liver weight induction than TCA.

Not only were PCO activity, peroxisome number, and cytoplasmic volume occupied by peroxisomes analyzed, but also CAT activity as a measure of peroxisome proliferation. For TCA and DCA, the results were opposite those reported for PCO activity. In Sprague-Dawley rats, control levels of CAT were reported to be 1.81 nmoles of carnitine transferred/min/mg/protein. Exposure to 5 g/L TCA was reported to increase CAT activity by 3.21-fold of control, while 5 g/L DCA was reported to induce CAT activity to 10.33-fold of control levels in Sprague-Dawley rats. However, while PCO activity was reported to be the same as controls and peroxisomal volume decreased, 5 g/L TCA increased CAT activity 3.21-fold of control in these rats. The level of CAT induced by 5 g/L DCA was over 10-fold of control in the rat while peroxisome number was only 2.5-fold of control and cytoplasmic volume 1.4-fold of control. Thus, the fold increases for these three measures were not the same for DCA treatment and for TCA in rats. Nevertheless for CAT, DCA was a stronger inducer in rats than was TCA.

In $B6C3F_1$ mice, 5 g/L TCA and 5 g/L DCA induced CAT activity to a similar extent (4.50- and 5.61-fold of control, respectively). The magnitude of CAT induction was similar to that of peroxisome number for both 5 g/L DCA and 5 g/L TCA and lower than PCO activity in DCA-treated mice and cytoplasmic volume in TCA-treated mice by about half. Thus, using CAT as the marker of peroxisome proliferation, the rat was more responsive than the mouse to DCA and nearly as responsive to TCA as the mouse at this high dose in these two specific strains. These data illustrate the difficulty of using only one measure for peroxisome proliferation and show that the magnitude of increased PCO activity is not necessarily predictive of the peroxisome number or cytoplasmic volume or CAT activity. The difficulty of interpretation of the data from so few animals and sections for the electron microscopy analysis, and the low number of animals for PCO activity and CAT activity (n = 3–6), the high dose studied (5 g/L), and the selection of a rat strain that appears to be more resistant to this activity but more susceptible to toxicity than the others tested, should be taken into account before conclusions can be made about differences between these chemicals for peroxisome activity between species.

Of note is that PCO activity was also shown to be increased by corn oil alone in F344 rats and to potentiate the induction of PCO activity of TCA. After 10 days of exposure to either water, corn oil, 200 mg/kg-day TCA in corn oil, or 200 mg/kg TCA in water via gavage dosing, there was 1.40-fold PCO activity from corn oil treatment alone in comparison to water, a 1.79-fold PCO activity from TCA in water treatment in comparison to water, and a 3.14-fold PCO activity from TCA in corn oil treatment in comparison to water.

The authors provided data for three concentrations of DCA and TCA for Sprague-Dawley and for one experiment in the $B6C3F_1$ mouse for examination of changes in body and percent liver/body weight ratios (1, 2, or 5 g/L DCA or TCA) after 14 days of exposure. As noted above, not only did the 5 g/L exposure concentration of DCA result in mortality in the Sprague-Dawley strain of rat, but the 5 and 2 g/L concentrations of DCA were reported to decrease body weight (~20 and 25%, respectively). The 5 g/L dose of TCA was also reported to induce a statistically significant decrease in body weight in the Sprague-Dawley rat. There were no differences in final body weight in any of the mice exposed to TCA or DCA.

As noted above, no TCA or DCA exposure group of Sprague-Dawley rats was reported to have a statistically significant increase in percent liver/body weight ratio over control. For the $B6C3F_1$ male mice, the percent liver/body weight ratio was 1.22-, 1.27-, and 1.55-fold of control after exposure to 1, 2, and 5 g/L DCA, respectively, and 1.08-, 1.16-, and 1.39-fold of control after exposure to 1, 2, and 5 g/L TCA, respectively. Thus, for DCA, there was only a 20% increase in liver weight corresponding to the twofold increase between the 1 and 2 g/L exposure levels of DCA. Between the 2 and 5 g/L exposure concentrations of DCA, there was a 2-fold increase in liver weight corresponding to a 2.5-fold increase in exposure concentration. For TCA, the magnitude of increase in dose was reported to be proportional to the magnitude of increase in percent liver/body weight ratio in the $B6C3F_1$ male mouse. As stated above, the correspondence between magnitude of dose and percent liver weight for TCA exposure in this experiment differed from the other experiment reported for this strain of mouse and also differed from the other three strains of mice examined in this study where the magnitude in liver weight gain was much less than exposure concentration.

E.2.3.2. Subchronic and Chronic Studies of DCA and TCA

Several experiments have been conducted with exposure to DCA and TCA, generally at very high levels with a limited dose range, for less periods of time than standard carcinogenicity bioassays, and with very limited information on any endpoints other than the liver tumor induction. Caldwell and Keshava (2006) and Caldwell et al. (2008b) have examined these studies for inferences of modes of action for TCE. Key studies are briefly described below for comparative purposes of results reported in TCE studies.

E.2.3.2.1. Snyder et al. (1995)

Studies of TCE have reported either no change or a slight increase in apoptosis only after a relatively high exposure level (Channel et al., 1998; Dees and Travis, 1993). Inhibition of apoptosis, which has been suggested to prevent removal of "initiated" cells from the liver and lead to increased survival of precancerous cells, has been proposed as part of the mode of action for peroxisome proliferators (see Section E.3.4). The focus of this study was to examine whether DCA, which has been shown to inhibit DNA synthesis after an initial transient increase (see

Section E.2.3.1.1), also alters the frequency of spontaneous apoptosis in mice. This study exposed 28-day-old male B6C3F$_1$ male mice (n = 5) to 0, 0.5 or 5.0 g/L buffered DCA in drinking water for up to 30 days (Phase I = 5–15 days exposure and Phase II = 20–30 days treatment).

Portions of the left lobe of the liver were prepared for histological examination after H&E staining. Hepatocyte number was determined by counting nuclei in 50 fields with nonparenchymal cell nuclei excluded on the basis of nuclear size. Multinucleate cells were counted as one cell. Apoptotic cells were visualized by in situ TDT nick end-labeling assay from 2 to 4 different liver sections from each control or treated animal. The average number of apoptotic cells was then determined for each animal in each group. The authors reported that in none of the tissues examined were necrotic foci observed, there was no any indication of lymphocyte or neutrophil infiltration indicative of an inflammatory response, and suggested that no necrotic cells contributed to the responses in their analysis.

Control animals were reported to exhibit apoptotic frequencies ranging from ~0.04 to 0.085% and that over the 30-day period the frequency rate declined. The authors suggested that this result is consistent with reports of the livers of these young animals undergoing rapid changes in cell death and proliferation. They note that animals receiving 0.5 g/L DCA also had a similar trend of decreasing apoptosis with age, supportive of the decrease being a physiological phenomenon. The 0.5 g/L exposure level of DCA was reported to decrease the percentage of apoptotic hepatocytes as the earliest time point studied and to remain statistically significantly decreased from controls from 5 to 30 days of exposure. The rate of apoptosis ranged from ~0.025 to 0.060% after 0.5 g/L DCA exposure during the 30-day period (i.e., and ~30–40% reduction). Animals receiving the 5.0 g/L DCA dose exhibited a significant reduction at the earliest time point that was sustained at a similar level and statistically significant throughout the time-course of the experiment (percent apoptosis ranged from 0.015 to 0.030%).

The results of this study not only provide a baseline of apoptosis in the mouse liver, which is very low, but also show the importance of taking into account the effects of age on such determinations. The authors reported that the for rat liver, the estimated frequency of spontaneous apoptosis to be ~0.1%, and therefore, greater than that of the mouse. The significance of the DCA-induced reduction in apoptosis, of a level that is already inherently low in the mouse, for the mode of action for induction of cancer is difficult to discern.

E.2.3.2.2. Mather et al. (1990)

This 90-day study in male Sprague-Dawley rats examined the body and organ weight changes, liver enzyme levels, and PCO activity in livers from rats treated with estimated concentrations of 3.9, 35.5, 345 mg/kg-day DCA or 4.1, 36.5, or 355 mg/kg-day TCA from drinking water exposures (i.e., 0, 50, 500, and 5,000 ppm or 0.05, 0.5, or 5.0 g/L DCA or TCA in the drinking water). All dose levels of DCA and TCA were reported to result in a dose-

dependent decrease in fluid intake at 2 months of exposure. The rats were 9 (DCA) or 10 (TCA) weeks old at the beginning of the study (n = 10/group). Animals with body weights that varied >20% of mean weights were discarded from the study. The DCA and TCA solutions were neutralized. The mean values for initial weights of the animals in each test group varied <3%.

DCA treatment induced a dose-related decrease in body weight that was statistically significant at the two highest levels (i.e., a 6, 9.5, and 17% decrease from control). TCA treatment also resulted in lower body weights that were not statistically significant (i.e., 2.1, 4.4, and 5.9%). DCA treatments were reported to result in a dose-related increase in absolute liver weights (1.01-, 1.13-, and 1.36-fold of control that were significantly different at the highest level) and percent liver/body weight ratios (1.07-, 1.24-, and 1.69-fold of control that were significant at the two highest dose levels). TCA treatments were reported to not result in changes in either absolute liver weights or percent liver/body weight ratios with the exception of statistically significant increase in percent liver/body weight ratios at the highest level of treatment (1.02-fold of control).

Total serum protein levels were reported to be significantly depressed in all animals treated with DCA with animals in the two highest dose groups also exhibiting elevations of ALP. Alanine-aminotransferase levels were reported to be elevated only in the highest treatment group. No consistent treatment-related effect on serum chemistry was reported to be observed for the TCA-treated animals with data not shown.

In terms of PCO activity, there was only a mild increase at the highest dose of 15% for TCA and a 2.5-fold level of control for DCA treatment that were statistically significant. The difference in PCO activity between control groups for the DCA and TCA experiments was reported to be 33%. No treatment effect was reported to be apparent for hepatic microsomal enzymes, or measures of immunotoxicity for either DCA or TCA, but data were not shown. Focal areas of hepatocellular enlargement in both DCA- and TCA-treated rats were reported to be present with intracellular swelling more severe with the highest dose of DCA treatment. Livers from DCA treated rats were reported to stain positively for PAS, indicating significant amounts of glycogen with TCA treated rats reported to display "less evidence of glycogen accumulation." Of note is that, in this study of rats, DCA was reported to induce a greater level of PCO activity than did TCA.

E.2.3.2.3. Parrish et al. (1996)

Parrish et al. (1996) exposed male B6C3F$_1$ mice (8 weeks old and 20–22 g upon purchase) to TCA or DCA (0, 0.01, 0.5, and 2.0 g/L) for 3 or 10 weeks (n = 6). Livers were excised and nuclei isolated for examination of 8-OHdG and homogenates examined for cyanide insensitive acyl-CoA oxidase (ACO) and laurate hydroxylase activity. The authors noted that control values between experiments varied as much as a factor of twofold for PCO activity and that data were presented as percent of concurrent controls. Initial body weights for treatment

groups were not presented and thus, differences in mean values between the groups cannot be ascertained.

Final body weights were reported to not be statistically significantly changed by DCA or TCA treatments at 21 or 71 days of treatment (all were within ~8% of controls). The mean percent liver/body ratios were reported to be 5.4, 5.3, 6.1, and 7.2% for control, 0.1, 0.5, and 2.0 g/L TCA, respectively, and 5.4, 5.5, 6.7, and 7.9% for control, 0.1, 0.5, and 2.0 g/L DCA, respectively, after 21 days of exposure. This represents 0.98-, 1.13-, and 1.33-fold of control levels with these exposure levels of TCA and 1.02-, 1.24-, and 1.46-fold of control levels with DCA after 21 days of exposure. For 71 days of exposure, the mean percent liver/body ratios were reported to be 5.1, 4.6, 5.8, and 6.9% for control, 0.1, 0.5, and 2.0 g/L TCA, respectively and 5.1, 5.1, 5.9, and 8.5% for control, 0.1, 0.5, and 2.0 g/L DCA, respectively. This represents 0.90-, 1.14-, and 1.35-fold of control with TCA exposure and 1.0-, 1.15-, and 1.67-fold of control with DCA exposure after 71 days of exposure. The magnitude of difference between the 0.1 and 0.5 g/L TCA doses is 5, and between 0.5 and 2.0 g/L doses is fourfold.

For the 21- and 71-day exposures the magnitudes of the increases in percent liver/body weight over control values were greater for DCA than TCA exposure at same concentration with the exception of 0.5 g/L doses at 71 days in which both TCA and DCA induced similar increases. For TCA, the 0.01 g/L dose produces a similar 10% decrease in percent liver/body weight. Although there was a fourfold increase in magnitude between the 0.5 and 2.0 g/L TCA exposure concentrations, the magnitude of increase for percent liver/body weight increase was 2.5-fold between them at both 21 and 71 days of exposure. For DCA, the 0.1 g/L dose was reported to have a similar value as control for percent liver/body weight ratio. Although there was a 4-fold difference in dose between the 0.5 and 2.0 g/L DCA exposure concentrations, there was a ~2-fold increase in percent liver/body weight increase at 21 days and ~4.5-fold increase at 71 days.

As a percentage of control values, TCA was reported to induce a dose-related increase in PCO activity at 21 days (~1.5-, 2.2-, and ~4.1-fold of control, for 0.1, 0.5, and 2 g/L TCA exposures). Only the 2.0 g/L dose of DCA was reported to induce a statistically significant increase at 21 days of exposure of PCO activity over control (~1.8-fold of control) with the 0.1 and 0.5 g/L exposure PCO activity to be slightly less than control values (~20% less). Thus, although there was no increase in percent liver/body weight at 0.1 g/L TCA, the PCO activity was reported to be increased by ~50% after 21 days. A 13% increase in liver weight at 0.5 g/L TCA was reported to be associated with 2.2-fold of control level of PCO activity and a 33% increase in liver weight after 2.0 g/L TCA to be associated with 4.1-fold of control level of PCO activity.

Thus, increases in PCO activity were not necessarily correlated with concurrent TCA-induced increases in liver weight and the magnitudes of increase in liver weight between 0.5 and 2.0 g/L TCA (2.5-fold) was greater than the corresponding increase in PCO activity

(1.8-fold of control). Although there was a 20-fold difference in TCA dose, the magnitude of increase in PCO activity between 0.1 and 2.0 g/L TCA was ~2.7-fold. As stated above, the 4-fold difference in TCA dose at the two highest levels resulted in a 2.5-fold increase in liver weight. For DCA, the increases in liver weight at 0.1 and 0.5 g/L DCA exposures were not associated with increased PCO activity after 21 days of exposure. The 2.0 g/L DCA exposure concentration was reported to induce 1.8-fold of control PCO activity.

After 71 days of treatment, TCA induced a dose-related increase in PCO activity that was approximately twice the magnitude as that reported at 21 days (i.e., ~9-fold greater at 2.0 g/L). After 71 days, for DCA the 0.1 and 0.5 g/L doses produced a statistically significant increase in PCO activity (~1.5- and 2.5-fold of control, respectively). The administration of 1.25 g/L clofibric acid in drinking water was used as a positive control and reported to induce approximately six- to sevenfold of control PCO activity at 21 and 71 days of exposure.

Laurate hydroxylase activity was reported to be elevated significantly only by TCA at 21 days (2.0 g/L TCA dose only) and to increased to approximately the same extent (~1.4–1.6-fold of control values) at all doses tested. For 0.1 g/L DCA, the laurate hydroxylase activity was reported to be similar to that of 0.1 g/L TCA (~1.4-fold of control) but to be ~1.2-fold of control at both the 0.5 and 2.0 g/L DCA exposures. At 71 days, both the 0.5 and 2.0 g/L TCA exposures induced a statistically significant increase in laurate hydroxylase activity (i.e., 1.6- and 2.5-fold of control, respectively) with no change after DCA exposure. The actual data rather than percent of control values were reported for laurate hydroxylase activity. The control values for laurate hydroxylase activity varied 1.7-fold between 21 and 71 days experiments.

The results for 8-OHdG levels are discussed in Section E.3.4.2.3. Of note is that the increases in PCO activity noted for DCA and TCA were not associated with 8-OHdG levels (which were unchanged, see Section E.3.4.2.3) and also not with changes laurate hydrolase activity or percent liver/body weight ratio increases observed after either DCA or TCA exposure. A strength of this study is that it examined exposure concentrations that were lower than those examined in many other short-term studies of DCA and TCA.

E.2.3.2.4. Bull et al. (1990)

The focus of this study was the determination of "dose-response relationships in the tumorigenic response to these chemicals [sic DCA and TCA] in $B6C3F_1$ mice, determine the nature of the nontumor pathology that results from the administration of these compounds in drinking water, and test the reversibility of the response." Male and female $B6C3F_1$ mice (age 37 days) were treated from 15 to 52 weeks with neutralized TCA and TCA. A highly variable number and generally low number of animals were reported to be examined in the study with n = 5 for all time periods except for 52 weeks where in males the n = 35 for controls, n =11 for 1 g/L DCA, n = 24 for 2 g/L DCA, n = 11 for 1 g/L TCA, and n = 24 for 2 g/L TCA exposed

mice. Female mice were only examined after 52 weeks of exposure and the number of animals examined was n = 10 for control, 2 g/L DCA, and 2 g/L TCA exposed mice.

"Lesions to be examined histologically for pathological examination were selected by a random process" with lesions reported to be selected from 31 of 65 animals with lesions at necropsy. 73 of 165 lesions identified in 41 animals were reported to be examined histologically. All hyperplastic nodules, adenomas, and carcinomas were lumped together and characterized as hepatoproliferative lesions. Accordingly, there were only exposure concentrations available for dose-response analyses in males and only "multiplicity of hepatoproliferative lesions" were reported from random samples. Thus, these data cannot be compared to other studies and are unsuitable for dose-response with inadequate analysis performed on random samples for pathological examination.

The authors state that some of the lesions taken at necropsy and assumed to be proliferative were actually histologically normal, necrotic, or an abscess as well. It is also limited by a relatively small number of animals examined in regard to adequate statistical power to determine quantitative differences. Similar concerns were raised by Caldwell et al. (2008b) with a subsequent study (eg., Bull et al., 2002). For example, the authors report that 5/11 animals had "lesions" at 1 g/L TCA at 52 weeks and 19/24 animals had lesions at 2 g/L TCA at 52 weeks. However, while 7 lesions were examined in 5 mice bearing lesions at 1 g/L TCA, only 16 of 30 lesions from 11 of the 19 animals bearing lesions examined in the 2 g/L TCA group. Therefore, almost half of the mice with lesions were not examined histologically in that group along with only half of the "lesions."

The authors reported the effects of DCA and TCA exposure on liver weight and percent liver/body changes ($m \pm$ SEM) and these results gave a pattern of hepatomegaly generally consistent with short-term exposure studies. The authors report "no treatment produced significant changes in the body weight or kidney weight of the animals (data not shown)."

In male mice (n = 5) at 37 weeks of exposure, liver weights were reported to be 1.6 ± 0.1, 2.5 ± 0.1, and 1.9 ± 0.1 g for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively. The percent liver/body weights were reported to be 4.1 ± 0.3, 7.3 ± 0.2, and 5.1 ± 0.1% for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively. In male mice at 52 weeks of exposure, liver weights were reported to be 1.7 ± 0.1, 2.5 ± 0.1, 5.1 ± 0.1, 2.2 ± 0.1, and 2.7 ± 0.1 g for control (n = 35), 1 g/L DCA (n = 11), 2 g/L DCA (n = 24), 1 g/L TCA (n = 11), and 2 g/L TCA (n = 24) exposed mice, respectively. In male mice at 52 weeks of exposure, percent liver/body weights were reported to be 4.6 ± 0.1, 6.5 ± 0.2, 10.5 ± 0.4, 6.0 ± 0.3, and 7.5 ± 0.5% for control, 1 g/L DCA, 2 g/L DCA, 1 g/L TCA, and 2 g/L TCA exposed mice, respectively. For female mice (n = 10) at 52 weeks of exposure, liver weights were reported to be 1.3 ± 0.1, 2.6 ± 0.1, and 1.7 ± 0.1 g for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively. The percent liver/body weights were reported to be 4.8 ± 0.3, 9.0 ± 0.2, and 6.0 ± 0.3% for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively.

Although the number of animals examined varied threefold between treatment groups in male mice, the authors reported that all DCA and TCA treatments were statistically increased over control values for liver weight and percent body/liver weight in both genders of mice. In terms of percent liver/body weight ratio, female mice appeared to be as responsive as males at the exposure concentration tested. Thus, hepatomegaly reported at these exposure levels after short-term exposures appeared to be further increased by chronic exposure with equivalent levels of DCA inducing greater hepatomegaly than TCA.

Interestingly, after 37 weeks of treatment and then a cessation of exposure for 15 weeks, liver weights were assessed in control male mice, 2 g/L DCA treated mice, and 2 g/L TCA treated mice (n = 11 for each group but results for controls were pooled and therefore, n = 35). Liver weights were reported to be 1.7 ± 0.1, 2.2 ± 0.1, and 1.9 ± 0.1 g for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively. The percent liver/body weights were reported to be 4.6 ± 0.1, 5.7 ± 0.3, and 5.4 ± 0.2% for control, 2 g/L DCA, and 2 g/L TCA exposed mice, respectively. After 15 weeks of cessation of exposure, liver weight and percent liver/body weight were reported to still be statistically significantly elevated after DCA or TCA treatment.

The authors partially attributed the remaining increases in liver weight to the continued presence of hyperplastic nodules in the liver. The authors stated that because of the low incidence of lesions in the control group and the two groups that had treatments suspended, all of the lesions from these groups were included for histological sectioning. However, the authors presented a table indicating that, of the 23 lesions detected in seven mice exposed to DCA for 37 weeks, 19 were examined histologically. Therefore, groups that were exposed for 52 weeks had a different procedure for tissue examination as those at 37 weeks.

In terms of liver tumor induction, the authors stated that "statistical analysis of tumor incidence employed a general linear model ANOVA with contrasts for linearity and deviations from linearity to determine if results from groups in which treatments were discontinued after 37 weeks were lower than would have been predicted by the total dose consumed." The multiplicity of tumors observed in male mice exposed to DCA or TCA at 37 weeks and then sacrificed at 52 weeks were reported by the authors to have a response in animals that received DCA very close to that which would be predicted from the total dose consumed by these animals. The response to TCA was reported by the authors to deviate significantly ($p = 0.022$) from the linear model predicted by the total dose consumed.

Multiplicity of lesions per mouse and not incidence was used as the measure. Most importantly, the data used to predict the dose response for "lesions" used a different methodology at 52 weeks than those at 37 weeks. Not only were not all animal's lesions examined but foci, adenomas, and carcinomas were combined into one measure. Therefore, foci, of which a certain percentage have been commonly shown to spontaneously regress with time, were included in the calculation of total "lesions." Pereira and Phelps (1996) note that in initiated mice treated with DCA, the yield of altered hepatocytes decreases as the tumor yields

increase between 31 and 51 weeks of exposure suggesting progression of foci to adenomas. Initiated and noninitiated control mice also had fewer foci/mouse with time.

Because of differences in methodology and the lack of discernment between foci, adenomas, and carcinomas for many of the mice exposed for 52 weeks, it is difficult to compare differences in composition of the "lesions" after cessation of exposure. For TCA treatment, the number of animals examined for determination of which "lesions" were foci, adenomas, and carcinomas was 11/19 mice with "lesions" at 52 weeks, while all 4 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were examined.

For DCA treatment, the number of animals examined was only 10/23 mice with "lesions" at 52 weeks while all 7 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were examined. Most importantly, when lesions were examined microscopically, they did not all turn out to be preneoplastic or neoplastic. Two lesions appeared "to be histologically normal" and one necrotic. Not only were a smaller number of animals examined for the cessation exposure than continuous exposure, but only the 2 g/L exposure levels of DCA and TCA were studied for cessation. The number of animals bearing "lesions" at 37 and then 15 week cessation weeks was 7/11 (64%) while the number of animals bearing lesions at 5 weeks was 23/24 (96%) after 2 g/L DCA exposure. For TCA, the number of animals bearing lesions at 37 weeks and then 15 weeks cessation was 4/11 (35%), while the number of animals bearing lesions at 52 weeks was 19/24 (80%). While suggesting that cessation of exposure diminished the number of "lesions," conclusions regarding the identity and progression of those lesions with continuous vs. noncontinuous DCA and TCA treatment are tenuous.

Macroscopically, the "livers of many mice receiving DCA in their drinking water displayed light colored streaks on the surface" at every sacrifice period and "corresponded with multi-focal areas of necrosis with frequent infiltration of lymphocytes." At the light microscopic level, the lesions were described to also be present in the interior of the liver as well. For TCA-treated mice, "similar necrotic lesions were also observed… but at a much lower frequency, making it difficult to determine if they were treatment-related." Control animals were reported not to show degenerative changes. "Marked cytomegaly" was reported for mice treated with either 1 or 2 g/L DCA "throughout the liver." In regard to cell size, the authors did not give any description in the methods section of the paper as to how sections were selected for morphometric analysis or what areas of the liver acinus were examined but reported after 52 weeks of treatment the long axis of hepatocytes measured (mean ± S.E.) 24.9 ± 0.3, 38.5 ± 1.0, and 29.3 ± 1.4 μm in control, DCA-, and TCA-treated mice, respectively.

Mice treated with TCA (2 g/L) for 52 weeks were reported to have livers with "considerable dose-related accumulations of lipofuscin." However, no quantitative analyses were presented. A series of figures representative of treatment showed photographs (1,000×) of lipofuscin fluorescence indicating greater fluorescence in TCA treated liver than control or DCA treated liver.

A series of photographs of H&E sections in the report (see Figures 2a, b, and c) were shown as representative histology of control mice, mice treated with 2 g/L DCA and 2 g/L TCA. The area of the liver from which the photographs were taken did not include either portal tract or central veins and the authors did not give the zone of the livers from which they were taken. The figure representing TCA treatment shows only a mild increase in cell volume in comparison to controls, while for DCA treatment, the hepatocyte diameter was greatly enlarged, pale stained so that cytoplasmic contents appear absent, nuclei often pushed to the cell perimeter, and the sinusoids appearing to be obscured by the swollen hepatocytes. The apparent reduction of sinusoidal volume by the enlarged hepatocytes raises the possibility of decreased blood flow through the liver, which may have been linked to focal areas of necrosis reported for this high exposure level.

In a second set of figures, glycogen accumulation was shown with PAS staining at the same level of power (400×) for the same animals. In control animals, PAS-positive material was not uniformly distributed between or within hepatocytes but tended to show a zonal pattern of moderate intensity. PAS positive staining (which the authors reported to be glycogen) appeared to be slightly less than controls but with a similar pattern in the photograph representing TCA exposure. However, for DCA, the photograph showed a uniform and heavy stain within each hepatocyte and across all hepatocytes.

The authors stated in the results section of the paper that "the livers of TCA-treated animals displayed less evidence of glycogen accumulation and it was more prominent in periportal than centrilobular portions of the liver acinus." In their abstract they state "TCA produced small increases in cell size and a much more modest accumulation of glycogen." Thus, the statement in the text, which is suggestive that TCA induced an increase in glycogen over controls that was not as much as that induced by DCA, and the statement in the abstract, which concludes TCA exposure increased glycogen is not consistent with the photographs. In the photograph shown for TCA, there is less not more PAS-positive staining associated with TCA treatment in comparison to controls.

In Sanchez and Bull (1990), the authors report that "TCA exposure induced a much less intense level of PAS staining that was confined to periportal areas" but do not compare PAS staining to controls but only to DCA treatment. In the discussion section of the paper, the authors state "Except for a small increase in liver weight and cell size, the effects produced by DCA were not observed with TCA." Thus, there seems to be a discrepancy with regard to what the effects of TCA are in relation to control animals from this report that has caused confusion in the literature. Kato-Weinstein et al. (2001) reported that in male mice exposed to DCA and TCA the DCA increased glycogen and TCA decreased glycogen content of the liver using chemical measurement of glycogen in liver homogenates and using ethanol-fixed sections stained with PAS, a procedure designed to minimize glycogen loss.

E.2.3.2.5. Nelson et al. (1990)

Nelson et al. (1990) reported that they used the same exposure paradigm as Herren-Freund et al. (1987), with little description of methods used in treatment of the animals. Male $B6C3F_1$ mice were reported to be exposed to DCA (1 or 2 g/L) or TCA (1 or 2 g/L) for 52 weeks. The number of animals examined for nontumor tissue was 12 for controls. The number of animals varied from two to eight for examination of nontumor tissue, hyperplastic nodules, and carcinoma tissues for c-Myc expression. There was no description for how hyperplastic nodules were defined and whether they included adenomas and foci. For the 52-week experiments, the results were pooled for lesions that had been obtained by exposure to the higher or lower concentrations of DCA or TCA (i.e., the TCA results are for lesions induced by either 1.0 or 2.0 g/L TCA).

A second group of mice were reported to be given either DCA or TCA for 37 weeks and then normal drinking water for the remaining time until 52 weeks with no concentrations given for the exposures to these animals. Therefore, it is impossible to discern what dose was used for tumors analyzed for c-Myc expression in the 37-week treatment groups and if the same dose was used for 37 and 52 week results.

Autoradiography was described for three different sections per animal in five different randomly chosen high power fields per section. The number of hyperplastic nodules or the number of carcinomas per animal induced by these treatments was not reported nor the criteria for selection of lesions for c-Myc expression. Apparently, a second experiment was performed to determine the expression of c-H-ras. Whereas in the first experiment, there were no hyperplastic nodules, in the second, one control animal was reported to have a hyperplastic nodule. The number of control animals reported to be examined for nontumor tissue in the second group was 12. The numbers of animals in the second group was reported to vary from one to seven for examination of nontumor tissue, hyperplastic nodules, and carcinoma tissues for c-H-ras expression. The number of animals per group for the investigation of H-ras did not match the numbers reported for that of c-Myc. The number of animals treated to obtain the "lesion" results was not presented (i.e., how many animals were tested to get a specific number of animals with tumors that were then examined). The number of lesions assessed per animal was not reported.

At 52 weeks of exposure, hyperplastic nodules (n = 8 animals) and carcinomas (n = 6 animals) were reported to have approximately twofold expression of c-Myc relative to nontumor tissue (n = 6 animals) after DCA treatment. After 37 weeks of DCA treatment and cessation of exposure, there was a ~30% increase in c-Myc in hyperplastic nodules (n = 4 animals) that was not statistically significant. There were no carcinomas reported at this time.

After 52 weeks of TCA exposure, there was approximately twofold of nontumor tissue reported for c-Myc in hyperplastic nodules (n = 6 animals) and approximately threefold reported for carcinomas (n = 6 animals). After 37 weeks of TCA exposure, there was ~2-fold c-Myc in

hyperplastic nodules (n = 2 animals) that was not statistically significant and ~2.6-fold increase in carcinomas (n = 3 animals) that was reported to be statistically significant over nontumor tissue. There was no difference in c-Myc expression between untreated animals and nontumor tissue in the treated animals.

The authors reported that c-Myc expression in TCA-induced carcinomas was "almost 6 times that in control tissue (corrected by subtracting nonspecific binding)," and concluded that c-Myc in TCA-induced carcinomas was significantly greater than in hyperplastic nodules or carcinomas and hyperplastic nodules induced by DCA. However, the c-Myc expression reported as the number of grains per cells was ~2.6-fold in TCA-induced carcinomas and ~2-fold in DCA-induced carcinomas than control or nontumor tissue at 52 weeks. The hyperplastic nodules from DCA and TCA treatments at 52 weeks gave identical ratios of approximately twofold. In three animals per treatment, c-Myc expression was reported to be similar in "selected areas of high expression" for either DCA or TCA treatments of 52 weeks.

There did not appear to be a difference in c-H-ras expression between control and nontumor tissue from DCA- or TCA-treated mice. The levels of c-H-ras transcripts were reported to be "slightly elevated" in hyperplastic nodules induced by DCA (~67%) or TCA (~43%) but these elevations were not statistically significant in comparison to controls. However, carcinomas "derived from either DCA- or TCA-treated animals were reported to have significantly increased c-H-ras levels relative to controls." The fold increase of nontumor tissue at 52 weeks for DCA-induced carcinomas was ~2.5-fold, and for TCA induced carcinomas, ~2.0-fold. Again, the authors stated that "if corrected for nonspecific hybridization, carcinomas expressed approximately 4 times as much c-H-ras than observed in surrounding tissues" Given that control and nontumor tissue results were given as the controls for the expression increases observed in "lesions," it is unclear what the usefulness of this "correction" is. The authors reported that "focal areas of increased expression of c-H-ras were not observed within carcinomas."

The limitations of this experiment include uncertainty as to what doses were used and how many animals were exposed to produce animals with tumors. In addition, results of differing doses were pooled and the term hyperplastic nodule was undefined. The authors state that c-Myc expression in itself is not sufficient for transformation and that its overexpression commonly occurs in malignancy. They also state that "Unfortunately, the limited amount of tissue available prevented a more serious pursuit of this question in the present study." In regard to the effects of cessation of exposure, the authors do not present data on how many animals were tested with the cessation protocol, what doses were used, and how many lesions comprised their results and thus, comparisons between these results and those from 52 weeks of continuous exposure are hard to make. Quantitatively, the small number of animals, whose lesions were tested, was n = 2–4 for the cessation groups. Bull et al. (1990) is given as the source of data for the cessation experiment (see Section E.2.3.2.4).

E.2.3.2.6. DeAngelo et al. (1999)

The focus of this study was to "determine a dose response for the hepatocarcinogenicity of DCA in male mice over a lifetime exposure and to examined several modes of action that might underlie the carcinogenic process." As DeAngelo et al. (1999) pointed out, many studies of DCA had been conducted at high concentrations and were less-than-lifetime studies, and therefore, were of suspect relevance to environmental concentrations. This study is one of the few that examined DCA at a range of exposure concentrations to determine a dose-response in mice. The authors concluded that DCA-induced carcinogenesis was not dependent on peroxisome proliferation or chemically sustained proliferation. The number of HCCs/animals was reported to be significantly increased over controls at all DCA treatments including 0.05 g/L and a NOEL was not observed. Peroxisome proliferation was reported to be significantly increased at 3.5 g/L DCA only at 26 weeks and did not correlate with tumor response. No significant treatment effects on labeling of hepatocytes (as a measure of proliferation) outside proliferative lesions were reported, and thus, the DCA-induced liver cancer was not dependent on peroxisome proliferation or chemically sustained cell proliferation.

Male B6C3F_1 mice were 28–30 days of age at the start of study and weighed 18–21 g (or ~14% range). They were exposed to 0, 0.05, 0.5, 1.0, 2.0, and 3.5 g/L DCA via drinking water as a neutralized solution. The time-weighted mean daily water consumption calculated over the 100-week treatment period was reported to be 147, 153, 158, 151, 147, and 124 (84% of controls) mL/kg/day for 0, 0.05, 0.5, 1, 2, and 3.5 g/L DCA, respectively. The number of animals used for interim sacrifices was 35, 30, 30, 30, and 30 for controls, 0.5, 1.0, 2.0, and 3.5 g/L DCA-treated groups respectively (i.e., 10 mice per treatment group at interim sacrifices of 26, 52, and 78 weeks). The number of animals at final sacrifice was reported to be 50, 33, 24, 32, 14 and 8 for controls, 0.05, 0.5, 1.0, 2.0, and 3.5 g/L DCA-treated groups respectively. The number of animals with unscheduled deaths before final sacrifice was reported to be 3, 2, 1, 9, 11, and 8 for controls, 0.05, 0.5, 1.0, 2.0, and 3.5 g/L DCA-treated groups respectively. The Authors reported that early mortality tended to occur from liver cancer.

The number of animals examined for pathology were reported to be 85, 33, 55, 65, 51, and 41 for controls, 0.05, 0.5, 1.0, 2.0, and 3.5 g/L DCA treated groups, respectively. The experiment was conducted in two parts with control, 0.5, 1.0, 2.0, and 3.5 g/L groups treated and then 1 months later, a second group consisting of 30 control group mice and 35 mice in a 0.05 g/L DCA exposure group were studied.

The authors reported no difference in prevalence and multiplicity of hepatocellular neoplasms in the two groups so that data were summed and reported together. The number of animals reported as examined for tumors were n = 10 animals, with controls reported to be 35 animals split among three interim sacrifice times—exact number per sacrifice time is unknown. The number of animals reported "with pathology" and assumed to be included in the

tumor analyses from Table 1, and the sum of the number of animals "scheduled for sacrifice that survived until 100 weeks" and "interim sacrifices" do not equal each other. For the 1 g/L DCA exposure group, 30 animals were sacrificed at interim periods 32 animals were sacrificed at 100 weeks and 9 animals were reported to have unscheduled deaths, but of those 71 animals, only 65 animals were reported to have pathology for the group. Therefore, some portion of animals with unscheduled deaths must have been included in the tumor analyses. The exact number of animals that may have died prematurely but included in analyses of pathology for the 100-week group is unknown.

In Figure 3 of the study, the authors reported prevalence and multiplicity of HCCs following 79–100 weeks of DCA exposure in their drinking water. The number of animals in each dose group used in the tumor analysis for 100 weeks was not given by the authors. Given that the authors included animals that survived past the 78-week interim sacrifice period but died unscheduled deaths in their 100-week results, the number must have been greater than those reported as present at final sacrifice. A comparison of the data for the 100-week data presented in Table 3a and Figure 3 shows that the data reported for 100 weeks is actually for animals that survived from 79 to 100 weeks.

The authors report a dose-response that is statistically significant from 0.5 to 3.5 g/L DCA for HCC incidence and a dose-response in HCC multiplicity that is significantly increased over controls from 0.05 to 0.5 g/L DCA that survived 79–100 weeks of exposure (i.e., 0, 8-, 84-, 168-, 315-, and 429 mg/kg-day dose groups with prevalences of 26, 33, 48, 71, 95, and 100%, respectively, and multiplicities of 0.28, 0.58, 0.68, 1.29, 2.47, and 2.90, respectively). Hepatocellular adenoma incidence or multiplicity was not reported for the 0.05 g/L DCA exposure group.

In Table 3 of the report, the time course of HCCs and adenoma development are given and summarized in Table E-2.

Table E-2. Prevalence and multiplicity data from DeAngelo et al. (1999)

Prevalence	Multiplicity (lesions/animal *m* ± SEM)	
	Carcinomas	Adenomas
52 wks control = 0% carcinomas, 0% adenoma	0	0
0.5 g/L DCA = 0/10 carcinoma, 1/10 adenomas	0	0.10 ± 0.09
1.0 g/L DCA = 0/10 carcinomas, 1/10 adenomas	0	0.10 ± 0.09
2.0 g/L DCA = 2/10 carcinomas, 0/10 adenomas	0.20 ± 0.13	0
3.5 g/L DCA = 5/10 carcinomas, 5/10 adenomas	0.70 ± 0.25	0.80 ±0.31
78 wks control = 10% carcinomas, 10% adenomas	0.10 ± 0.10	0.10 ± 0.09
0.5 g/L DCA = 0/10carcinoma, 1/10 adenomas	0	0.10 ± 0.09
1.0 g/L DCA = 2/10 carcinomas, 2/10 adenomas	0.20 ± 0.13	0.20 ± 0.13
2.0 g/L DCA = 5/10 carcinomas, 5/10 adenomas	1.0 ± 0.47	1.00 ± -0.42
3.5 g/L DCA = 7/10 carcinomas, 5/10 adenomas	1.20 ± 0.37	1.00 ± 0.42
100 wks control = 26% carcinoma, 10% adenoma	0.28 ± 0.07	0.12 ± 0.05
0.5 g/L DCA = 48% carcinoma, 20% adenomas	0. 68 ± 0.17	0.32 ± 0.14
1.0 g/L DCA = 71% carcinomas, 51.4% adenomas	1.29 ± 0.17	0.80 ± 0.17
2.0 g/L DCA = 95% carcinomas, 42.9% adenomas	2.47 ± 0.29	0.57 ± 0.16
3.5 g/L DCA = 100% carcinomas, 45% adenomas	2.90 ± 0.40	0.64 ± 0.23

The authors reported HCCs and number of lesions/animal in mice that survived 79–100 weeks of exposure. They combined exposure groups to be animals after the week 78 sacrifice time that did and did not make it to 100 weeks. These are the same data reported above for the 100-week exposure with the inclusion of the 0.05 g/L DCA data. The difference between number of animals at interim and final sacrifices and those "with pathology" and used in the tumor analysis but most likely coming from unscheduled deaths is reported in Table E-3 as "extra" and varied across treatment groups.

Table E-3. Difference in pathology by inclusion of unscheduled deaths from DeAngelo et al. (1999)

Dose = prevalence of hepatocellular carcinoma	Number hepatocellular carcinoma/animal	n = at 100 wks	Extra added in
Control = 26%	0.28	50	0
0.05 g/L = 33%	0.58	33	0
0.5 g/L = 48%	0.68	24	1
1 g/L = 71%	1.29	32	3
2 g/L = 95%	2.47	14	7
3.5 g/L = 100%	2.9	8	3

These data show a dose-related increase in tumor formation and decrease in time-to-tumor associated with DCA exposure at the lowest levels examined. These findings are limited

by the small number of animals examined at 100 weeks but especially those examined at "interim sacrifice" periods (n = 10). The data illustrate the importance of examining multiple exposure levels at lower concentrations at longer durations of exposure and with an adequate number of animals to determine the nature of a carcinogenic response.

Preneoplastic and non-neoplastic hepatic changes were reported to have been described previously and summarized as large preneoplastic foci observed at 52 weeks with multiplicities of 0.1, 0.1, 0.2 and 0.16 for 0.5, 1, 2, and 3.5 g/L DCA exposure, respectively. At 100 weeks, all values were reported to be significant (0.03, 0.06, 0.14, 0.27 for 0.5, 1, 2, and 3.5 g/L DCA exposure respectively). Control values were not reported by the authors.

The authors reported that the prevalence and severity of hepatocellular cytomegaly and of cytoplasmic vacuolization with glycogen deposition to be dose-related and considered significant in all dose groups examined when compared to control liver. However, no quantitative data were shown.

The authors reported a severity index of 0 = none, 1 = ≤25%, 2 = 50–75%, and 4 = 75% of liver section for hepatocellular necrosis and report, at 26 weeks, scores (n = 10 animals) of 0.10 ± 0.10, 0.20 ± 0.13, 1.20 ± 0.38, 1.20 ± 0.39, and 1.10 ± 0.28 for control, 0.5, 1, 2, and 3.5 g/L DCA treatment groups, respectively. Thus, there appeared to be a treatment-related, but not dose-related, increase in hepatocellular necrosis that does not involve most of the liver from 1 to 3.5 g/L DCA at this time point. At 52 weeks of exposure, the score for hepatocellular necrosis was reported to be 0, 0, 0.20 ± 0.13, 0.40 ± 0.22, and 1.10 ± 0.43 for control, 0.5, 1, 2, and 3.5 g/L DCA treatment groups, respectively. At 78 weeks of exposure, the score for hepatocellular necrosis was reported to be 0, 0, 0, 0.30 ± 0.21, and 0.20 ± 0.13 for control, 0.5, 1, 2, and 3.5 g/L DCA treatment groups, respectively. Finally, at the final sacrifice time when more animals were examined, the extent of hepatocellular necrosis was reported to be 0.20 ± 0.16, 0.20 ± 0.08, 0.42 ± 0.15, 0.38 ± 0.20, and 1.38 ± 0.42 for control, 0.5, 1, 2, and 3.5 g/L DCA treatment groups, respectively.

Thus, there was no reported increase in hepatocellular necrosis at any exposure period for 0.5 g/L DCA treatment, and the mild hepatocellular necrosis seen at the three highest exposure concentrations at 26 weeks had diminished with further treatment except for the highest dose at up to100 weeks of treatment. Clearly, the pattern of hepatocellular necrosis did not correlate with the dose-related increases in HCCs reported by the authors and was not increased over control at the 0.5 g/L DCA level where there was a DCA-related tumor increase.

The authors cited previously published data and state that CN-insensitive palmitoyl CoA oxidase activity (a marker of peroxisome proliferation) data for the 26-week time point plotted against 100-week HCC prevalence of animals bearing tumors was significantly enhanced at concentrations of DCA that failed to induce "hepatic PCO" activity. The authors reported that neither 0.05 nor 0.5 g/L DCA had any marked effect on PCO activity and that it was "only significantly increased after 26 weeks of exposure to 3.5 g/L DCA and returned to control level

at 52 weeks (data not shown)." In regards to hepatocyte labeling index after treatment for 5 days with tritiated thymidine, the authors reported that animals examined in the dose-response segment of the experiment at 26 and 52 weeks were examined but no details of the analysis were reported. The authors commented on the results from this study and a previous one that included earlier time points of study and stated that there were "no significant alterations in the labeling indexes for hepatocytes outside of proliferative lesions at any of the DCA concentrations when compared to the control values with the exception of 0.05 g/L DCA at 4 weeks (4.8 ± 0.6 vs. 2.7 ± 0.4 control value; data not shown)."

The effects of DCA on body weight, absolute liver weight, and percent liver/body weight were given in Table 2 of the paper for 26, 52, 78, and 100 weeks of exposure. For 52- and 78-week studies, 10 animals per treatment group were examined. Liver weights were not determined for the lowest exposure concentration (0.05 g/L DCA) except for the 100-week exposure period. At 26 weeks of exposure, there was not a statistically significant change in body weight among the exposure groups (i.e., 35.4 ± 0.7, 37.0 ± 0.8, 36.8 ± 0.8, 37.9 ± 0.6, and 34.6 ± 0.8 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). Absolute liver weight was reported to have a dose-related significant increase in comparison to controls at all exposure concentrations examined, with liver weight reaching a plateau at the 2 g/L concentration (i.e., 1.86 ± 0.07, 2.27 ± 0.10, 2.74 ± 0.08, 3.53 ± 0.07, and 3.55 ± 0.1 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). The percent liver/body weight ratio increases due to DCA exposure were reported to have a similar pattern of increase (i.e., 5.25 ± 0.11, 6.12 ± 0.16, 7.44 ± 0.12, 9.29 ± 0.08, and 10.24 ± 0.12% for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). This represented a 1.17-, 1.41-, 1.77-, and 1.95-fold of control percent liver/body weight at these exposures at 26 weeks.

At 52 weeks of exposure, there was not a statistically significant change in body weight among the exposure groups except for the 3.5 g/L exposed group in which there was a significant decrease in body weight (i.e., 39.9 ± 0.8, 41.7 ± 0.8, 41.7 ± 0.9, 40.8 ± 1.0, and 35.0 ± 1.1 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). Absolute liver weight was reported to have a dose-related significant increase in comparison to controls at all exposure concentrations examined with liver weight reaching a plateau at the 2 g/L concentration (i.e., 1.87 ± 0.13, 2.39 ± 0.04, 2.92 ± 0.12, 3.47 ± 0.13, and 3.25 ± 0.24 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). The percent liver/body weight ratio increases due to DCA exposure were reported to have a similar pattern of increase (i.e., 4.68 ± 0.30, 5.76 ± 0.12, 7.00 ± 0.15, 8.50 ± 0.26, and 9.28 ± 0.64% for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively).

For liver weight and percent liver/body weight, there was much larger variability between animals within the treatment groups compared to controls and other treatment groups. There were no differences reported for patterns of change in body weight, absolute liver weight, or percent liver/body weight between animals examined at 26 weeks and those examined at 52 weeks.

At 78 weeks of exposure, there was not a statistically significant change in body weight among the exposure groups except for the 3.5 g/L exposed group in which there was a significant decrease in body weight (i.e., 46.7 ± 1.2, 43.8 ± 1.5, 43.4 ± 0.9, 42.3 ± 0.8, and 40.2 ± 2.2 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). Absolute liver weight was reported to have a dose-related increase in comparison to controls at all exposure concentrations examined, but none were reported to be statistically significant (i.e., 2.55 ± 0.14, 2.16 ± 0.09, 2.54 ± 0.36, 3.31 ± 0.63, and 3.93 ± 0.59 g for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively). The percent liver/body weight ratio increases due to DCA exposure were reported to have a similar pattern of increase over control values but only the 3.5 g/L exposure level was reported to be statistically significant (i.e., 5.50 ± 0.35, 4.93 ± 0.09, 5.93 ± 0.97, 7.90 ± 1.55, and 10.14 ± 1.73% for control, 0.5, 1, 2, and 3.5 g/L DCA, respectively).

Finally, for the animals reported to be sacrificed between 90 and 100 weeks, there was not a statistically significant change in body weight among the exposure groups except for the 2.0 and 3.5 g/L exposed groups in which there was a significant decrease in body weight (i.e., 43.9 ± 0.8, 43.3 ± 0.9, 42.1 ± 0.9, 43.6 ± 0.7, 36.1 ± 1.2, and 36.0 ± 1.3 g for control, 0.05, 0.5, 1, 2, and 3.5 g/L DCA, respectively). Absolute liver weight did not show a dose-response pattern at the two lowest exposure levels but was elevated with the three highest doses with the two highest being statistically significant (i.e., 2.59 ± 0.26, 2.74 ± 0.20, 2.51 ± 0.24, 3.29 ± 0.21, 4.75 ± 0.59, and 5.52 ± 0.68 g for control, 0.05, 0.5, 1, 2, and 3.5 g/L DCA, respectively). The percent liver/body weight ratio increases due to DCA exposure were reported to have a similar pattern of increase over control values but only the 2.0 and 3.5 g/L exposure levels were reported to be statistically significant (i.e., 6.03 ± 0.73, 6.52 ± 0.55, 6.07 ± 0.66, 7.65 ± 0.55, 13.30 ± 1.62, and 15.70 ± 2.16% for control, 0.05, 0.5, 1, 2, and 3.5 g/L DCA, respectively).

It must be recognized that liver weight increases, especially in older mice, will reflect increased weight due to tumor burden and thus, DCA-induced hepatomegaly will be somewhat obscured at the longer treatment durations. However, by 100 weeks of exposure, there did not appear to be an increase in liver weight at the 0.05 and 0.5 g/L exposures, while there was an increase in tumor burden reported. Examination of the 0.5 g/L exposure group from 26 to 100 weeks shows that slight hepatomegaly, reported as either absolute liver weight increase over control or change in percent liver/body ratio, was present by 26 weeks (i.e., 22% increase in liver weight and 17% increase in percent liver/body weight), decreased with time, and while similar at 52 weeks, was not significantly different from control values at 78- or 100-week durations of exposure. However, tumor burden was increased at this low concentration of DCA.

The authors present a figure comparing the number of HCCs per animal at 100 weeks compared with the percent liver/body weight at 26 weeks and show a linear correlation (r^2 = 0.9977). Peroxisome proliferation and DNA synthesis, as measured by tritiated thymidine, were reported to not correlate with tumor induction profiles and were also not correlated with early liver weight changes induced by DCA exposure. Most importantly, in a paradigm that

examined tumor formation after up to 100 weeks of exposure, DCA-induced tumor formation was reported to occur at concentrations that did not also cause cytotoxicity and at levels 20–40 times lower than those used in "less than lifetime" studies reporting concurrent cytotoxicity.

E.2.3.2.7. Carter et al. (2003)

The focus of this study was to present histopathological analyses that included classification, quantification, and statistical analyses of hepatic lesions in male B6C3F$_1$ mice receiving DCA at doses as low as 0.05 g/L for 100 weeks and at 0.5, 1.0, 2.0, and 3.5 g/L for between 26 and 100 weeks. This analysis used tissues from the DeAngelo et al. (1999) (two blocks from each lobe and all lesions found at autopsy).

This study used the following diagnostic criteria for hepatocellular changes. Altered hepatic Foci (AHF) were defined as histologically identifiable clones that were groups of cells smaller than a liver lobule that did not compress the adjacent liver. Large foci of cellular alteration (LFCA) were defined as lesions larger than the liver lobule that did not compress the adjacent architecture [previously referred to as hyperplastic nodules by Bull et al. (1990)] but had different staining. These are not non-neoplastic proliferative lesions termed "hepatocellular hyperplasia" that occur secondary to hepatic degeneration or necrosis. Adenomas showed growth by expansion resulting in displacement of portal triad and had alterations in both liver architecture and staining characteristics. Carcinomas were composed of cells with a high nuclear-to-cytoplasmic ration and with nuclear pleomorphism and atypia that showed evidence of invasion into the adjacent tissue. They frequently showed a trabecular pattern characteristic of mouse hepatocellular carcinomas.

The report grouped lesions as eosinophilic, basophilic and/or clear cell, and dysplastic. "Eosinophilic lesions included lesions that were eosinophilic but could also have clear cell, spindle cell or hyaline cells. Basophilic lesions were grouped with clear cell and mixed cell (i.e., mixed basophilic, eosinophilic, hyaline, and/or clear cell) lesions." The authors reported that:

> this grouping was necessary because many lesions had both a basophilic and clear cell component and a few <10 % had an eosinophilic or hyaline component…Lesions with foci of cells displaying nuclear pleomorphism, hyperchromasia, prominent nucleoli, irregular nuclear borders and/or altered nuclear to cytoplasmic ratios were considered dysplastic irrespective of their tinctorial characteristics.

Therefore, Carter et al. (2003) lumped mixed phenotype lesions into the basophilic grouping so that comparisons with the results of Bull et al. (2002) or Pereira (1996), which segregate mixed phenotype from those without mixed phenotype, cannot be done.

This report examined type and phenotype of preneoplastic and neoplastic lesions pooled across all time points. Therefore, conclusions regarding what lesions were evolving into other

lesions have left out the factor of time. Bannasch (1996) reported that examining the evolution of foci through time is critical for discerning neoplastic progression and described foci evolution from eosinophilic or basophilic lesions to more basophilic lesions. Carter et al. (2003) suggested that size and evolution into a more malignant state are associated with increasing basophilia, a conclusion consistent with those of Bannasch (1996). The analysis presented by Carter et al. (2003) also suggested that there was more involvement of lesions in the portal triad, which may give an indication where the lesions arose. Consistent with the results of DeAngelo et al. (1999), Carter et al. (2003) reported that "DCA (0.05 – 3.5 g/L) increased the number of lesions per animal relative to animals receiving distilled water and shortened the time to development of all classes of hepatic lesions." They also concluded that:

> although this analysis could not distinguish between spontaneously arising lesions and additional lesions of the same type induced by DCA, only lesions of the kind that were found spontaneously in control liver were found in increased numbers in animals receiving DCA…Development of eosinophilic, basophilic and/or clear cell and dysplastic AHF was significantly related to DCA dose at 100 weeks and overall adjusted for time.

The authors concluded that the presence of isolated, highly dysplastic hepatocytes in male $B6C3F_1$ mice chronically exposed to DCA suggested another direct neoplastic conversion pathway other than through eosinophilic or basophilic foci.

It appears that the lesions being characterized as carcinomas and adenomas in DeAngelo et al. (1999) were not the same as those by Carter et al. (2003) at 100 weeks even though they were from the same tissues (see Table E-4). Carter et al. (2003) identified all carcinomas as dysplastic despite tincture of lesion and subdivided adenomas by tincture. If the differing adenoma multiplicities are summed for Carter et al. (2003), they do not add up to the same total multiplicity of adenoma given by DeAngelo et al. (1999).

Table E-4. Comparison of data from Carter et al. (2003) and DeAngelo et al. (1999)

Exposure level of DCA at 79–100 wks (g/L)	Total adenoma multiplicity (Carter)	Total adenoma multiplicity (DeAngelo)	Total carcinoma multiplicity (Carter)	Total carcinoma multiplicity (DeAngelo)	Sum of adenomas and carcinoma multiplicity (Carter)	Sum of adenomas and carcinoma multiplicity (DeAngelo)
0	0.22	0.12	0.05	0.28	0.27	0.40
0.05	0.48	-	<0.025	0.58	~0.50	-
0.5	0.44	0.32	0.20	0.68	0.64	1.0
1.0	0.52	0.80	0.30	1.29	0.82	2.09
2.0	0.60	0.57	1.55	2.47	2.15	3.27
3.5	1.48	0.64	1.30	2.90	2.78	3.54

It is unclear how many animals were included in the differing groups in both studies for pathology. The control and high-dose groups differ in respect to "animals with pathology" between DeAngelo et al. (1999) and the "number of animals in groups" examined for lesions in Carter et al. (2003). Neither report gave how many animals with unscheduled deaths were treated in regards to how the pathology data were included in presentation of results. Given that DeAngelo et al. (1999) represents animals at 100 weeks as also animals from 79 to 100 weeks of exposure, it is probable that the animals that died after 79 weeks were included in the group of animals sacrificed at 100 weeks. However, the number of animals affecting that result (which would be a mix of exposure times) for either DeAngelo et al. (1999) or Carter et al. (2003) is unknown from published reports.

In general, it appears that Carter et al. (2003) reported more adenomas/animal for their 100 week animals than DeAngelo et al. (1999) did, while DeAngelo et al. (1999) reported more carcinomas/animal.

In order to compare these data with others (eg., Pereira and Phelps, 1996) for estimates of multiplicity by phenotype or tincture it would be necessary to add foci and LFCA together as foci, and adenomas and carcinomas together as tumors. It would also be necessary to lump mixed foci together as "basophilic" from other data sets as was done for Carter et al. (2003) in describing "basophilic lesions." If multiplicity of carcinomas and adenomas are summed from each study to control for differences in identification between adenoma and carcinoma, there are still differences in the two studies in multiplicity of combined lesions/animal with DeAngelo et al. (1999) giving consistently higher estimates. However, both studies show a dose response of tumor multiplicity with DCA and a difference between control values and the 0.05 DCA exposure level. Error is introduced by having to transform the data presented as a graph in Carter et al. (2003). Also no SEM is given for the Carter data.

In regard to other histopathological changes, the authors report that:

> necrosis was found in 11.3% of animals in the study and the least prevalent toxic or adaptive response. No focal necrosis was found at 0.5 g/L. The incidence of focal necrosis did not differ from controls at 52 or 78 weeks and only was greater than controls at the highest dose of 3.5 g/L at 100 weeks. Overall necrosis was negatively related to the length of exposure and positively related to the DCA dose. Necrosis was an early and transitory response. There was no difference in necrosis 0 and 0.05 g/L or 0.5 g/L. There was an increase in glycogen at 0.5 g/L at the perioportal area. There was no increase in steatosis but a dose-related decrease in steatosis. Dysplastic LFCA were not related to necrosis indicating that these lesions do not represent, regenerative or reparative hyperplasia. Nuclear atypia and glycogen accumulation were associated with dysplastic adenomas. Necrosis was not related to occurrence of dysplastic adenomas. Necrosis was of borderline significance in relation to presence of hepatocellular carcinomas. Necrosis was not associated with dysplastic LFCAs or Adenomas.

They concluded that "the degree to which hepatocellular necrosis underlies the carcinogenic response is not fully understood but could be significant at higher DCA concentrations (≥1 g/L)."

E.2.3.2.8. Stauber and Bull (1997)

This study was designed to examine the differences in phenotype between altered hepatic foci and tumors induced by DCA and TCA. Male B6C3F_1 mice (7 weeks old at the start of treatment) were treated with 2.0 g/L neutralized DCA or TCA in drinking water for 38 or 50 weeks, respectively. They were then treated with additional exposures (n = 12) of 0, 0.02, 0.1, 0.5, 1.0, or 2.0 g/L DCA or TCA for an additional 2 weeks. Three days prior to sacrifice in DCA-treated mice or 5 days for TCA-treated mice, animals had miniosmotic pumps implanted and administered BrdU.

Immunohistochemical staining of hepatocytes from randomly selected fields (minimum of 2,000 nuclei counter per animal) from five animals per group were reported for 14- and 28-day treatments. It was unclear how many animals were examined for 280- and 350-day treatments from the reports. The percentage of labeled cells in control livers was reported to vary between 0.1 and 0.4% (i.e., fourfold).

There was a reported ~3.5-fold of control level for TCA labeling at a 14-day time period and a ~5.5-fold for DCA. At 28 days, there was ~2.5-fold of control for TCA, but a ~2.3-fold decrease of control for DCA. At 280 days, there was no data reported for TCA, but for DCA, there was a ~2-fold decrease in labeling over control. At 350 days, there were no data for DCA, but a reported ~2.3-fold decrease in labeling of control with TCA. The authors reported that the increases at day 14 for TCA and DCA exposure and the decrease at day 28 for DCA exposure were statistically significant, although a small number of animals were examined. Thus, although there may be some uncertainty in the exact magnitude of change, there was, at most, ~5-fold of control labeling for DCA within after 14 days of exposure that was followed by a decrease in DNA synthesis by day 28 of treatment. These data show that hepatocytes undergoing DNA synthesis represented a small population of hepatocytes with the highest level with either treatment <1% of hepatocytes. Rates of cell division were reported to be less than control for both DCA and TCA by 40 and 52 weeks of treatment.

In this study, the authors reported that there was no necrosis with the 2.0 g/L DCA dose for 52 weeks and concluded that necrosis is a recurring but inconsistent result with chronic DCA treatment. Histological examination of the livers involved in the present study found little or no evidence of such damage or overt cytotoxicity. It was assumed that this effect has little bearing on data on replication rates.

Foci and tumors were combined in reported results, and therefore, cannot be compared the results Bull et al. (2002) or to DeAngelo et al. (1999). Prevalence rates were not reported.

Data were reported in terms of "lesions" with DCA-induced "lesions" containing a number of smaller lesions that were heterogeneous and more eosinophilic with larger "lesions" tending to less numerous and more basophilic. For TCA results using this paradigm, the "lesions" were reported to be less numerous, more basophilic, and larger than those induced by DCA. The DCA-induced larger "lesions" were reported to be more "uniformly reactive to c-Jun and c-Fos but many nuclei within the lesions displaying little reactivity to c-Jun." The authors stated that while most DCA-induced "lesions" were homogeneously immunoreactive to c-Jen and C-Fos (28/41 lesions), the rest were stained heterogeneously. For TCA-induced lesions, the authors reported not difference in staining between "lesions" and normal hepatocytes in TCA-treated animals. Again, of note is that not only were "lesions" comprised of foci and tumors at different stages of progression reported in these results, but that also DCA and TCA results were reported for different durations of exposure.

E.2.3.2.9. Pereira (1996)

The focus of this study was to report the dose-response relationship for the carcinogenic activity of DCA and TCA in female B6C3F_1 mice and the characteristics of the lesions. Female B6C3F_1 mice (7–8 weeks of age) were given drinking water with either DCA or TCA at 2.0, 6.67, or 20 mmol/L and neutralized with sodium hydroxide to a pH or 6.5–7.5. The control received 20 mmol/L sodium chloride. Conversion of mmol/L to g/L was as follows: 20.0 mmol/L DCA = 2.58 g/L, 6.67 mmol/L DCA = 0.86 g/L, and 2.0 mmol/L = 0.26 g/L; 20.0 mmol/L TCA = 3.27 g/L, 6.67 mmol/L TCA = 1.10 g/L, and 2.0 mmol/L TCA = 0.33 g/L. The concentrations were reported to be chosen so that the high concentration was comparable to those previously used by us to demonstrate carcinogenic activity. The mice were exposed until sacrifice at 360 (51 weeks), or 576 days (82 weeks) of exposure.

Whole liver was reported to be cut into ~3 mm blocks and along with representative sections of the visible lesions, fixed and embedded in paraffin and stained with H&E for histopathological evaluation of foci of altered hepatocytes, hepatocellular adenomas, and HCCs. The slides were reported to be evaluated blind. Foci of altered hepatocytes in this study were defined as containing six or more cells and hepatocellular adenomas were distinguished from foci by the occurrence of compression at >80% of the border of the lesion.

Body weights were reported to be decreased only the highest dose of DCA from 40 weeks of treatment onward. For TCA, there were only two examination periods (weeks 51 and 82) that had significantly different body weights from control and only at the highest dose. Liver/body weight percentage was reported in comparison to concentration graphically and shows a dose-response for DCA with steeper slope than that of TCA at 360 and 576 days of exposure. The authors reported that all three concentrations of DCA resulted in increased vacuolation of hepatocytes. Such vacuolization was probably due to glycogen removal from tissue processing. Using a score of 1–3 (with 0 indicating the absence of vacuolization,

+1 indicating vacuolated hepatocytes in the periportal zone, + 2 indicating distribution of vacuolated hepatocytes in the midzone, and +3 indicating maximum vacuolization of hepatocytes throughout the liver), the authors also reported that "the extent of vacuolization of the hepatocytes in the mice administered 0, 2.0, 6.67 or 20.0 mmol/l DCA was scored as 0.0, 0.80 ± 0.08, 2.32 ± 0.11, or 2.95 ± 0.05, respectively."

Cell proliferation was reported to be determined in treatment groups containing 10 mice each and exposed to either DCA or TCA for 5, 12, or 33 days with animals implanted with miniosmotic pumps 5 days prior to sacrifice and administered BrdU. Tissues were immunohistochemically stained for BrdU incorporation. At least 2,000 hepatocytes/mouse were reported to be evaluated for BrdU-labeled and unlabeled nuclei and the BrDU-labeling index was calculated as the percentage of hepatocytes with labeled nuclei.

Pereira (1996) reported a dose-related increase in BrDU labeling in 2,000 hepatocytes that was statistically significant at 6.67 and 20.mmol/L DCA at 5 days of treatment but that labeling at all exposure concentrations decreased to control levels by days 12 and 33 of treatment. The largest increase in BrdU labeling was reported to be twofold of controls at the highest concentration of DCA after 5 days of exposure. For TCA, all doses (2.0, 6.67, and 20 mmol/L) gave a similar and statistically significant increase in BrDU labeling by 5 days of treatment (~3-fold of controls) but by days 12 and 33, there were no increases above control values at any exposure level. Given the low level of hepatocyte DNA synthesis in quiescent control liver, these results indicate a small number of hepatocytes underwent increased DNA synthesis after DCA or TCA treatment and that by 12 days of treatment, these levels were similar to control levels in female B6C3F_1 mice.

Incidence of foci and tumors in mice administered DCA or TCA (prevalence or number of animals with tumors of those examined at sacrifice) in this report are given in Tables E-5 and E-6.

Table E-5. Prevalence of foci and tumors in mice administered NaCl, DCA, or TCA from Pereira (1996)

Treatment	N	Foci		Adenomas		Carcinomas	
		Number	%	Number	%	Number	%
82 wks							
20.0 mmol NaCl	90	10	11.1	2	2.2	2	2.2
20.0 mmol DCA	19	17	89.5[a]	16	84.2[a]	5	26.3[a]
6.67 mmol DCA	28	11	39.3[a]	7	25.0[a]	1	3.6
2.0 mmol DCA	50	7	14.0	3	6.0	0	0
20.0 mmol TCA	18	11	61.1[a]	7	38.9[a]	5	27.8[a]
6.67 mmol TCA	27	9	33.3[a]	3	11.1	5	18.5[a]
2.0 mmol TCA	53	10	18.9	4	7.6	0	0
51 wks							
20.0 mmol NaCl	40	0	0	1	2.5	0	0
20.0 mmol DCA	20	8	40.0[a]	7	35[a]	1	5
6.67 mmol DCA	20	1	5	3	15	0	0
2.0 mmol DCA	40	0	0	0	0	0	0
20.0 mmol TCA	20	0	0	2	15.8	5	25[a]
6.67 mmol TCA	19	0	0	3	7.5	0	0
2.0 mmol TCA	40	3	7.5	3	2.5	0	0

[a]$p < 0.05$.

NaCl = sodium chloride control

Table E-6. Multiplicity of foci and tumors in mice administered NaCl, DCA, or TCA from Pereira (1996)

Treatment	N	Foci/mouse	Adenomas/mouse	Carcinomas/mouse
82 wks				
20.0 mmol NaCl	90	0.11 ± 0.03	0.02 ± 0.02	0.02 ± 0.02
20.0 mmol DCA	19	7.95 ± 2.00[a]	5.58 ± 1.14[a]	0.37 ± 0.17[b]
6.67 mmol DCA	28	0.39 ± 0.11[b]	0.32 ± 0.13[b]	0.04 ± 0.04
2.0 mmol DCA	50	0.14 ± 0.05	0.06 ± 0.03	0
20.0 mmol TCA	18	1.33 ± 0.31[a]	0.61 ± 0.22[b]	0.39 ± 0.16[b]
6.67 mmol TCA	27	0.41 ± 0.13[b]	0.11 ± 0.06	0.22 ± 0.10[b]
2.0 mmol TCA	53	0.26 ± 0.08	0.08 ± 0.04	0
51 wks				
20.0 mmol NaCl	40	0	0.03 ± 0.03	0
20.0 mmol DCA	20	0.60 ± 0.22[a]	0.45 ± 0.17[a]	0.10 ± 0.10
6.67 mmol DCA	20	0.05 ± 0.05	0.20 ± 0.12	0
2.0 mmol DCA	40	0	0	0
20.0 mmol TCA	20	0	0.15 ± 0.11	0.50 ± 0.18[b]
6.67 mmol TCA	19	0	0.21 ± 0.12	0
2.0 mmol TCA	40	0.08 ± 0.04	0.08 ± 0.04	0

[a]$p < 0.01$.
[b]$p < 0.05$.

These data show the decreased power of using fewer than 50 mice, especially at shorter durations of exposure. By 82 weeks of exposure, increased adenomas and carcinomas induced by TCA or DCA treatment are readily apparent.

The foci of altered hepatocytes and the tumors obtained from this study were reported to be basophilic, eosinophilic, or mixed containing both characteristics and are shown in Tables E-7 and E-8. DCA was reported to induce a predominance of eosinophilic foci and tumors, with over 80% of the foci and 90% of the tumors in the 6.67 and 20.0 mmol/L concentration groups being eosinophilic. Only approximately half of the lesions were characterized as eosinophilic with the rest being basophilic in the group administered 2.0 mmol/L DCA. The eosinophilic foci and tumors were reported to consistently stained immunohistochemically for the presence of GST-π, while basophilic lesions did not stain for GST-π, except for a few scattered cells or small areas comprising <10% of foci.

Table E-7. Phenotype of foci reported in mice exposed to NaCl, DCA, or TCA by Pereira (1996)

Treatment at 51 and 82 wks	N	% Foci		
		Basophilic	Eosinophilic	Mixed
20.0 mmol NaCl	10	70	30	0
20.0 mmol DCA	150	3.3	96.7	0
6.67 mmol DCA	11	18.2	81.8	0
2.0 mmol DCA	7	42.8	57.2	0
20.0 mmol TCA	22	36.4	54.6	9.1
6.67 mmol TCA	11	45.5	54.5	0
2.0 mmol TCA	13	38.5	61.5	0

Table E-8. Phenotype of tumors reported in mice exposed NaCl, DCA, or TCA by Pereira (1996)

Treatment at 51 and 82 wks	N	Tumors		
		Basophilic	Eosinophilic	Mixed
20.0 mmol NaCl	4	50	25	25.5
20.0 mmol DCA	105	2.9	96.1	1
6.67 mmol DCA	10	10	90	0
2.0 mmol DCA	3	0	100	0
20.0 mmol TCA	18	61.1	22.2	16.7
6.67 mmol TCA	6	100	0	0
2.0 mmol TCA	4	100	0	0

The foci of altered hepatocytes in the TCA treatment groups were approximately equally distributed between basophilic and eosinophilic in tincture. However, the tumors were predominantly basophilic, lacking GST-pi (21 of 28 or 75%) including all 11 HCCs. The limited numbers of lesions (i.e., 14) in the sodium chloride (vehicle control) group were characterized as 64.3, 28.6, and 7.1% basophilic, eosinophilic, and mixed, respectively.

These data for female $B6C3F_1$ mice show that DCA and TCA treatment induced a mixture of basophilic or eosinophilic foci. The pooling of the data between time and adenoma vs. carcinoma decreases the ability to ascertain the phenotype of tumor due to treatment or the progression of phenotype with time as well as the small number of tumor examined at lower exposure concentrations. Foci that occurred at 51 and 82 weeks were presented as one result. Adenoma and carcinoma data were pooled as one endpoint (n = number of total foci or tumors examined). Therefore, evolution of phenotype between less to more malignant stages of tumor were lost.

E.2.3.2.10. Pereira and Phelps (1996)

The focus of this study was to determine tumor response and phenotype in methyl nitrosourea (MNU)-treated mice after DCA or TCA exposure. The concentrations of DCA or TCA were the same as Pereira (1996). For Pereira (1996), the animals were reported to be 7–8 weeks of age when started on treatment and sacrificed after 360 or 576 days of exposure (51 or 82 weeks). For this study and Tao et al. (2004b), animals were reported to be 6 weeks of age when exposed to DCA or TCA via drinking water and to be 31 or 52 weeks of age at sacrifice. Thus, exposure time would be ~24 or 45 weeks. A control group of non-MNU treated animals was presented for female B6C3F_1 mice treated for 31 or 52 weeks and are discussed in Table E-9.

Table E-9. Multiplicity and incidence data (31 week treatment) from Pereira and Phelps (1996)

Treatment	Number	Foci/mouse	incidence %	Adenomas/mouse	incidence %
20.0 mmol NaCl	15	0.13 ± 0.13	6.7	0.13 ± 0.13	not reported
20.0 mmol DCA	10	0.40 ± 0.16	40	0	0
6.67 mmol DCA	10	0.10 ± 0.10	10	0	0
2.0 mmol DCA	15	0	0	0	0
20.0 mmol TCA	10	0	0	0	0
6.67 mmol TCA	10	0	0	0	0
2.0 mmol TCA	15	0	0	0	0

Although this paradigm appears to be the same paradigm as those reported in Pereira (1996), fewer animals were studied. The number of animals in each group varied between 8 controls and 14 animals in the 2.0 mmol/L treatment groups. In mice that were not treated with MNU but were treated with either DCA or TCA at 31 weeks, there were no reported statistically significant treatment-related effects upon the yield of foci or altered hepatocytes and liver tumors but the number of animals examined was small and therefore, of limited power to detect a response. The results below indicate a DCA-related increase in foci and percentage of mice with foci.

See Section E.4.2.3 for further discussion of the results of co-exposures to MNU and DCA or TCA from this study.

E.2.3.2.11. Ferreira-Gonzalez et al. (1995) HCCs induced by TCA or DCA in male B6C3F_1 mice. Mice (28-day

The focus of this study was the investigation of differences in H-ras mutation spectra in old) were exposed for 104 weeks to 0. 1.0, or 3.5 g/L DCA or 4.5 g/L TCA that was pH adjusted. Tumors observed from this treatment were diagnosed as either hepatocellular adenomas or carcinomas. DNA was extracted from either spontaneous, DCA- or TCA-induced HCCs.

Samples for analysis were chosen randomly in the treatment groups, of which 19% of untreated mice had spontaneous liver HCCs (0.26 carcinomas/animal).

DCA treatment induced 100% prevalence at 3.5 g/L (5.06 carcinomas/animal) and 70.6% carcinomas at 1.0 g/L (1.29 carcinomas/animal). TCA treatment was reported to induce 73.3% prevalence at 4.5 g/L (1.5 carcinomas/animal). The number of samples analyzed was 32 for spontaneous carcinomas, 33 for mice treated with 3.5 g/L DCA, 13 from mice treated with 1.0 g/DCA, and 11 from mice treated with 4.5 g/L TCA.

This study has the advantage of comparison of tumor phenotype at the same stage of progression (HCC), for allowance of the full expression of a tumor response (i.e., 104 weeks), and an adequate number of spontaneous control lesions for comparison with DCA or TCA treatments. However, tumor phenotype at an endstage of tumor progression reflects of tumor progression and not earlier stages of the disease process.

There were no ras mutations detected except at H-61 in DNA from spontaneously arising tumors of control mice. Only 4/57 samples from carcinogen-treated mice were reported to demonstrate mutation other than in the second exon of H-ras. In spontaneous liver carcinomas, 58% were reported to show mutations in H-61 as compared with 50% of tumor from 3.5 g/L DCA-treated mice and 45% of tumors from 4.5.g/L TCA-treated mice. Thus, there was a heterogeneous response for this phenotypic marker for the spontaneous, DCA-, and TCA-treatment induced HCCs.

All samples positive for mutation in the exon 2 of H-ras were sequenced for the identification of the base change responsible for the mutation. The authors noted that H-ras mutations occurring in spontaneously developing HCCs from $B6C3F_1$ male mice are largely confined to codon 61 and involve a change from CAA to either AAA or CGA or CTA in a ratio of 4:2:1. They noted that in this study, all of the H-ras second codon mutations involved a single base substitution in H-61 changing the wild-type sequence from CAA to AAA (80%), CGA (20%), or CTA for the 18 HCCs examined.

In the 16 HCCs from 3.5 g/L DCA treatment with mutations, 21% were AAA transversions, 50% were CGA transversions, and 29% were CTA transversions. For the six HCCs from 1.0 g/L DCA with mutations, 16% were an AAA transversion, 50% were a CGA transversion, and 34% were a CTA transversion. For the five HCCs from 4.5 g/L TCA with mutations, 80% were AAA transversions, 20% CGA tranversions, and 0% were CTA transversions. The authors note that the differences in frequency between DCA and TCA base substitutions did not achieve statistical significance due to the relatively small number of tumors from TCA-treated mice. They note that the finding of essentially equal incidence of H-ras mutations in spontaneous tumors and in tumors of carcinogen-treated mice did not help in determining whether DCA and TCA acted as “genotoxic” or “nongenotoxic” compounds.

E.2.3.2.12. Pereira et al. (2004a)

Pereira et al. (2004a) exposed 7–8-week-old female B6C3F$_1$ mice treated with "AIN-76A diet" to neutralized 0 or 3.2 g/L DCA in the drinking water and 4.0 or 8.0 g/kg L methionine added to their diet. The final concentration of methionine in the diet was estimated to be 11.3 and 15.3 g/kg. Mice were sacrifice 8 and 44 weeks after exposure to DCA, with body and liver weights evaluated for foci, adenomas, and HCCs. No histological descriptions were given by the authors other than tinctorial phenotype of foci and adenomas for a subset of the data. The number of mice examined was 36 for the DCA + 8.0 g/kg methionine or 4.0 g/kg methionine group sacrificed at 44 weeks. However, for the DCA-only treatment group, the number of animals examined was 32 at 44 weeks and for those groups that did not receive DCA but either methionine at 8.0 or 4.0 g/kg, there were only 16 animals examined. All groups examined at 8 weeks had eight animals per group.

Liver glycogen was reported to be isolated from 30 to 50 mg of whole liver. Peroxisomal acyl-CoA oxidase activity was reported to be determined using lauroyl-CoA as the substrate and was considered a marker of peroxisomal proliferation. Whole-liver DNA methylation status was analyzed using a 5-MeC antibody.

Methionine (8.0 g/kg) and DCA co-exposure was reported to result in the death of three mice, while treatment with methionine (4.0 g/kg) and DCA or methionine (8.0 g/kg) alone was reported to kill one mouse in each group. The authors reported that "There was an increased in body weight during weeks 12 to 36 in the mice that received 8.0 g/kg methionine without DCA. There was no other treatment-related alteration in body weight." However, the authors do not present the data and initial or final body weights were not presented for the differing treatment groups.

DCA treatment was reported to increase percent liver/body weight ratios at 8 and 44 weeks to about the same extent (i.e., ~2.4-fold of control at 8 weeks and 2.2-fold of control at 44 weeks). Methionine co-exposure was reported to not affect that increase (~2.4-, 2.2-, and 2.1-fold of control after DCA treatment alone, DCA/4 g/kg methionine, and DCA/8 mg/kg methionine treatment for 8 weeks, respectively). There was a slight increase in percent liver/body weight ratio associated with 8.0 g/kg methionine treatment alone in comparison to controls (~7%) at 8 weeks with no difference between the two groups at 44 weeks.

After 8 weeks of only DCA exposure, the amount of glycogen in the liver was reported to be ~2.09-fold of the value for untreated mice (115 vs. 52.5 mg/g glycogen in treated vs. control, respectively, at 8 weeks). Both 4 and 8 g/kg methionine co-exposure reduced the amount of DCA-induced glycogen increase in the liver (~1.64-fold of control for DCA/4.0 g/kg methionine and ~1.54-fold of control for DCA/8.0 mg/kg methionine). Thus, for treatment with DCA alone or with the two co-exposure levels of methionine, the magnitude of the increase in liver weight was greater than that of the increase in liver glycogen (i.e., 2.42- vs. 2.09-fold of control percent liver/body weight vs. glycogen content for DCA alone, 2.20- vs. 1.64-fold of control percent

liver/body weight vs. glycogen content for DCA/4.0 g/kg methionine, 2.10- vs. 1.54-fold of control percent liver/body weight vs. glycogen content for DCA/8.0 g/kg methionine). Thus, the magnitudes of treatment-related increases were higher for percent liver/body weight than for glycogen content in these groups.

In regard to percentage of liver mass that glycogen represented, the control value for this study is similar to that presented by Kato-Weinstein et al. (2001) in male mice (~60 mg glycogen/g liver) and represents ~6% of liver mass. Therefore, a doubling of the amount of glycogen is much less than the twofold increases in liver weight observed for DCA exposure in this paradigm. These data suggest that DCA-related increases in liver weight gain are not only the result of increased glycogen accumulation, and that methionine co-exposure is affecting glycogen accumulation to a much greater extent than the other underlying processes that are contributing to DCA-induced hepatomegaly after 8 weeks of exposure. The authors reported that 8-weeks of DCA exposure alone did not result in a significant increase in cell proliferation as measured by PCN index (neither data nor methods were shown). This is consistent with other data showing that DCA effects on DNA synthesis were transient and had subsided by 8 weeks of exposure.

The levels of lauroyl-CoA oxidase activity were reported to be increased (~1.33-fold of control) by DCA treatment alone at 8 weeks and to be slightly reduced by 8 g/kg methionine treatment alone (~0.83-fold of control). Methionine co-exposure was reported to have little effect on DCA-induced increases in lauroyl-CoA oxidase activity. The levels of DNA methylation were reported to be increased by 8.0 g/kg methionine only treatment at 8 weeks ~1.32-fold of control, and reduced by DCA only treatment to ~0.44-fold of control. DCA and 4.0 g/kg methionine co-exposure gave similar results as controls (within 2%). Co-exposures of DCA and 8.0 g/kg methionine treatments were reported to increase DNA methylation 1.22-fold of controls after 8 weeks of co-exposure.

In the 44-week study, the authors reported that foci and hepatocellular adenomas were found. However, the authors do not report the incidences of these lesions in their study groups (how many of the treated animals developed lesions). As noted above, the numbers of animals in these groups varied widely between treatments (e.g., n = 36 for DCA and co-exposure to 8.0 g/kg methionine but only n = 16 for 8 g/kg methionine treatment alone). Although reporting unscheduled deaths in the 8.0 g/kg methionine and DCA co-exposure groups, the authors did not indicate whether these mortalities occurred in the 44- or 8-week study groups.

Multiplicities of foci and adenoma data were presented. DCA was reported to induce 2.42 ± 0.38 foci/mouse and 1.28 ± 0.31 adenomas/mouse (mean ± SE) after 44 weeks of treatment. The DCA-induced foci and adenomas were reported to stain as eosinophilic with "relatively large hepatocytes and nuclei." The authors did not present data on the percent of foci and adenomas that were eosinophilic using this paradigm. The addition of 4.0 or 8.0 g/kg methionine to the AIN-76A diet was reported to reduce the number of DCA-induced

adenomas/mouse to 0.167 ± 0.093 and 0.028 ± 0.028, respectively. However, the addition of 4.0 g/kg methionine to the DCA treatment was reported to increase the number of foci/mouse (3.4 ± 0.46 foci/mouse). The addition of 8.0 g/kg methionine to the DCA treatment was reported to yield 0.94 ± 0.24 foci/mouse. There were no foci or tumors in the 16 mice that received either the control diet or the 8.0 g/kg methionine treatment without DCA. The authors did not report whether methionine treatment had an effect on the tincture of the foci or adenomas induced by DCA.

Therefore, a very high level of methionine supplementation to an AIN-760A diet, was shown to affect the number of foci and adenomas (i.e., decrease them) after 44 weeks of co-exposure to very high exposure concentration of DCA. However, a lower level of methionine co-exposure increased the incidence of foci at the same concentration of DCA. Methionine treatment alone at the 8 g/kg level was reported to increase liver weight, decrease lauroyl-CoA activity, and increase DNA methylation.

No histopathology was given by the authors to describe the effects of methionine alone. Co-exposure of methionine with 3.2 g/L DCA was reported to decrease by ~25% DCA-induced glycogen accumulation and increase mortality, but not to have much of an effect on peroxisome enzyme activity (which was not elevated by >33% over control for DCA exposure alone). The authors suggested that their data indicate that methionine treatment slowed the progression of foci to tumors. Whether these results would be similar for lower concentrations of DCA and lower concentrations of methionine that were administered to mice for longer durations of exposure cannot be ascertained from these data. It is possible that in a longer-term study, the number of tumors would be similar. Whether methionine treatment co-exposure had an effect on the phenotype of foci and tumors was not presented by the authors in this study. Such data would have been valuable to discern if methionine co-exposure at the 4.0 mg/kg level that resulted in an increase in DCA-induced foci, resulted in foci of a differing phenotype or resulted in a more heterogeneous composition than DCA treatment alone.

E.2.3.2.13. DeAngelo et al. (2008)

In this study, neutralized TCA was administered in drinking water to male B6C3F$_1$ mice (28–30 days old) in three studies. In the first study, control animals received 2 g/L sodium chloride while those in the second study were given 1.5 g/L neutralized acetic acid (HAC) to account for any taste aversion to TCA dosing solutions. In a third study, deionized water served as the control.

No differences in water uptake were reported. Mean initial weights were reported to not differ between the treatment groups (19.5 ± 2.5 g – 21.4 ± 1.6 g or ~10% difference). The first study was reported to be conducted at the U.S. EPA laboratory in Cincinnati, Ohio in which mice were exposed to 2 g/L sodium chloride, or 0.05, 0.5, or 5 g/L TCA in drinking water for 60 weeks. There were five animals at each concentration that were sacrificed at 4, 15, 31, and

45 weeks with 30 animals sacrificed at 60 weeks of exposure. There were 3 unscheduled deaths in the 0.05 g/L TCA group leaving 27 mice at final necropsy. For the other exposure groups, there were 29 or 30 animals at final necropsy.

In the second study, also conducted in the same laboratory, mice were reported to be exposed to 1.5 g/L neutralized acetic acid or 4.5 g/L TCA for 104 weeks. Serial necropsies were conducted (5 animals per group) at 15, 30, and 45 weeks of exposure and 10 animals in the control group at 60 weeks. For this study, a total of 25 animals were sacrificed in interim necropsies in the 1.5 g/L HAC group and 15 in the 4.5 g/L TCA group. There were 7 unscheduled deaths in the HAC group and 12 in the 4.5 g/L TCA group, leaving 25 and 30 animals in the final necropsy groups, respectively.

Study 3 was conducted at the U.S. EPA laboratory in Research Triangle Park, North Carolina. Mice were exposed to deionized water or 0.05 or 0.5 g/L TCA in the drinking water for 104 weeks with serial necropsies (n = 8 per group) conducted at 26, 52, and 78 weeks. There were 19–21 animals reported at interim sacrifices and 17 unscheduled deaths in the deionized water group, 24 unscheduled deaths in the 0.05 g/L TCA group, and 24 unscheduled deaths in the 0.5 g/L TCA group. This left 34 mice at final necropsy in the control group, 29 mice in the 0.05 g/L TCA group, and 27 mice in the 0.5 g/L group.

At necropsy, liver, kidneys, spleen, and testes weights were reported to be taken and organs examined for gross lesions. Tissues were prepared for light microscopy and stained with H& E. At termination of the exposure periods, a complete rodent necropsy was reported to be performed. Representative blocks of tissue were examined only in five mice from the high-dose and control groups with the exception of gross lesions, liver, kidney, spleen, and testis at interim and terminal sacrifices. If the number of any histopathologic lesions in a tissue was "significantly increased above that in control animals," then that tissue was reported to be examined in all TCA dose groups.

For Study #3, a second contract pathologist reviewed 10% of the described hepatic lesions. No "major differences" were reported between the two pathologic diagnoses.

The prevalence and multiplicity of hepatic tumors were reported to be derived by performing a histopathologic examination of surface lesions and four sections cut from each of four tissue blocks excised from each liver lobe. Tumor prevalence was reported to be calculated as the percentage of the animals with a neoplastic lesion compared to the number of animals examined. Tumor multiplicity was reported to be calculated by dividing the number of each lesion or combined adenomas and carcinomas by the number of animals examined. Preneoplastic large foci of cellular alteration were also observed over the course of the study.

The prevalence and severity of hepatocellular cytoplasmic alterations, inflammation, and necrosis were reported to be determined using a scale based on the amount of liver involved of 1 = minimal (occupying 25%), 2 = mild (occupying 25–50%), 3 = moderate (occupying 50–

75%), and 4 = marked (occupying >75%). The only "significant change outside of the liver" was reported to be testicular degeneration.

LDH was determined in arterial blood collected at 30 and 60 weeks (Study 1) and 4, 30, and 104 weeks (Study 2). Cyanide insensitive PCO was also reported to be measured. Five days prior to sacrifice, tritiated thymidine (Studies 1 and 2) or BrdU (Study 3) was administered via miniosmotic pumps and the number of hepatocyte nuclei with grain counts >6 were scored in 1,000 cells or chromogen pigment over nuclei (BrdU). The labeling index was calculated by dividing the number of labeled hepatocyte nuclei by the total number of hepatocytes scored.

Total neoplastic and preneoplastic lesions (multiplicity) were counted individually or combined (adenomas and carcinomas) for each animal. The analysis of tumor prevalence data was reported to include only those animals examined at the scheduled necropsies or animals surviving to week 60 (Study 1) or longer than 78 weeks (Studies 2 and 3). The data from all of the scheduled necropsies were combined for an overall test of treatment-related effect.

For Study #1 (60-week exposure), all TCA-treated groups experienced a decrease in drinking water consumption, with the decreases in drinking water for the 0.5 and 5 g/L TCA exposure groups reported as statistically significant by the authors. The water consumption in mL/kg-day was reported to be reduced by 11, 17, and 30% in the 0.05, 0.5, and 5 g/L TCA treated groups compared to 2 g/L sodium chloride control animals as measured by time-weighted mean daily water consumption measured over the study. The control value was reported to be 171 mL/kg/day. Although the 0.05 g/L exposure concentrations were not measured, the 0.5 and 5 g/L solutions were within 4% of target concentrations. The authors estimated that the mean daily doses were 0, 8, 68, and 602 mg/kg-day.

For the 102-week studies, the mean water consumption with deionized water was reported to be 112 and 132 mL/kg-day for control animals given 1.5 g/L HAC. Therefore, there appeared to be a 35% decrease in water consumption between the controls in Study #1 given 2 g/L sodium chloride and controls in Study #3 given deionized water but conducted at a different laboratory. There appeared to be a 23% reduction in water consumption between animals given 2 g/L sodium chloride and those given 1.5 g/L HAC at the same laboratory (Study #2).

As the concentrations of TCA were increased, there would be a corresponding increase in the amount of sodium hydroxide needed to neutralize the solutions and a corresponding increase in salts in the solution as well as TCA. The authors did not address nor discuss the differences in drinking water consumption between the differing control solutions between the studies.

DeAngelo et al. (1999) reported mean drinking water consumption of 147 mL/kg/day in control mice of over 100 weeks and that the highest dose of DCA (3.5 g/L) reduced drinking water consumption by 26%. Carter et al. (1995) reported that DCA at 5 g/L decreased drinking water consumption by 64 and 46%, but 0.5 g/L DCA did not affect drinking water consumption. In this study, while reporting that Study #1 showed that increasing TCA concentration decreased

drinking water consumption, the drinking water consumption in Studies #2 and #3 were similar between controls and TCA exposure groups with both being less than the control and low TCA concentration values reported in Study #1 (i.e., in Study #2, the 1.5 g/L HAC and 4.5 g/L TCA drinking water consumption was ~130 mL/kg/day and in Study #3, the drinking water consumption was ~112 mL/kg/day for the deionized water control and 0.05 and 0.5 g/L TCA exposure groups). Thus, the drinking water concentrations for Study #3 was ~35% less than for the control values for Study #1 and was also ~25% less than for DeAngelo et al. (1999). The reasons for the apparently lower drinking water averages for Study #3 and the lack of effect of the addition of 0.5 g/L TCA that was reported in Study #1 and in other studies, was not discussed by the authors.

In Study #1, there was little difference between exposure groups (n = 5) noted for the final body weights (mean range of 27.6–28.1 g) in mice sacrificed after 4 weeks of exposure. However, absolute liver weight and percent liver/body weight ratios increased with TCA dose. The percent liver/body weight ratios were 5.7 ± 0.4, 6.2 ± 0.3, 6.6 ± 0.4, and 7.7 ± 0.6% for the 2 g/L sodium chloride control, 0.05, 0.5, and 5 g/L TCA exposure groups, respectively. These represent 1.09-, 1.16-, and 1.35-fold of control levels that were statistically significant.

At 15 weeks of exposure the fold increases in percent liver/body weight ratios were 1.14-, 1.16-, and 1.47-fold of controls for 0.05, 0.5, and 5 g/L TCA. At 31 weeks of exposure, the fold increases in percent liver/body weight ratios were 0.98-, 1.09-, and 1.59-fold of controls for 0.05, 0.5, and 5 g/L TCA. At 45 weeks of exposure, the fold increases in percent liver/body weight ratios were 1.13-, 1.45-, and 1.98-fold of controls for 0.05, 0.5, and 5 g/L TCA. At 60 weeks of exposure, the percent liver/body weight ratios were 0.94-, 1.25-, 1.60-fold of controls for 0.05, 0.5, and 5 g/L TCA.

Thus, the range of increase at the lowest level of TCA exposure (i.e., 0.05 g/L) was 0.94–1.14-fold of controls. These data consistently show TCA-induced increases in liver weight from 4 to 60 weeks of the study that were dose-related. For the 0.5 g/L exposure group, the magnitude of the increase compared to control was reported to be about the same between weeks 4 and 30, with the highest increase reported to be at week 45 (1.45-fold of control). In regard to the correspondence with magnitude of difference in dose of TCA and liver weight increase, there was ~2-fold increase in liver weight gain corresponding to 10-fold increases in TCA concentration at 4 weeks of exposure. For the 4- and 15-week exposures, there were ~3.3- and 3.9-fold difference in liver weight that corresponded to a 100-fold difference in exposure concentration of TCA (i.e., 0.05 vs. 5.0 g/L TCA).

The small number of animals examined, n = 5, limit the power of the study to determine the change in percent liver/body weight up to 45 weeks, especially at the lowest dose. However, the 0.05 g/L TCA exposure groups at 4 and 15 weeks were reported to significantly increased percent liver/body weight ratios.

The percent liver/body weight ratios for all of the treatment groups and the ability to detect significant changes were affected by changes in final body weight and changing numbers of animals. After 4–30 weeks of exposure, the final body weights of mice increased in control animals but were within 11% of each other between weeks 31 and 60. The percent liver/body weight ratios in controls decreased from 4 to 31 weeks and were slightly elevated by 60 weeks compared to the 31-week level. Although control values were changing, there appeared to be no difference between control values and treated values in final body weight for any duration of exposure with the exception of the 5 g/L TCA exposure group after 60 weeks of exposure, which was decreased by ~15%. At the 31- and 60-week exposure durations, the 0.05 g/L TCA groups did not have increased percent liver/body weight ratios over controls.

In Study #2, conducted in the same laboratory but with a 1.5 g/L HAC solution used for control groups, there was <5% difference in final body weights between control mice give HAC and those treated with 4.5 g/L TCA up to 45 weeks. However, final body weight was reduced by TCA treatment by 104 weeks by ~15%. Between the interim sacrifices of 15, 30, and 45 weeks, the percent liver/body weight ratios in control mice were similar at 15 and 45 weeks (~4.8%) but greater in the 30-week control group (5.3 or ~10% greater than other interim control groups). The TCA-induced increases in body weight were 1.60-, 1.40-, and 1.79-fold of control for the 15-, 30-, and 45-week groups exposed to 4.5 g/L TCA in Study #2. The smaller magnitude of TCA-induced liver weight increase at 30 weeks than that for 15 and 45 weeks, was a reflection of the increased percent liver/body weight ratio reported for the HAC control at that time point.

Comparisons can be made between Studies #1 and #2 for 4.5 or 5.0 g/L TCA exposure levels and controls for 15, 30/31, and 45 weeks of exposure to ascertain the consistency of response from the same laboratory. Although the two studies had differing control solutions and reported different drinking water consumption overall, they were exposing the TCA groups to almost the same concentration of TCA in the same buffered solutions for the same periods of time with the same number of mice per group.

Between Studies #1 and #2, there were consistent percent liver/body weight ratios induced by either 5.0 or 4.5 g/L TCA at weeks 15 and 30/31 (i.e., within 3% of each other). The percent liver/body ratios for these exposure groups ranged from 7.3 to 7.7% between weeks 15 and 30/31 for the ~5.0 g/L TCA exposure in both studies. Final body weights were within 10%. While the percent liver/body weight ratios induced by ~5.0 g/L TCA were similar, the magnitude of increase in comparison to the controls was 1.47- and 1.59-fold of control for Study #1, and 1.60- and 1.40-fold of control for Study #2 after 15 and 30/31 weeks of exposure, respectively. At 45 weeks, the percent liver/body weight ratios were within 11% of each other (9.4 vs. 8.4%) and final body weights were within 2% of each for this exposure concentration between the two studies giving a 1.98- and 1.79-fold of control percent liver/body weight, respectively. Thus, the apparent magnitude of TCA-induced increase in percent liver/body weight was affected by control values used as the basis for comparison. The percent liver/body weights reported for

either 4.5 or 5.0 g/L TCA exposure groups for weeks 15 and 30/31 was similar between the two studies conducted in the same laboratory.

Study #3 was conducted in a separate laboratory, interim sacrifice times were not the same as for Study #1, the number of animals examined differed (n = 5 for Study #1 and n = 8 for Study #3), and control animals studied for comparative purposes were given different drinking water solutions (deionized water vs. 2 g/L sodium chloride). Most importantly, the body weights reported at 52 weeks were much greater than that reported at 45 weeks for Studies #1 and #2.

However, a comparison of TCA-induced liver weight gain and the effects of final body weight can be made between the 0.05 and 0.5 g/L TCA exposure groups at 30 weeks (Study #1) and 26 weeks (Study #3), at 45 weeks and 60 weeks (Study #1), and 52 weeks (Study #3). At 31 weeks, there was <2% difference in mean final body weights between control and the two TCA-treatment groups in Study #1. There was also little difference between the TCA-treated groups at week in Study #3 at week 26 and the TCA treatment groups in at week 31 in Study #1 (i.e., range of 42.6–43.5 g for 0.05 and 0.5 g/L TCA treatments in Studies #1 and #3). However, in Study #3, the control value was 12% lower than that of Study #1 for mean final body weight. Based on final body weights, there would be an expectation of similar results between the two studies at the 26- and 30-week time points.

At the 45-week (Study #1), 52-week (Study #3), and 60-week (Study #1) durations of exposure, the mean final body weights varied little between their corresponding control groups at each sacrifice time (<4% variation between control and TCA-treated groups). However, there was variation in mean final body weights between the differing sacrifice times. Control and TCA-treated groups were reported to have lower mean final body weights at 45 weeks of exposure in Study #1 than at either 30 or 60 weeks. The 45-week mean final body weights in Study #1 were also reported to be lower than those at 52 weeks in Study #3. Control mean body weight values were 28% higher at 52 weeks in Study #3 than 45 weeks in Study #1 and 15% higher for 60 weeks in Study #1. In essence, for Study #1, mean final body weights went down between 31 and 45 weeks of exposure and then went back up at 60 weeks of exposure for control mice (~43, ~40, and ~44 g for 31, 45, and 60 weeks, respectively) as well as for both TCA concentrations. However, for Study #3, final mean body weights went up between 26 and 52 weeks of exposure for control mice (~39 vs. ~51 g) and for both TCA concentrations.

While for Study #1, the percent liver/body weight ratios were 0.98- and 1.09-fold of control at 31 weeks of exposure, at week 45, the ratios were 1.13- and 1.45-fold of control, and at week 60, they were 0.94- and 1.25-fold of controls for the 0.05 and 0.5 g/L TCA exposure levels, respectively. For Study #3, the pattern differed than that of Study #1. There was a 1.07- and 1.18-fold of control percent liver/body weight for 26 weeks but a 0.92- and 1.04-fold of control percent liver/body weight change at 52 weeks of exposure at 0.05 and 0.5 g/L TCA exposure, respectively.

Thus, there appeared to be differences in control and the treatment groups at the 26-week sacrifice groups in Study #3 that was not apparent at the 52-week sacrifice time. Overall, the final body weights appeared to be similar between controls and TCA treatment groups at the 52-week sacrifice time in Study #3 and at the 31-, 45-, and 60-week sacrifice times in Study #1. However, although consistent within sacrifice times, the final body weights differed between the various sacrifice times in Studies #1 and #3. The patterns of percent liver/body weight at differing and similar sacrifice times appeared to differ between the Studies #1 and #3 at the same concentrations of TCA. The largest difference appeared to be between week 45 group in Study #1 and week 52 group in Study #3 where both concentrations of TCA were reported to induce increases in percent liver/body weight in one study but to have little difference in the other. The differences in mean final body weights between these two sacrifice times were also the largest although control and TCA-treatment groups had little difference on this parameter. Similar to the work of Kjellstrand and colleagues with TCE (Kjellstrand et al., 1983a), the groups with the lower body weight appeared to have the greatest response in liver weight increase.

These data illustrate the variability in findings of percent liver weight induction between laboratories, studies, choice of controls solutions, and the effects of final body weights on this parameter. They also illustrate the limitations for determining either the magnitude or pattern of liver weight increases using a small number of test animals. As animals age, the size of their liver changes, but also during the latter parts of the lifespan, foci and spontaneously occurring liver tumors can affect liver weight. The results of Study #1 show a consistent dose-response in TCA liver weight increases at 4- and 15-week time periods over a range of concentration from 0.05 to 5 g/L TCA.

In regard to non-neoplastic pathological changes, the authors reported that:

> Increased incidences and severity of centrilobular cytoplasmic alterations, inflammation, and necrosis were the only nonproliferative changes seen in livers of animals exposed to TCA for 60 weeks (Tables 7-9; Study 1. Incidences were between 21 and 93%; severity ranged from minimal to mild; and some lesions were transient. Centrilobular cytoplasmic alterations (Table 7) were the most prominent nonproliferative lesion. The incidence and severity were dose related and significantly increased at all TCA concentrations. Centrilobular alterations are a low-grade degeneration of the hepatocytes characterized by an intense eosinophilic cytoplasm with deep basophilic granularity (microsomes) and slight hepatomegaly. The distribution ranged from centrilobular to diffuse. The incidence of inflammation was increased significantly in the 5 g/L TCA treatment group (Table 8), but was significantly lower in the 0.05- and 0.5 g/L groups between 31 and 45 weeks, but abated by 60 weeks. There was a significant dose-related trend, but a significant increase in severity was only found at 5 g/L. No alteration in the severity of this lesion was observed. The occurrence and severity of nonproliferative lesions in animals exposed to 0.5 and 4.5 g/L TCA for 104 weeks were similar to those observed at 60 weeks (data not shown). No

> pathology outside the liver was observed except for a significant dose-related trend and incidence of testicular tubular degeneration at 0.5 and 5 g/L TCA.

The results shown in Table 7 by the authors for the 60-week TCA-exposed mice did not show a dose-response for either incidence or severity of centrilobular cytoplasmic alterations. They reported a 7, 48, 21, and 93% incidence and a 0.10 ± 0.40, 0.70 ± 0.82, 0.34 ± 0.72, and 1.60 ± 0.62 mean severity score for control, 0.05, 0.5, and 5.0 g/L TCA exposure groups, respectively. Thus, for control, 0.05, and 0.5 g/L TCA exposure, there was less than minimal (i.e., score of 1 or occupying <25% of the microscopic field) severity of this finding for the 27–30 mice examined in each group. Only slight hepatomegaly is noted by the authors to be included in their description of the centrilobular cytoplasmic alteration. Interestingly, the elevation of this parameter for both incidence and severity in the 0.05 g/L TCA exposed group compared to 0.5 g/L exposure group did not correspond to an increase in percent liver/body weight for this same exposure group. While the percent liver/body weight ratio was 32% higher, the incidence and severity of this lesion were reported to be half that in the 0.5 vs. 0.05 g/L exposure groups after 60 days of TCA exposure. Thus, TCA-induced hepatomegaly did not appear to be associated with this centrilobular cytoplasmic change.

Similarly the incidence of hepatic inflammation was reported to be 10, 0, 7, and 24% and severity, 0.11 ± 0.40, 0.09 ± 0.30, 0.12 ± 0.33, and 0.29 ± 0.48 for control, 0.05, 0.5, and 5.0 g/L TCA exposure groups, respectively. Thus, at no TCA exposure concentration was the incidence >24%, and the severity was considerably less than minimal. The reported results for hepatic necrosis were pooled from data from the five mice exposed for either 30 or 45 weeks (n = 10 total). No incidences of necrosis were reported for either control or 0.05 g/L TCA exposed mice. At 0.5 g/L, TCA 3/10 mice were reported to have necrosis but at a severity level of 0.50 ± 0.97. At 5.0 g/L, TCA 5/10 mice were reported to have necrosis but at a severity level of 1.30 ± 1.49. The limitations of the small number of animals pooled in these data are obvious. However, there does not appear to be much more than minimal necrosis at the highest dose of TCA between 30 and 45 weeks and this response is reported by the authors to be transient.

Serum LDH activity was reported by the authors for 31- and 60-week TCA exposures in Study #1. They state that:

> There was a dose-related trend at 31 weeks; serum LDH was significantly increased at 0.5 and 5 g/L TCA (161 ± 39 and 190 ± 44, respectively vs. 100 ± 28 IU for the control). LDH activity returned to control levels at 60 weeks. Similarly, elevated LDH levels were observed at early time periods for 0.5 and 4.5 g/L TCA during the 104 week exposure (data not shown: Studies 2 and 3).

The data presented by the author for Study #1 are from 5 animals/group for the 30-week results and 30 animals/group for the 60-week results. Of interest is for the 60-week data, there appears to be 50% decreased in LDH activity at 0.05 and ~25% decrease in LDH activity at

0.5 g/L TCA treatment with the LDH level reported to be the same as control for the 5 g/L TCA exposure group. For the 31-week data, in which only five animals were tested in each treatment group, there appeared to be a slight increase at the 0.5 g/L (60% increase over control) and 5 g/L (90% increase over control) treatment groups. The data for necrosis detected by light microscopy and by LDH level is consistent with no changes from control detected at the 0.05 g/L TCA treatment group and less than minimal necrosis of on a 60% increase in LDH level over control reported for 0.5 g/L TCA treatment. Even at the highest dose of 5.0 g/L TCA, there is still little necrosis or LDH release reported over control.

Data for testicular tubular degeneration was reported for Study #1 after 60 weeks of TCA exposure. The incidence of testicular tubular degeneration was reported to be 7, 0, 14, and 21% for mice exposed to 2.0 g/L sodium chloride, 0.05, 0.5, and 5.0 g/L TCA. The severity of the lesions was reported to be 0.10 ± 0.40, 0, 0.17 ± 0.47, and 0.21 ± 0.41 with a significant trend with dose reported by the authors for severity and for the 0.5 and 5 g/L treatment groups to be significantly increased over control incidence levels. Of note, similar to the percent liver/body weight ratios and hepatic inflammation values for this data set, the values for testicular tubular degeneration were slightly higher in control mice than 0.05 g/L TCA exposed mice. In regard to mean severity levels for testicular degeneration, although still minimal, there was little difference between the results for reported for the 0.5 and 5.0 g/L TCA exposed mice.

In regard to peroxisome proliferation, liver PCO activity was presented for up to 60 weeks (Study #1) and 104 weeks (Study #2). Similar to the data for LDH activity, ~30 animals were examined at the 60-week time point but only 5 animals per exposure group were examined for 4-, 15-, 31-, and 45-week results. The data are presented in a figure, and in some instances, it is hard to determine the magnitude of change.

Similar to other reports, the baseline level of PCO activity was variable between control groups and ranged 2.7-fold (~1.49–4.06 nmol NAD reduced/minute/mg protein given by the authors). There appeared to be little change in PCO activity between the 0.05 g/L TCA exposure and control levels for up to 45 weeks of exposure (i.e., the groups with n = 5) in Study #1. For the 60-week group, the 0.05 g/L TCA group PCO activity was ~1.7-fold of control but was not statistically significant. For the 0.5 g/L TCA treatment groups, the increase ranged from ~1.3- to 2.7-fold of control after 4, 15, 31, and 45 weeks of exposure with the largest differences reported at 4 and 60 weeks (i.e., 2.2- and 2.7-fold of control, respectively). For the 5.0 g/L TCA exposure groups, the increase ranged from ~3.2- to ~5.7-fold of control after 4, 15, 31, and 45 weeks of exposure.

While the data at 60 weeks had the most animals examined (~30 vs. 5) with ~1.7-, 2.7-, and 4.5-fold of control PCO activity, at this time period, the authors report the occurrence of tumors had already occurred. At the earlier time points of 4 and 15 weeks, there was a difference in the magnitude of TCA-induced increases in PCO activity. As displayed graphically, at 4 weeks, the PCO increases were ~1.3-, 2.4-, and 5.3-fold of control for 0.05, 0.5, and 5.0 g/L

TCA, respectively, while at 15 weeks, the PCO levels were decreased by 5%, increased to 1.3-fold, and increased to 3.2-fold of control with only the 5.0 g/L treatment group difference to be statistically significant.

For Study #2, the authors present a figure (Figure #4) that states that PCO values were given for mice given HAC or 4.5 g/L TCA for 4–60 weeks. However, the data presented in #4 appears to be for 15-, 30-, 45-, and 104-week exposures. The number of mice is not given in the figure but the methods section states that serial sections were conducted on 5 mice/group for these interim sacrifice periods. The number of mice examined for PCO activity at 104 weeks was not given by the authors but the number of mice at final sacrifice was given as 25. The levels of PCO in the control tissues varied by ~33% for weeks 15–45 but there was a ~5-fold difference between the level reported at 104 weeks and that for the earlier time periods in control mice shown in the figures (~2.23 vs. 0.41 nmol NAD reduced/min/mg protein as given by the authors). The increase over control induced by 4.5 g/L TCA in Study #2 was shown to be ~6.9-, 4.8-, 3.6-, and 19-fold of controls for 15, 30, 45 and 104 weeks, respectively.

Therefore, at a comparable level of TCA exposure (~5.0 g/L), number of mice examined (n = 5), and durations of exposure (15, 30, and 45 weeks), the increase in PCO activity induced by ~5.0 g/L TCA varied between 3.2- and 5.7-fold of control in Study #1 and between 3.6- and 6.9-fold of control in Study #2. There was not a consistent pattern between the two studies in regard to level of PCO induction from ~5 g/L TCA and duration of exposure. The lowest TCA-induced PCO activity increase was recorded at 15 weeks in Study #1 (i.e., 3.2-fold of control) and highest PCO activity increase was recorded at 15 weeks in Study #2 (i.e., 6.9-fold of control). No PCO data were reported for data in Study #3 with the exception of the authors stating that "PCO activity was significantly elevated for the 0.5 g/L TCA exposure over the 104 weeks (study 3). The extent of the increases was similar to those measured for 0.5 g/L TCA (200–375%: data not shown) in Study 1." No other details are given for PCO activity in Study #3.

Hepatocyte proliferation was reported by the authors to be assessed by either incorporation of tritiated thymidine (Studies #1 and #2) or BrdU (Study #3) into hepatocyte nuclei. As noted previously, these techniques measure DNA synthesis and not necessarily hepatocyte proliferation. The authors did not report whether specific areas of the liver were analyzed by autoradiographs or how many autoradiographs were examined in the analyses they conducted. For later time points of examination (60–104 weeks), the authors did not indicate whether hepatocytes in foci or adenomas were excluded from DNA synthesis reports. The authors present data for what are clearly, 31-, 45-, and 60-week exposures for Study #1 as the percent tritiated thymidine labeled nuclei. An early time point that appears to be 8 weeks is also given.

However, for Study #1, only 4- and 15-week durations were tested, so it cannot be established what time period the earlier time point represents. What is very apparent from the

data presented for Study #1 is that the baseline level of tritiated thymidine incorporation was relatively high and was highly variable for the five animals examined (~8% of hepatocytes were labeled). There did not appear to be an apparent pattern of TCA treatment groups at this timepoint, with the 0.05 and 5.0 g/L TCA groups having a similar percentage of labeled hepatocytes and for 0.5 g/L TCA reported to have a 60% reduction in labeled hepatocytes.

After 31 weeks of exposure, the control values were reported to be 2% of hepatocytes labeled. The authors report that only the 5.0 g/L TCA group had a statistically significant increase of control and was elevated to ~6% of hepatocytes. The two lower exposure concentrations of TCA had similar reported incidences of labeled hepatocytes of 4.5% that were not reported to be statistically significant.

For the 45-week exposure period in Study #1, the control value was reported to be 1.2%, with only the 5.0 g/L TCA value reported to be statistically significantly increased at 3.2% and the other two TCA groups to be similar to control. Finally, for the 60-week group from Study #1, the control value was reported to be 0.6% of hepatocytes labeled and only the 0.5 g/L TCA dose reported to be statistically significantly increased over control at 3.2%.

What is clear from this study is that the control value for the unidentified early time point is much higher than the other values. There should not be such a large difference in mature mice nor such a high level. The difference in control values between the earlier time point and the 31-week time point was fourfold. The difference between the earlier time point and the 45-week time point was approximately sevenfold. There did not appear to be an increase in hepatocyte tritiated thymidine labeling due to any concentration of TCA at the early unidentified time point (approximately week 10 from the figure) from Study #1. There was no dose-response apparent for the other study periods and the percent of hepatocytes labeled were ≤3%. These results indicated that DNA synthesis was not increased by 10–60-week exposures to TCA exposure that induced increased liver tumor response.

For Study #2, results were reported for tritiated thymidine incorporation into hepatocytes in a figure that was labeled as 4.5 g/L TCA and control tissue for 104 weeks but showed data for 15, 30, and 45 weeks of exposure. Of note is that the control values for this study were much lower than that reported for Study #1. The percent of hepatocytes labeled with tritiated thymidine was reported to be ~2% for the 15-week exposure period and <1% for the 30- and 45-week exposure periods. For the 4.5 g/L TCA exposures, the percent hepatocytes labeled with tritiated thymidine were ~2–4% at all time points with only the 45-week period identified by the authors as statistically significant.

For Study #3, rather than tritiated thymidine, BrdU was used as a measure of DNA synthesis. The results are presented in Figure #8 of the report in which the 0.5 g/L TCA concentration is mislabeled as 0 g/L and the figure is mislabeled as having a duration of 104 weeks, but the data are presented for 26, 52, and 78 weeks of exposure. The percent of hepatocytes at 26 weeks was reported to be ~1–2% for the control, 0.05, and 0.5 g/L TCA

groups. At 52 weeks, the control value was ~1%, the 0.05 g/L TCA value was <0.1% and the 0.5 g/L TCA value was ~3.5% but was not statistically significant. At 78 weeks of exposure, the control value was reported to be ~0.2% with only the 0.05 g/L TCA group having a statistically significant increase over control.

From these data, the estimated control values for DNA synthesis at similar time points of exposure ranged from 0.4 to 2% at 26–31 weeks and ~0.1–1.2% at 45–52 weeks. The results for Studies #1 and #2 were inconsistent in regard to the magnitude of tritiated thymidine incorporation, but were consistent in that there was a lot of variability in these measurements, not a consistent pattern with time that was TCA-dose related, and, even at the highest dose of TCA, did not indicate much of an increase in cell proliferation at 15–45 weeks of exposure. Similarly, the results for Studies #1 and #3 indicate that at the two lower doses of TCA, there were not generally statistically significant increases in DNA synthesis from 15 to 45 weeks of exposure, although there was an increase in liver tumor response at later time points.

The authors reported that "all gross and microscopic histopathological alterations were consistent across the three studies." However, the histological descriptions that follow were focused on the liver for both neoplastic and non-neoplastic parameters. As stated above, only a few animals (n = 5) from the control and high TCA dose level were examined for lesions other than liver, kidneys, spleen, and testes. Thus, whether other neoplastic lesions were induced by TCA exposure cannot be determined from this set of studies.

Study #1 was conducted for 60 weeks. Although of short duration and using ≤30 animals, the authors reported in the text that:

> a significant trend with dose was found for liver cancer. The prevalence and multiplicity of adenomas (38%; 0.55 ± 0.15) or carcinoma (38%; 0.42 ± 0.11) were statistically significant at 602 mg/kg-day TCA compared to control (7%; 0.07 ± 0.05) [sic for both adenoma and carcinoma the same value was given, mean ± SD]. When either an adenoma or a carcinoma was present, statistical significant was seen at both 5 g/L (55%; 1.00 ± 0.19) and 0.5 g/L (38%: 0.52 ± 0.14 TCA exposure groups compared to control (13%; 0.13 ± 0.06).

No significant changes in liver neoplasia were reported to be observed by the authors at 0.05 g/L TCA. Preneoplastic large foci of cellular alteration (24%) were seen in the 5 g/L TCA group compared to control.

Although not statically significant, there was an incidence of 15% adenoma in the 0.05 g/L TCA treatment group (n = 27) and a multiplicity of 0.15 ± 0.07 adenomas/mouse reported, with both values being twice that of the values given for the controls (n = 30). The incidence and multiplicity for carcinomas was approximately the same for the 0.05 g/L TCA treatment group and the control group. Given the small number of animals examined, the study was limited in its ability to determine statistical significance for the lower TCA exposure level.

The fold increases of incidence and multiplicity of adenomas at 60 weeks was 2.1-, 3.0-, and 5.4-fold of control incidence and 2.1-, 3.4-, and 7.9-fold of control multiplicity for 0.05, 0.5, and 5 g/L exposure to TCA. For multiplicity of adenomas and carcinomas combined, there was a 1.46-, 4.0-, and 7.68-fold of control values. Analysis of tumor prevalence data for this study included only animals examined at scheduled necropsy. Since most animals survived until 60 weeks, most were included and a consistent time point for tumor incidence was reported.

There are significant discrepancies for reporting of data for tumor incidences in this report for the 104-week data. While the methods section and table describing the dose calculation and animal survival indicate that Study #3 control animals were administered deionized water and those from Study#2 were given HAC, Table 6 of the report gives 2 g/L sodium chloride as the control solution given for Study #2 and 1.5 g/L HAC for Study #3. A comparison of the descriptions of animal survival and tumor incidence and multiplicity between the results given in DeAngelo et al. (2008) and George et al. (2000) (see Table E-10) shows not only that the control data presented in DeAngelo et al. (2008) for Study #3 are the same data as that presented by George et al. (2000) previously, but also indicates that rather than 1.5 g/L HAC, the tumor data presented in DeAngelo et al. (2008) is for mice exposed to deionized water. DeAngelo et al. (2008) did not report that these data were from a previous publication.

Table E-10. Comparison of descriptions of control data between George et al. (2000) and DeAngelo et al. (2008)

Descriptor	George et al. (2000)	DeAngelo et al. (2008)
Species	Mouse	Mouse
Strain	$B6C3F_1$	$B6C3F_1$
Gender	Male	Male
Age	28–30 d	28–30 d
Source	Charles River, Portage	Charles River, Portage
Mean initial body weight	19.5 ± 2.5 g	19.5 ± 2.5 g
Water consumption	111.7 mL/kg-d	112 mL/kg-d
Laboratory	Research Triangle Park, North Carolina	Research Triangle Park, North Carolina
Number of animals at start	72	72
Number of animals at interim sacrifice	22	21
Number of unscheduled deaths	16	17
Number of animals at final sacrifice	34	34
Number of animals for pathology	65	63
Adenoma incidence	21.40%	21%
Adenoma multiplicity	0.21 ± 0.06	0.21 ± 0.06
Carcinoma incidence	54.80%	55%
Carcinoma multiplicity	0.74 ± 0.12	0.74 ± 0.12

For Studies #2 and #3, tumor prevalence data were reported in the methods section of the report to include necropsies of animals that survived >78 weeks and thus, included animals that were scheduled for necropsy but also those that were moribund and sacrificed at differing times.

Thus, for the longer times of study, there was a mixture of exposure durations that included animals that were ill and sacrificed early and those that survived to the end of the study. Animals that were allowed to live for longer periods or did not die before scheduled sacrifice times had a greater opportunity to develop tumors. However, animals that died early may have died from tumor-related causes.

The mislabeling of the tumor data in DeAngelo et al. (2008) has effects on the interpretation of results; if the tumor results table was not mislabeled, it would indicate that 17 animals were included in the liver tumor analysis that were not included in the final necropsy and that the seven unscheduled deaths could not account for the total number of "extra" mice included in the tumor analysis, so some of the animals had to have come from interim sacrifice times (≤78 weeks) and that for Study #3, the data from 9 animals at terminal sacrifice were not used in the tumor analysis. Not only does it appear that the control data was mislabeled for Study #3, but the control data were also apparently mislabeled for Study #2 as being 2.0 g/L sodium chloride rather than 1.5 g/L HAC. Of the 42 animals used for the tumor analysis in Study #3, only 34 were reported to have survived to interim sacrifice so that 8 animals were included from unscheduled deaths. However, the authors report that there were 17 unscheduled deaths in the study, but not all were included in the tumor analysis. The basis for the selection of the eight animals for tumor analysis was not given by the authors.

Not only are the numbers of control animals used in the tumor analysis different between two studies (25 mice in Study #2 and 42 mice in Study #3), but the liver tumor results reported for Study #2 and Study #3 were very different. Of the 42 "control" mice examined from Study #3, the incidence and multiplicity of adenomas were reported to be 21% and 0.21 ± 0.06, respectively. For carcinomas, the incidence and multiplicity were reported to be 55% and 0.74 ± 0.12, respectively, and the incidence and multiplicity of adenomas and carcinomas combined were reported to be 64% and 0.93 ± 0.12, respectively. For the 25 mice reported by the authors for Study #2 to have been treated with "2.0g/L NaCl" but were probably exposed to 1.5 g/L HAC, the incidence and multiplicity of adenomas was 0%. For carcinomas, the incidence and multiplicity were reported to be 12% and 0.20 ± 0.12, respectively, and the incidence and multiplicity of adenomas and carcinomas combined were reported to be 12% and 0.20 ± 0.12, respectively. Therefore, while ~64% the 42 control mice in Study #3 were reported to have adenomas and carcinomas, only 12% of the 25 mice were reported to have adenomas and carcinomas in Study #2 for 104 weeks.

While the effect of using fewer mice in one study vs. the other will be to reduce the power of the study to detect a response, there are additional factors that raise questions regarding the tumor results. Not only were the tumor incidences reported to be higher in control mice from

Study #3 than Study #2, but the number of unscheduled deaths was reported to also be twofold higher. The age, gender, and strain of mouse were reported to be the same between Studies #2 and #3 with only the vehicles differing and weight of the mice to be reported to be different. Although the study by George et al. (2000) described the same control data set as for Study #3 as being for animals given deionized water, there is uncertainty as to the identity of the vehicle used for the tumor results reported for Study #3 and there are some discrepancies in reporting between the two studies. As discussed below in Section E.2.5, the differences in the weight of the mice between Studies #1, #2, and #3 is critical to the issue of differences in background tumor rate and hence interpretability of the study.

As noted by Leakey et al. (2003a), the greatest correlation with liver tumor incidence and body weight appears between the ages of 20 and 60 weeks in male mice. As reported in Section E.2.5, the mean 45-week body weight reported for control male $B6C3F_1$ mice in the George et al. (2000) study, which is the same control data as DeAngelo et al. (2008) was ~50 g. This is a much greater body weight than reported for Study #1 at 45 weeks (i.e., 39.6 g) and for Study #2 at 45 weeks (i.e., 39.4 g). Using probability curves presented by Leakey et al. (2003a), the large background rate of 64% of combined adenomas and carcinomas for Study #3 is in the range predicted for such a large body weight (i.e., ~65%). Such a high background incidence compromises a 2-year bioassay, as it prevents demonstration of a positive dose-response relationship. Thus, Study #3 of DeAngelo et al. (2008) is not comparable to the results in Studies #1 and #2 for the determination of the dose-response for TCA.

The accurate determination of the background liver tumor rate is very important in determining a treatment-related effect. The very large background level of tumor incidence reported for Study #3 makes the detection of a TCA-related change in tumor incidence at low exposure levels very difficult to determine. Issues also arise as to what the source of the tumor data were in the TCA-treatment and control groups in Study #3. While 29 mice exposed to 0.05 g/L TCA were reported to have been examined at terminal sacrifice, 35 mice were used for liver tumor analysis. Similarly, while 27 mice exposed to 0.5 g/L TCA were reported to have been examined at terminal sacrifice, 37 mice were used for tumor analysis. Finally, for the 42 control animals examined for tumor pathology in the control group, 34 were examined at terminal sacrifice. Clearly, more animals were included in the analyses of tumor incidence and multiplicity than were sacrificed at the end of the experiment. What effect differential addition of the results from mice not sacrificed at 104 weeks and the selection bias that may have resulted from their inclusion on these results cannot be determined. Not only were the background levels of tumors reported to be increased in the control animals in Study #3 compared to Study #2 at 104 weeks, but the rate of unscheduled deaths was doubled. This is also an expected consequence of using much larger mice (Leakey et al., 2003a).

For the 35 mice examined after 0.05 g/L TCA in Study #3, the incidence and multiplicity of adenomas were reported to be 23% and 0.34 ± 0.12, respectively. For carcinomas, the

incidence and multiplicity were reported to be 40% and 0.71 ± 0.19, respectively, and the incidence and multiplicity of adenomas and carcinomas combined were reported to be 57% and 1.11 ± 0.21, respectively. For the 37 mice examined after 0.5 g/L TCA in Study #3, the incidence and multiplicity of adenomas were reported to be 51% and 0.78 ± 0.15, respectively. For carcinomas, the incidence and multiplicity were reported to be 78% and 1.46 ± 0.21, respectively, and the incidence and multiplicity of adenomas and carcinomas combined were reported to be 87% and 2.14 ± 0.26, respectively.

Thus at 0.5 g/L TCA, the results presented for this study for the "104 week" liver tumor data were significantly increased over the reported control values. However, these results are identical to those reported in Study #3 for a 10-fold higher concentration of TCA (4.5 g/L TCA) for the same 104 weeks of exposure but in the much larger mice. Of the 36 animals exposed to 4.5 g/L TCA in Study #2 and included in the tumor analysis, 30 animals were reported to be examined at 104 weeks. The incidence and multiplicity of adenomas were reported to be 59% and 0.61 ± 0.16, respectively. For carcinomas, the incidence and multiplicity were reported to be 78% and 1.50 ± 0.22, respectively, and the incidence and multiplicity of adenomas and carcinomas combined were reported to be 89% and 2.11 ± 0.25, respectively.

The importance of selection and determination of the control values for comparative purposes of tumor induction are obvious from these data. The very large difference in control values between Study #2 and Study #3 is the determinant of the magnitude of the dose response for TCA after 104 weeks of exposure. The tumor response for 0.5 and 4.5 g/L TCA exposure between the two experiments was identical. Therefore, only the background tumor rate determined the magnitude of the response to treatment. If similar control values (i.e., a historical control value) were used in these experiments, there would appear to be no difference in TCA-tumor response between 0.5 and 4.5 g/L TCA at 104 weeks of exposure. DeAngelo et al. (1999) report for male B6C3F$_1$ mice exposed only water for 79–100 weeks the incidence of carcinomas to be 26% and multiplicity to be 0.28 lesions/mouse. For 100-week data, the incidence and prevalence of adenomas were reported to be 10% and 0.12 ± 0.05 and to be 26% and 0.28 ± 0.07 for carcinomas.

Issues with reporting for that study have already been discussed in Section E.2.3.2.6. However, the data for DeAngelo et al. (1999) are more consistent with the control data for "1.5 g/L HAC" for Study #2 in which there were 0% adenomas and 12% carcinomas with a multiplicity of 0.20 ± 0.12, than for the control data for Study #3 in which 64% of the control mice were reported to have adenomas and carcinomas and the multiplicity was 0.93 ± 0.12. If either the control data from DeAngelo et al. (1999) or Study #2 were used for comparative purposes for the TCA-treatment results of Study #2 or #3, there would be a dose-response between 0.05 and 0.5 g/L TCA but no difference between 0.5 and 4.5 g/L TCA after 100 weeks of exposure. The tumor incidence would have peaked at ~90% in the 0.5 and 4.5 g/L TCA exposure groups. These results would be more consistent with the 60-week results in Study #1

in which 0.5 and 5 g/L TCA exposure groups already had incidences of 38 and 55% of adenomas and carcinomas combined, respectively, compared to the 13% control level. With increased time of exposure, the differences between the two highest TCA exposure concentrations may diminish as tumor progression is allowed to proceed further. However, the use of the larger and more tumor prone mice in Study #3 also increases the tumor incidence at the longer period of study.

The authors also presented data for multiplicity of combined adenomas or carcinomas for mice sacrificed at weeks 26, 52, and 78 for Study #3 (n = 8 per group). No indication of variability of response, incidence data, statistical significance, or data for adenomas vs. carcinomas, or the incidence of adenomas was reported. The authors reported that "neoplastic lesions were first found in the control and 0.05 g/L TCA groups at 52 weeks. At 78 weeks, adenomas or carcinomas were found in all groups (0.29, 0.20, and 0.57 tumors/animals for control, 0.05 g/L TCA, and 0.5 g/L TCA, respectively)." Because no other data were presented at the 52 and 78 week time points in this study, these results cannot be compared to those presented for Study #1, which was conducted for 60 weeks. Of note, the results presented from Study #1 for 60 weeks of exposure to control, 0.05, or 0.5 g/L TCA exposure in 27–30 mice show a 13, 15, and 38% incidence of hepatocellular adenomas and carcinomas and a multiplicity of 0.13 ± 0.06, 0.19 ± 0.09, and 0.52 ± 0.14, respectively. Both the incidence and multiplicity of adenomas were twofold higher in the 0.05 g/L TCA treatment group than for the control. However, the interim data presented by the authors from Study #3 for 52 weeks of exposure in only eight mice per group gives a higher multiplicity of adenomas and carcinomas for control animals (~0.25) than for either 0.05 or 0.5 g/L TCA treatments. Again, comparisons between Studies #2 and #3 are difficult due to difference in mouse weight.

Of note, there are no descriptions given in this report in regard to the phenotype of the tumors induced by TCA or for the liver tumors reported to occur spontaneously in control mice. Such information would have been of value, as this study reports results for a range of TCA concentration and for 60 and 100 weeks of exposure. Insight could have been gained as to the effects of differing concentrations of TCA exposure and whether TCA-induced liver tumors had a similar phenotype as those occurring spontaneously, as well as information in regard to effects on tumor progression and heterogeneity.

Although only examining tissues from five mice from the control and high-dose groups only at 104 weeks at organ sites other than the liver, the authors report that:

> neoplastic lesions at 104 weeks (Studies #2 and #3) at organ sites other than the liver were found in the lung, spleen, lymph nodes, duodenum (lymphosarcoma), seminal vesicles, skin, and thoracic cavity of control and treated animals. All were considered spontaneous for the male B6C3F_1 mouse and did not exceed the tumor incidences when compared to a historical control database (NIEHS, 1998; Haseman et al., 1984).

No data were shown. The limitations involved in examining only five animals in the control and high-dose groups, and the need to examine the concurrent control data in each experiment, especially given the large variation in liver tumor response between long-term studies carried out in the two different laboratories used for Study #2 and Study #3 using the same strain and gender of mouse, make assertions regarding extrahepatic carcinogenicity of TCA from this study impossible to support.

A key issue raised from this study is whether changes in any of the parameters measured in interim sacrifice periods before the appearance of liver tumors (i.e., 4–15 weeks) corresponded to the induction of liver tumors. The first obstacle for determining such a relationship is the experimental design of these studies in which only a full range of TCA concentrations is treated for 60 weeks of exposure with a small number of animals available for determination of a carcinogenic response (i.e., ≤30 animals in Study #1) and a very small number of animals (n = 5 group) examined for other parameters. Also as stated above, PCO activity was highly variable between controls and between treatment groups (e.g., the PCO activity for Studies #1 and #2 at ~5 g/L exposure for 15 weeks).

On the other hand, most of the animals that were examined at terminal sacrifice were also utilized for the tumor results without the differential deletion or addition of "extra" animals for the tumor analysis. For the 60-week data in Study #1, there appeared to be a consistent dose-related increase in the incidence and multiplicity of tumors after TCA exposure (Table E-11). The TCA-induced increases in liver tumor responses can be compared with both increased liver weight and PCO activity that were also reported to be increased with TCA dose as earlier events. Although the limitations of determining the exact magnitude of responses has already been discussed, as shown below, the incidence and multiplicity of adenomas show a dose-related increase at 60 weeks. However, the magnitude of differences in TCA concentrations was not similar to the magnitude of increased liver tumor induction by TCA after 60 weeks of exposure.

First of all, the greater occurrence of TCA-induced increases in adenomas than carcinomas reported after 60 weeks of exposure would be expected for this abbreviated duration of exposure as they would be expected to occur earlier than carcinomas. For adenoma induction, there was an approximately twofold increase between the 0.05 g/L dose of TCA and the control group for incidence (7 vs. 15%) and multiplicity (0.07 vs. 0.15 tumors/animals). However, an additional 10-fold increase in TCA dose (0.5 g/L) only resulted in a reported 1.8-fold greater incidence (15 vs. 21%) and 2.2-fold increase in multiplicity (0.15 vs. 0.24 tumors/animal) of control adenoma levels. An additional 10-fold increase in dose (5.0 vs. 0.5 g/L TCA) resulted in a 2.2-fold increase in incidence (21 vs. 38%) and 2.9-fold increase in multiplicity (0.24 vs. 0.55 tumors/animal) of control adenoma levels.

Thus, a 100-fold difference in TCA exposure concentration resulted in differences of fourfold of control incidence and sixfold of control multiplicity for adenomas. For adenomas or carcinomas combined (a parameter that included carcinomas for which only the two highest

exposure levels of TCA were reported to increase incidence and multiplicity), the incidences were reported to be 13, 15, 38, and 55%, and the multiplicity was reported to be 0.13, 0.19, 0.52, and 1.00 for control, 0.05, 0.5, and 5.0 g/L TCA at 60 weeks. For multiplicity of adenomas or carcinomas, the 0.05 g/L TCA exposure induced a 1.5-fold increase over control. An additional 10-fold increase in TCA (0.5 g/L) induced a 6-fold increase in tumors/animal. An additional 10-fold increase in TCA (5.0 vs. 0.5 g/L) induced an additional 2.2-fold increase in tumors/animal. Therefore, using combinations of adenomas or carcinomas, there was a 13-fold increase in multiplicity that corresponded with a 100-fold increase in dose.

Table E-11. TCA-induced increases in liver tumor occurrence and other parameter over control after 60 weeks (Study #1)

Dose TCA g/L	Adenomas		Adenomas or carcinomas		% liver/body weight		PCO activity	
Sodium chloride	Incidence 7%	Multiplicity 0.07	Incidence 13%	Multiplicity 0.13	4-wk	15-wk	4-wk	15-wk
0.05	15% (2.1-fold)	0.15 (2.1-fold)	15% (1.2-fold)	0.19 (1.5-fold)	1.09-fold	1.14-fold	1.3-fold	1.0 -fold
0.5	21% (3.0-fold)	0.24 (3.4-fold)	38% (2.9-fold)	0.52 (4.0-fold)	1.16-fold	1.16-fold	2.4-fold	1.3-fold
5.0	38% (5.4-fold)	0.55 (7.9-fold)	55% (4.2-fold)	1.00 (7.7-fold)	1.35-fold	1.47-fold	5.3-fold	3.2-fold

The results for adenoma induction at 60 weeks of TCA exposure (i.e., ~2-fold increased incidences and 2–3-fold increases in multiplicity with 10-fold increases in TCA dose) are similar to the ~2-fold increase in liver weight gain resulting from 10-fold differences in dose reported at 4 weeks of exposure. For PCO activity, there was a ~30% increase in PCO activity from control at 0.05 g/L TCA. A 10-fold increase in TCA exposure concentration (0.5 g/L) resulted in an additional ~5-fold increase in PCO activity. However, another 10-fold increase in TCA concentration (0.5 vs. 5 g/L) resulted in a 3-fold increase in PCO activity. The 100-fold increase in TCA dose (0.05 vs. 5 g/L TCA) was correlated with a 14-fold increase in PCO activity. For 15 weeks of TCA exposure, there was no difference in 0.05 g/L and control PCO activity and only a 30% difference between the 0.05 and 0.5 g/L TCA exposures. There was a sevenfold difference in PCO activity between the 0.5 and 5.0 g/L TCA exposure concentrations. The increases in PCO activity and liver weight data at 15 weeks did not fit the magnitude of increases in tumor multiplicity or incidence data at 60 weeks as well as did the 4-week data. However, the TCA-induced increase in tumors at 60 weeks (especially adenomas) seemed to correlate more closely with the magnitude of liver weight increase than for PCO activity at both 4 and 15 weeks.

In regard to Studies #1 and #2, there were consistent periods of study for percent liver/body weight with the consistency of the control values being a large factor in the magnitude of TCA-induced liver weight increases. As discussed above, there were differences in the magnitude of percent liver/body weight increase at the same concentration between the two studies (e.g., a 1.47-fold of control percent liver/body weight in the 5 g/L TCA exposed group in Study #1 and 1.60-fold of control in Study #2 at 15 weeks). For the two studies that had extended durations of exposure (Studies #2 and #3), the earliest time period for comparison of percent liver/body weight is 26 weeks (Study #3) and 30 weeks (Study #2). If those data sets (26 weeks for Study #3 and 30 weeks for Study #2) are combined, then 0.05, 05, and 4.5 g/L TCA gives a percent liver body/weight increase of 1.07-, 1.18-, and 1.40-fold over concurrent control levels. Using this parameter, there appears to be a generally consistent pattern as that reported for Study #1 at weeks 4 and 15. Generally, a 10-fold increase in TCA exposure concentration resulted in ~2.5-fold increased in additional liver weight observed at ~30 weeks of exposure, which correlated more closely with adenoma induction at 60 weeks than did changes in PCO activity. A similar comparison between Studies of longer duration (Studies #2 and #3) could not be made for PCO activity as data were not reported for Study #3.

For 104-week studies of TCA-tumor induction (Studies #2 and #3), the lower TCA exposure levels (0.05 and 0.5 g/L TCA) were assayed in a separate experiment and by a separate laboratory than the high dose (5.0 g/L TCA) and most importantly in larger, more tumor prone mice. The total lack of similarity in background levels of tumors in Studies #2 and #3, the differences in the number of animals included in the tumor analyses, and the low number of animals examined in the tumor analysis at 104 weeks (<30 for the TCA treatment groups) makes the determination of a dose-response TCA-induced liver tumor formation after 104-weeks of

exposure problematic. The correlation of percent liver/body weight increases with incidence and multiplicity of liver tumors in Study #1 and the similarity of dose-response for early induction of percent liver/body weight gain between Study #1 suggest that there should be a similarity in tumor response. However, as noted above, the 104-week studies had very difference background rates of spontaneous tumors reported in the control mice between Studies #2 and #3.

Table E-12 shows the incidence and multiplicity data for Studies #2 and #3 along with the control data for DeAngelo et al. (1999) for the same paradigm. It also provides an estimate of the magnitude of increase in liver tumor induction by TCA treatments if the control values from the DeAngelo et al. (1999) data set were used as the background tumor rate. As shown below, the background rates for Study #2 are more consistent with those of DeAngelo et al. (1999). Whereas there was a 2:1 ratio of multiplicity for adenomas and adenomas and carcinomas between 0.5 and 5.0 g/L TCA after 60 weeks of exposure, there was no difference in any of the data (i.e., adenoma, carcinoma, and combinations of adenoma and carcinoma incidence and multiplicity) for these exposure levels in Studies #2 and #3 for 104 weeks. The difference in the incidences and multiplicities for all tumors was twofold between the 0.05 and 0.5 g/L TCA exposure groups in Study #2. These results are consistent with the two highest exposure levels, reaching a plateau of response with a long enough duration of exposure (~90% of animals having liver tumors) and with the 2-fold difference in liver tumor induction between concentrations of TCA that differed by 10-fold, reported in Study #1.

Table E-12. TCA-induced increases in liver tumor occurrence after 104 weeks (Studies #2 and #3)

Dose TCA	Adenomas		Carcinomas		Adenomas or carcinomas	
	Incidence	Multiplicity	Incidence	Multiplicity	Incidence	Multiplicity
Study #3						
1.5 g/L HAC (H_2O?)	21%	0.21	55%	0.74	64%	0.93
0.05 g/L TCA	23%	0.34	40%	0.71	57%	1.11
	(1.1-fold)	(1.6-fold)	(0.7-fold)	(1.0-fold)	(0.9-fold)	(1.2-fold)
0.5 g/L TCA	51%	0.78	78%	1.46	87%	2.14
	(2.4-fold)	(3.7-fold)	(1.4-fold)	(2.0-fold)	(1.4-fold)	(2.3-fold)
Study #2						
2.0 g/L NaCl (HAC?)	0%	0	12%	0.20	12%	0.20
4.5 g/L TCA	59%	0.61	78%	1.50	89%	2.14
	(?)	(?)	(6.5-fold)	(7.5-fold)	(7.4-fold)	(11-fold)
DeAngelo et al. (1999)						
H_2O	10%	0.12	26%	0.28		
0.05 g/TCA (S #3)	(2.3-fold)	(2.8-fold)	(1.5-fold)	(2.5-fold)		
0.5 g/L TCA (S #3)	(5.1-fold)	(6.5-fold)	(3.0-fold)	(5.2-fold)		
5.0 g/L TCA (S #2)	(5.9-fold)	(6.5-fold)	(3.0-fold)	(5.4-fold)		

H_2O = water

If either the control values for Study #2 or the control values from DeAngelo et al. (1999) were used for as the background rate of spontaneous liver tumor formation, the magnitude of liver tumor induction by the 0.05 g/L TCA over control levels differs dramatically from that reported as control tumor rates in Study #3. To put the 64% incidence data for carcinomas and adenomas reported in DeAngelo et al. (2008) for the control group of Study #3 in context, other studies cited in this review for $B6C3F_1$ mice show a much lower incidence in liver tumors in that: (1) the NCI (1976) study of TCE reports a colony control level of 6.5% for vehicle and 7.1% incidence of HCCs for untreated male $B6C3F_1$ mice (n = 70–77) at 78 weeks; (2) Herren-Freund et al. (1987) report a 9% incidence of adenomas in control male $B6C3F_1$ mice with a multiplicity of 0.09 ± 0.06 and no carcinomas (n = 22) at 61 weeks; (3) NTP (1990) reports an incidence of 14.6% adenomas and 16.6% carcinomas in male $B6C3F_1$ mice after 103 weeks (n = 48); and (4) Maltoni et al. (1986) report that $B6C3F_1$ male mice from the "NCI source" had a 1.1% incidence of "hepatoma" (carcinomas and adenomas) and those from "Charles River Co." had a 18.9% incidence of "hepatoma" during the entire lifetime of the mice (n = 90 per group). The importance of examining an adequate number of control or treated animals before confidence can be placed in those results in illustrated by Anna et al. (1994) in which at 76 weeks 3/10 control male $B6C3F_1$ mice that were untreated and 2/10 control animals given corn oil were reported to have adenomas but from 76 to 134 weeks, 4/32 mice were reported to have adenomas (multiplicity of 0.13 ± 0.06) and 4/32 mice were reported to have carcinomas (multiplicity of 0.12 ± 0.06).

Using concurrent control values reported in Study #3, there is no increase in incidence of multiplicity of adenomas and carcinomas for the 0.05 g/L exposure group. However, compared to either the control data from DeAngelo et al. (1999) or the control data from Study #3, there is a ~2–3- or ~5-fold increased in incidence or multiplicity of liver tumors, respectively. Thus, trying to determine a correspondence with either liver weight increases or increases in PCO activity at earlier time points will depend on the confidence placed in the concurrent control data reported in Study #3 in the 104 week studies. As noted previously, the use of larger, tumor prone mice in Study #3 limits its usefulness to determine the dose-response for TCA.

The authors provided a regression analysis for "tumors/animal" or multiplicity as a percent of control values and PCO activity for the 60- and 104-week data. Whether adenomas and carcinomas combined or individual tumor type were used was not stated. In addition, comparing PCO activity at the end of the experiments, when there was already a significant tumor response rather than at earlier time points, may not be useful as an indicator of PCO activity as a key event in tumorigenesis. A regression analysis of these data are difficult to interpret because of the dose spacing of these experiments as the control and 5 g/L exposure levels will basically determine the shape of the dose-response curve. The 0.05 and 0.5 g/L exposure groups in the regression were so close to the control value in comparison to the 5 g/L exposure, that the dose response will appear linear between control and the 5.0 g/L value with

the two lowest doses not affecting the slope of the line (i.e., "leveraging" the regression). The value of this analysis is limited by: (1) the use of tumor prone larger mice in Study #3 that had large background rates of tumors, which make inappropriate the apparent combination of results from Studies #2 and #3 for the multiplicity as percentages of control values; (2) the low and varying number of animals analyzed for PCO values and the variability in PCO control values; (3) the appropriateness of using PCO values from later time points; and (4) the dose-spacing of the experiment.

Similarly, the authors reported a regression analysis that compares "percent of hepatocellular neoplasia," which again, is indicated by tumor multiplicity with TCA dose as represented by mg/kg-day. This regression analysis also is of limited value for the same reasons as that for PCO with added uncertainty, as the exposure concentrations in drinking water have been converted to an internal dose and each study gave different levels of drinking water with one study showing a reduction of drinking water at the 5 g/L level. The authors attempted to identify a NOEL for tumorigenicity using tumor multiplicity and TCA dose. However, it is not an appropriate descriptor for these data, especially given that "statistical significance" of the tumor response is the determinant of the conclusions regarding a dose in which there is no TCA-induced effect. Only the 60-week experiment (i.e., Study #1) is useful for the determination of tumor dose-response due to the issues related to appropriateness of control in Study #3. A power calculation of the 60-week study shows that the type II error, which should be >50% and thus greater than the chances of "flipping a coin," was 41 and 71% for incidence and 7 and 15% for multiplicity of adenomas for the 0.05 and 0.5 g/L TCA exposure groups. For the combination of adenomas and carcinomas, the power was 8 and 92% for incidence and 6 and 56% for multiplicity at 0.05 and 0.5 g/L TCA exposure. Therefore, the designed experiment could accept a false null hypothesis, especially in terms of tumor multiplicity, at the lower exposure doses and erroneously conclude that there is no response due to TCA treatment.

E.2.3.2.14. DeAngelo et al. (1997)

The design of this study appears to be similar to that of DeAngelo et al. (2008) but to have been conducted in F344 rats. Rats (28–30 day old rats), reported to be of similar weights, were exposed to 2.0 g/L sodium chloride, 0.05, 0.5, or 5.0 g/L TCA in drinking water for 104 weeks. There were groups of animals sacrificed at 15, 30, 45, and 60 weeks (n = 6) for PCO analysis. There were 23, 24, 19, and 22, animals reported to be examined at terminal sacrifice at 104 weeks and 23, 24, 20, and 22 animals reported to be used in the liver tumor analysis reported by the authors for the control, 0.05, 0.5, and 5.0 g/L treatment groups, respectively. Complete pathological exams were reported to be performed for all tissues from animals in the high-dose TCA group at 104 weeks. No indication was given as to whether a complete necropsy and pathological exam was performed for controls at terminal sacrifice. Tritiated thymidine was reported to be administered at interim sacrifices 5 days prior to sacrifice and to be examined with

autoradiography. The 5 g/L TCA treatment group was reported to have a reduction in growth to 89.3% of controls.

For water consumption, TCA was reported to slightly decrease water consumption at all doses with a 7, 8, and 4% decrease in water consumption reported for 0.05, 0.5, and 5.0 g/L TCA, respectively. Body weight was decreased by 5.0 g/L TCA dose only through 78 weeks of exposure to 89.3% of the control value. All of the percent liver/body weight ratios were reported to be slightly decreased (1–4%) by all of the exposure concentrations of TCA but the data shown do not indicate if the liver weight data were taken at interim sacrifice times and appear to be only for animals at terminal sacrifice of 104 weeks.

No data were shown for hepatocyte proliferation but the authors reported no TCA treatment effects. For PCO, there was a 2.3-fold difference between control values between the 15- and 104-week data. For the 0.05 and 0.5 g/L TCA treatment groups, there was not a statistically significant difference reported between control and treated group PCO levels. At 15 weeks, the PCO activity was reduced by 55%, increased to 1.02-fold, and increased 2.12-fold of control for 0.05, 0.5, and 5.0 g/L TCA exposures, respectively. For the 30-week exposure groups, the 0.05 and 0.5 g/L TCA groups were reported to have PCO levels within 5% of the control level. However, for the 5.0 g/L TCA treatment groups, there was approximately twofold of control PCO activity at the 15, 30, 45, and 60 weeks and at 104 weeks, there was a fourfold of control PCO activity. Of note is that the control PCO value was lowest at 104 weeks, while the TCA treatment group was similar to interim values.

For analysis of liver tumors, there were 20–24 animals examined in each group. Unlike the study of DeAngelo et al. (2008), it appeared that most of the animals that were sacrificed at 104 weeks were used in the tumor analysis without addition of "extra" animals or deletion of animal data. The incidence of adenomas was reported to be 4.4, 4.2, 15, and 4.6% and the incidence of HCCs was reported to be 0, 0, 0, and 4.6% for the control, 0.05, 0.5, and 5.0 g/L TCA exposure groups. The multiplicity or tumors/animal was reported to be 0.04, 0.08, 0.15, and 0.05 for adenomas and 0, 0, 0, and 0.05 for carcinomas for the control, 0.05, 0.5, and 5.0 g/L TCA exposure groups.

Although there was an increase in the incidence of adenomas at 0.5 g/L and an increase in carcinomas at 5.0 g/L TCA, they were not reported to be statistically significant by the authors; neither were the increases in adenoma multiplicity at the 0.05 and 0.5 g/L exposures. However, using such a low number of animals per treatment group (n = 20–24) limits the ability of this study to determine a statistically significant increase in tumor response and to be able to determine that there was no treatment-related effect. A power calculation of the study shows that the type II error, which should be >50% and thus, greater than the chances of "flipping a coin," was <6% for incidence and multiplicity of tumors at all exposure DCA concentrations with the exception of the incidence of adenomas for 0.5 g/L treatment group (58.7%). Therefore, the designed experiment could accept a false null hypothesis, especially in terms of tumor

multiplicity, at the lower exposure doses and erroneously conclude that there is no response due to TCA treatment. Thus, while suggesting a lower response than for mice for TCA-induced liver tumors, the study is inconclusive for determination of whether TCA induces a carcinogenic response in the liver of rats. The experimental design is such that extrahepatic carcinogenicity of TCA in the male rat cannot be determined.

E.2.3.2.15. DeAngelo et al. (1996)

In this study, 28-day-old male F344 rats were given drinking water containing DCA at concentrations of 0, 0.05, 0.5, or 5.0 g/L with another group was provided water containing 2.0 g/L sodium chloride for 100 weeks. This experiment modified its exposure protocol due to toxicity (peripheral neuropathy) such that the 5.0 g/L group was lowered to 2.5 g/L at 9 weeks, then 2.0 g/L at 23 weeks, and finally to 1.0 g/L at 52 weeks. When the neuropathy did not reverse or diminish, the animals were sacrificed at 60 weeks and excluded from the results. Based on measured water intake in the 0, 0.05, and 0.5 g/L groups, the TWA doses were reported to be 0, 3.6, and 40.2 mg/kg-day respectively. This experiment was conducted at a U.S. EPA laboratory in Cincinnati and the controls for this group were given 2.0 g/L sodium chloride (Study #1). In a second study, rats were given either deionized water or 2.5 g/L DCA, which was also lowered to 1.5 g/L at 8 weeks and to 1.0 g/L at 26 weeks of exposure (Study #2).

Although 23 animals were reported to be sacrificed at terminal sacrifice that had been given 2 g/L sodium chloride, the number of animals reported to be examined in this group for hepatocellular lesions was 3. The incidence data for this group for adenomas was 4.4%, so this is obviously a typographical error. The number of rats included in the water controls for tumor analysis was reported to be 33, which was the same number as those at final sacrifice. The number of animals at final sacrifice was reported to be 23 for 2 g/L sodium chloride, 21 for 0.05 g/L DCA, 23 for 0.5 g/L DCA in experiment #1, and 33 for deionized water and 28 for the initial dose of 2.5 g/L DCA in experiment #2.

Although these were of the same strain, the initial body weight was 59.1 vs. 76 g for the 2.0 g/L control group vs. deionized water group. The treatment groups in both studies were similar to the deionized water group. The percent liver/body weights were greater (4.4 vs. 3.7% in the sodium chloride vs. deionized water control groups [~20%]). The number of unscheduled deaths was greater in Study #2 (22%) than in Study #1 (12%). Interim sacrifice periods were conducted.

As with the DeAngelo et al. (DeAngelo et al., 2008) study in mice, the number of animals reported at final sacrifice was not the same as the number examined for liver tumors in Study #1 (five more animals examined than sacrificed at the 0.05 g/L DCA and six more animals examined than sacrificed at the 0.5 g/L DCA exposure groups) with n = 23, n = 26, and n = 29 for the 2 g/L sodium chloride, 0.05 g/L DCA, and 0.5 g/L DCA groups utilized in the tumor analysis. For Study #2, the same number of rats was reported to be sacrificed as examined. The source of the

extra animals for tumor analysis in Study #1, whether from interim sacrifice or unscheduled deaths, was not given by the authors and is unknown. Carcinoma prevalence data were not reported for the control group or 0.05 g/L DCA group in Study #1 and multiplicity data were not reported for the control group or 0.05 g/L DCA group. Multiplicity was not reported for adenomas in the 0.05 g/L DCA group in Study #1.

There was a lack of hepatocyte DNA synthesis and necrosis reported at any dose group carried out to final sacrifice at 100 weeks. The authors reported the incidence of adenomas to be 4.4% in 2 g/L sodium chloride control, 0 in 0.05 g/L DCA, and 17.2% in the 0.5 g/L DCA exposure groups. For carcinomas, no data were reported for the control or 0.05 g/L DCA group but an incidence of 10.3% was reported for the 0.5 g/L DCA group. The authors reported increased hepatocellular adenomas and carcinomas in male F344 rats, although no data were reported for carcinomas in the control and 0.05 g/L exposure groups. They reported that for 0.5 g/L DCA, 24.1 vs. 4.4% adenomas and carcinomas combined (Study #1) and 28.6 vs. 3.0% (Study #2) at what was initially 2.5 g/L DCA but continuously reduced. Tumor multiplicity was reported to be significantly increased in the 0.5 g/L DCA group (0.04 adenomas and carcinomas/ animal in control vs. 0.31 in 0.5 g/L DCA in Study #1 and 0.03 in control vs. 0.36 in what was initially 2.5 g/L DCA in Study #2). The issues of use of a small number of animals, additional animals for tumor analysis in Study #1, and most of all, the lack of a consistent dose for the 2.5 g/L animals in Study #2, are obvious limitations for establishment of a dose-response for DCA in rats.

E.2.3.2.16. Richmond et al. (1995)

This study was conducted by the same authors as DeAngelo et al. (1996) and appears to report results for the same data set for the 2 g/L sodium chloride control, 0.05 g/L DCA and 0.5 g/L DCA exposed groups. Of note is that while DeAngelo et al. (1996) refer to the 28-day-old rats as ”weanlings,” the same aged rats are referred to as “adults” in this study. Male F344 rats were administered TWA concentrations of 0, 0.05, 0.5, or 2.4 g/L DCA in drinking water. Concentrations were kept constant but due to hind-limb paralysis, all 2.4 g/L DCA rats had been sacrificed by 60 weeks of exposure. In the 104-week sacrifice time, there were 23 rats reported to be analyzed for incidence of hepatocellular adenomas and carcinomas in the control group, 26 rats in the 0.05 g/L DCA group, and 29 rats in the 0.5 g/L DCA exposed group. This is the same number of animals included in the tumor analysis reported in DeAngelo et al. (1996). Tumor multiplicity was not given.

Richmond et al. (1995) reported that there was a 4% incidence of adenomas reported in the 2.0 g/L sodium chloride control animals, 0% at 0.05 g/L DCA, and 21% in the 0.5 DCA group at 104 weeks. These figures are similar to those reported by DeAngelo et al. (1996) for the same data set, with the exception of a 17.2% incidence of adenomas reported for the 0.5 g/L DCA group.

There were no HCCs reported in the control or 0.05 g/L exposure groups, but a 10% incidence reported in the 0.5 g/L DCA exposure group at 104 weeks of exposure. While carcinomas were not reported by DeAngelo et al. (1996) for the control and 0.05 g/L groups, they are assumed to be zero in the summary data for carcinomas and adenomas combined. The 10% incidence at 0.5 g/L DCA is similar to the 10.4% incidence reported for this group by DeAngelo et al. (1996).

At 60 weeks at 2.4 g/L DCA, the incidences of hepatocellular adenomas were reported to be 26% and HCCs to be 4%. This is not similar to the values reported by DeAngelo for 2.5 g/L DCA that was continuously decreased so that the estimated final concentration was 1.6 g/L DCA for 100 weeks. For those animals, the incidence of adenomas was reported by DeAngelo et al. (1996) to be 10.7% and carcinomas 21.4%, probably more a reflection of longer exposure time allowing for adenoma to carcinoma progression. The authors did not report any of the results of DCA-induced increases of adenomas and carcinomas to be statistically significant. As it appears the same data set was used for the 2.0 g/L sodium chloride control, 0.05 g/L DCA, and 0.5 g/L DCA exposure groups as was reported in DeAngelo et al. (1996), the same issues arise as regarding the differences in numbers of animals that were included in tumor analysis than were reported to have been present at final sacrifice. As stated previously for the DeAngelo et al. (1997) study of TCA in rats, the use of small numbers of rats limits the detection of and ability to determine whether there was no treatment-related effects, especially at the low concentrations of DCA exposure.

E.2.4. Summaries and Comparisons Between TCE, DCA, and TCA Studies

There are a number of studies of TCE that have reported effects on the liver. However, the study of this compound is difficult as its concentration does not remain stable in drinking water, some studies have been carried out using TCE with small quantities of a carcinogenic stabilizing agent, some studies have been carried out in whole-body inhalation chambers that resulted in additional oral administration and for which individual animal data were not recorded throughout the experiment, and the results of gavage studies have been limited by gavage-related deaths and vehicle effects. In addition, some studies have been conducted using the i.p. route of administration, which results in route-related toxicity and inflammation. For many studies, liver effects consisted of measured increases in liver weight with little or no description of attendant histological changes induced by TCE treatment. A number of studies were conducted at a few relatively high doses with attendant effects on body weight, indicative of systemic toxicity and affecting TCE-induced liver weight gain. Although many studies have been performed in male mice, the inhalation studies of Kjellstrand et al. (1981b, 1983a, 1983b) indicate that male mice, regardless of strain appear to have a greater variability in response, as measured by TCE-induced liver weight gain, and susceptibility to TCE-induced decreases in body weight than female mice. However, the body of the TCE literature is consistent in identifying the liver as a target of TCE-

induced effects, with the most commonly reported change to be a dose-related TCE-induced increase in liver weight in multiple species, strains, and genders from both inhalation and oral routes of exposure.

The following sections will not only summarize results for studies of TCE reported in Sections E.2.1–E.2.2, but provide comparison of studies of either TCA or DCA that have used similar paradigms or investigated similar parameters described in Sections E.2.3.1 and E.2.3.2. A synopsis of the results from studies of CH and in comparison with TCE results is presented in Section E.2.5. While the study of Bull et al. (2002), described in Section E.2.2.2.2, presents data for combinations of DCA or TCA exposure for comparisons of tumor phenotype with those induced by TCE, the examination of co-exposure studies of TCE metabolites in rodents that are also exposed to a number of other carcinogens, and descriptions of the toxicity data for brominated haloacetates that also occur with TCE in the environment, are presented in Section E.4.3.3.

E.2.4.1. Summary of Results For Short-term Effects of TCE

In regard to early changes in DNA synthesis, the data for TCE are very limited. The study by Mirsalis et al. (1989) used an in vivo-in vitro hepatocyte DNA repair and S-phase DNA synthesis in primary hepatocytes from male F344 rats (180–300 g) and male and female B6C3F_1 mice (20–29 g for male mice and 18–25 g female mice) administered TCE by gavage in corn oil. They reported negative results 2–12 hours after treatment from 50 to 1,000 mg/kg TCE in rats and mice (male and female) for UDS and repair using three animals per group. After 24 and 48 hours of 200 or 1,000 mg/kg TCE in male mice (n = 3) and after 48 hours of 200 (n = 3) or 1,000 (n = 4) mg/kg TCE in female mice, similar values of 0.30–0.69% of hepatocytes were reported as undergoing DNA synthesis in those hepatocytes in primary culture with only the 1,000 mg/kg TCE dose in male mice at 48 hours giving a result considered to be positive (~2.2%). No statistical analyses were performed on these measurements, which were obviously limited by both the number of animals examined and the relevance of the paradigm.

TCE-induced increases in liver weight have been reported to occur quickly. The inhalation study of Okino et al. (1991) in male rats demonstrates that liver weight and metabolism were increased with as little as 8 hours of TCE exposure (500 and 2,000 ppm) and as early as 22 hours after cessation of such exposures with little concurrent hepatic necrosis. Laughter et al. (2004) reported increase liver weight in SV129 mice in their 3-day study (see below). Tao et al. (2000) reported a 1.26-fold of control percent liver/body weight in female B6C3F_1 mice fed 1,000 mg/kg TCE in corn oil for 5 days. Elcombe et al. (1985) and Dees and Travis (1993) reported gavage results in mice and rats after 10 days exposure to TCE, which showed TCE-induced increases in liver weight (see below for more detail on dose-response). Tucker et al. (1982) reported that 14 days of exposure to 24 and 240 mg/kg TCE via gavage induced a dose-related increase in liver weight in male CD-1 mice but did not show the data.

TCE-induced increases in percent liver/body weight ratios have been studied most extensively in B6C3F$_1$ and Swiss mice. Both strains have been shown to have a TCE-induced increase in liver tumors from long-term exposure as well (see Section E.2.4.3). A number of studies have provided dose-response information for TCE-induced increases in liver weight from 10 days to 13 weeks of exposure in mice. Most studies have reported that the magnitude of increase in TCE exposure concentration is similar to the magnitude increase of percent liver/body weight increase. For example a twofold increase in TCE exposure has often resulted in a twofold increase in the percent change in liver/body weight over control (i.e., 500 mg/kg TCE induces a 20% increase in liver weight and 1,000 mg/kg TCE induces a 50% increase in liver weight as reported by Elcombe et al. (1985). The range in which this relationship is valid has been reported to vary from 100 mg/kg TCE at 10 days (Dees and Travis, 1993) to 1,600 mg/kg (Buben and O'Flaherty, 1985) at 6 weeks and up to 1,500 mg/kg TCE for 13 weeks (NTP, 1990). The consistency in the relationship between magnitude of liver weight increase and TCE exposure concentration has been reported for both genders of mice, across oral and inhalation routes of exposure, and across differing strains of mice tested. For rats, there are fewer studies with fewer exposure levels tested, but both Berman et al. (1995) and Melnick et al. (1987) report that short-term TCE exposures from 150 to ~2,000 mg/kg induced percent liver/body weight that increased proportionally with the magnitude of TCE exposure concentration.

Dependence of PPARα activation for TCE-liver weight gain has been investigated in PPARα null mice by both Nakajima et al. (2000) and Laughter et al. (2004). After 2 weeks of 750 mg/kg TCE exposure to carefully matched SV129 wild-type or PPARα-null male and female mice (n = 6 group), there was a reported 1.50-fold of control in wild-type and 1.26-fold of control percent liver/body weight in PPARα-null male mice by Nakajima et al. (2000). For female mice, there was ~1.25-fold of control percent liver/body weight ratios for both wild-type and PPARα-null mice. Ramdhan et al. (2010) also reported increased liver weight in male PPARα-null mice after high levels of inhalation exposure that were comparable to that in wild type mice after 7 days of exposure (up to 40–50% increases at the highest dose). Thus, TCE-induced liver weight gain was not dependent on a functional PPARα receptor in female mice, and data indicate that a significant portion of it may have also not have been PPARα receptor-dependent in male mice.

Nakajima et al. (2000) report that both wild-type male and female mice have similar increases in the number of peroxisome in the pericentral area of the liver after TCE exposure and, although increased twofold, were still only ~4% of cytoplasmic volume. Female wild-type mice were reported to have less TCE-induced elevation of very long chain acyl-CoA synthetase, D-type peroxisomal bifunctional protein, mitochondrial trifunctional protein α subunits α and β, and CYP 4A1 than males mice, even though peroxisomal volume was similarly elevated in male and female mice. The induction of PPARα protein by TCE treatment was also reported to be slightly less in female than male wild-type mice (2.17- vs. 1.44-fold of control, respectively).

Ramdhan et al. (2010) examined TCE-induced hepatice steatosis and toxicity using male wild type, PPARα-null, and human PPARα-inserted mice (humanized) exposed to high inhalation concentrations of TCE for 7 days. Significant differences were observed among control mice for each genotype with reduced body weight in untreated humanized mice. Liver/body weight ratios were 11% higher in untreated PPARα- null mice than wild type mice. Higher levels of liver triglycerides and hepatic steatosis were reported in the untreated humanized mice and PPARα-null mice than wild type mice. Background expression of a number of genes and protein expression levels were significantly different between the untreated strains. In particular, human PPARα protein levels were >10-fold greater in the humanized mice than mouse PPARα in untreated wild type mice. Insertion of human PPARα in the null mice did not return the mice to a normal state. Both PPARα-null and humanized mice were more susceptible to TCE toxicity as evidenced by serum AST and ALT (liver injury biomarkers), hepatic triglyceride levels, and hepatic steatosis. Hepatomegally was induced in all strains to a similar extent after TCE exposure. However, urinary TCA concentrations were reported to be significantly lower and TCOH levels significantly higher in TCE-treated PPARα-null mice in comparison to treated wild type mice. This difference was not related to changes in expression of metabolic enzymes. Thus, TCE-induced liver toxicity was not dependent on PPARα with dysregulation of the receptor in null or humanized mice, rendering them more susceptible to TCE-induced toxicity.

Laughter et al. (2004) also studied SV129 wild-type and PPARα-null male mice treated with 3 daily doses of TCE in 0.1% methyl cellulose for either 3 days (1,500 mg/kg TCE) or 3 weeks (0, 10, 50, 125, 500, 1,000, or 1,500 mg/kg TCE 5 days/week). However, not only is the paradigm not comparable to other gavage paradigms, but no initial or final body weights of the mice were reported and thus, the influence of differences in initial body weight on percent liver/body weight determinations could not be ascertained. In the 3-day study, while control wild-type and PPARα-null mice were reported to have similar percent liver/body weight ratios (~4.5%), at the end of the 3-week experiment, the percent liver/body weight ratios were reported to be increased in the PPARα-null male mice (5.1%).

TCE treatment for 3 days was reported to increase the percent liver/body weight ratio 1.4-fold of control in the wild-type mice and 1.07-fold of control in the null mice. In the 3-week study, wild-type mice exposed to various concentrations of TCE had percent liver/body weights that were reported to be within ~2% of control values except for the 1,000 and 1,500 mg/kg groups (~1.18- and 1.30-fold of control levels, respectively). For the PPARα-null mice, the variability in percent liver/body weight was reported to be greater than that of the wild-type mice in most of the groups, and the baseline level of percent liver/body weight ratio also 1.16-fold greater. TCE exposure was apparently more toxic in the null mice with death at the 1,500 mg/kg TCE exposure level, resulting in the prevention of recording of percent liver/body weights. At the 1,000 mg/kg TCE exposure level, there was a reported 1.10-fold of control percent liver/body weight in the PPARα-null mice.

None of the increases in percent liver/body weight in the null mice were reported to be statistically significant by Laughter et al. (2004). However, the statistical power of the study was limited due to low numbers of animals and increased variability in the null mice groups. The percent liver/body weight after TCE treatment that was reported in this study was actually greater in the null mice than the wild-type male mice at the 1,000 mg/kg TCE exposure level (5.6 ± 0.4 vs. 5.2 ± 0.5%, for null and wild-type mice, respectively). At 1-week and at 3-weeks, TCE appeared to induce increases in liver weight in PPARα-null mice, although not reaching statistical significance in this study. At a 1,000 mg/kg TCE exposure for 3 weeks, percent liver/body weights were reported to be 1.18-fold of control in wild-type and 1.10-fold of control in null mice. Although the experiments in Laughter et al. (2004) for DCA and TCA were not conducted using the same paradigm, the TCE-induced increase in percent liver/body weight more closely resembled the dose-response pattern for DCA than for DCA wild-type SV129 and PPARα-null mice.

Many studies have used cyanide-insensitive PCO as a surrogate for peroxisome proliferation. Of note is that several studies have shown that this activity is not correlated with the volume or number of peroxisomes that are increased as a result of exposure to TCE or its metabolites (Nakajima et al., 2000; Nelson et al., 1989; Elcombe et al., 1985). This activity appears to be highly variable both as a baseline measure and in response to chemical exposures. Laughter et al. (2004) presented data showing that WY-14,643 induced increases in PCO activity varied up to sixfold between experiments in wild-type mice. They also showed that PCO activity, in some instances, was up to sixfold of wild-type mice values in untreated PPARα-null mice. Parrish et al. (1996) noted that control values between experiments varied as much as a factor of 2-fold for PCO activity and thus, their data were presented as percent of concurrent controls. Goldsworthy and Popp (1987) reported that 1,000 mg/kg TCE induced a 6.25-fold of control PCO activity in $B6C3F_1$ mice in two 10-day experiments. However, for F344 rats, the increases over control between two experiments conducted at the same dose were reported to vary by >30%. Finally, Melnick et al. (1987) have reported that corn oil administration alone can elevate PCO activity as well as catalase activity.

For TCE there are two key 10-days studies (Dees and Travis, 1993; Elcombe et al., 1985) that examine the effects of short-term exposure in mice and rats via gavage exposure and attempt to determine the nature of the dose- response in a range of exposure concentrations that include levels below which there is concurrent decreased body weights. Although they have limitations, they reported generally consistent results. In regard to liver weight in mice, gavage exposure to TCE at concentrations ranging from 100 to 1,500 mg/kg TCE produced increases in liver/body weight that was dose-related (Dees and Travis, 1993; Elcombe et al., 1985).

Elcombe et al. (1985) reported a small decrease in DNA content with TCE treatment (consistent with hepatocellular hypertrophy) that was not dose-related, increased tritiated thymidine incorporation in whole mouse liver DNA that was that was treatment-related but not

dose-related (i.e., two-, two-, and fivefold of control values in mice treated with 500, 1,000, and 1,500 mg/kg TCE), and slightly increased numbers of mitotic figures that were treatment-related, but not dose-related and not correlated with DNA synthesis as measured by thymidine incorporation. Elcombe et al. (1985) reported an increase in peroxisome volume after TCE exposure that was correlated with the magnitude of increase in peroxisomal-associated enzyme activity at the only dose in which both were tested. Peroxisome increases after TCE treatment in mice livers were identified as being pericentral in location. After TCE treatment, increased peroxisomal volumes in $B6C3F_1$ mice were reported to be not dose-related (i.e., there was little difference between 500 and 1,500 mg/kg TCE exposures). The TCE-induced increases in peroxisomal volumes were also not correlated with the reported increases in thymidine incorporation or mitotic activity in mice.

Neither TCE-induction of peroxisomes nor hepatocellular proliferation, as measured by either mitotic index or thymidine incorporation, was correlated with TCE-induced liver weight increases. Elcombe et al. (1985) only measured PCO activity in a subset of $B6C3F_1$ mice at the 1,000 mg/kg TCE exposure level for 10 days of exposure and reported an 8-fold of control PCO activity and a 1.5-fold of control catalase activity. This result was similar to that of Goldsworthy and Popp (1987), who reported 6.25-fold of control PCO activity in male $B6C3F_1$ mice exposed to 1,000 mg/kg-day TCE for 10 days in two separate experiments.

Similar to Elcombe et al. (1985), who reported no difference in response between 500 and 1,000 mg/kg TCE treatments, (Dees and Travis, 1993) reported that incorporation of tritiated thymidine in DNA from mouse liver was elevated after TCE treatment and the mean peak level of tritiated thymidine incorporation occurred at 250 mg/kg TCE treatment level remaining constant for the 500 and 1,000 mg/kg treated groups. (Dees and Travis, 1993) specifically report that mitotic figures, although very rare, were more frequently observed after TCE treatment, most often in the intermediate zone, and in cells resembling mature hepatocytes. They reported that there was little tritiated thymidine incorporation in areas near the bile duct epithelia or close to the portal triad in liver sections from both male and female mice. They also reported no evidence of increased lipofuscin and that increased apoptosis from TCE exposure "did not appear to be in proportion to the applied TCE dose given to male or female mice" (i.e., the mean number of apoptosis 0, 0, 0, 1, and 8 for control, 100, 250, 500, and 1,000 mg/kg TCE treated groups, respectively). Both Elcombe et al. (1985) and (Dees and Travis, 1993) reported no changes in apoptosis other than increased apoptosis only at a treatment level of 1,000 mg/kg TCE.

Elcombe et al. (1985) reported increases in percent liver/body weight after TCE treatment in both the Osborne-Mendel and Alderley Park rat strain, although to a smaller extent than in mice. For both strains, Elcombe et al. (1985) reported no TCE-induced changes in body weight at doses ranging from 500 to 1,500 mg/kg. For male Osborne-Mendel rats, administration of TCE in corn oil gavage resulted in a 1.18-, 1.26-, and 1.30-fold of control percent liver/body weight at 500, 1,000, and 1,500 mg/kg-day exposures, respectively. For Alderley Park rats, those increases

were 1.14-, 1.17-, and 1.17-fold of control at the same respective exposure levels for 10 days of exposure.

In regard to liver weight increases, Melnick et al. (1987) reported a 1.13- and 1.23-fold of control percent liver/body weight in male F344 rats fed 600 and 1,300 mg/kg-day TCE in capsules, respectively. There was no difference in the extent of TCE-induced liver increase between the two lowest dosed groups administered TCE in corn oil gavage (~20% increase in percent liver/body weight at 600 and 1,300 mg/kg-day TCE) for 14 days. However, the magnitude of increases in percent liver/body weight in these groups was affected by difference between control groups in liver weight although initial and final body weights appeared to be similar. By either type of vehicle, Melnick et al. (1987) reported decreases in body weights in rats treated with concentrations of TCE ≥2,200 mg/kg-day for 14 days. Similarly, Nunes et al. (2001) reported decreased body weight in Sprague-Dawley rats administered 2,000 mg/kg-day for 7 days in corn oil. Melnick et al. (1987) reported that both exposures to either 600 or 1,300 mg/kg-day TCE in capsules did not result in decreased body weight and caused less than minimal focal necrosis randomly distributed in the liver. At 2,200 and 4,800 mg/kg TCE fed via capsule, Melnick et al. (1987) reported that although there was decreased body weight in rats treated at these exposures, there was little TCE-induced necrosis, and no evidence of inflammation, cellular hypertrophy or edema with TCE exposure. Similarly, Berman et al. (1995) reported increases in liver weight gain at doses as low as 50 mg/kg TCE, no necrosis up to doses of 1,500 mg/kg, and hepatocellular hyper trophy only at the 1,500 mg/kg level in female F344 rats.

For rats, Elcombe et al. (1985) reported an increase over untreated rats of 1.13-fold of control PCO activity in Alderley Park rats after 1,000 mg/kg-day TCE exposure for 10 days, while Goldsworthy and Popp (1987) reported a 1.8- and 2.39-fold of control in male F344 rats at the same exposure in two separate experiments. Melnick et al. (1987) reported PCO activity of 1.23- and 1.75-fold of control in male F344 rats fed 600 and 1,300 mg/kg-day TCE for 14 days in capsules. For rats treated by gavage with 600 or 1,200 mg/kg-day TCE corn oil, they reported 1.16- and 1.29-fold of control values. However, control levels of PCO were 16% higher in corn oil controls than in untreated controls. In addition, Melnick et al. (1987) reported little catalase increases in rats fed TCE via capsules in food (<6% increase) but a 1.18- and 1.49-fold of control catalase activity in rats fed 600 or 1,200 mg/kg/TCE via corn oil gavage, indicative of a vehicle effect.

The data from Elcombe et al. (1985) included reports of TCE-induced pericentral hypertrophy and eosinophilia for both rats and mice but with "fewer animals affected at lower doses." In terms of glycogen deposition, Elcombe et al. (1985) report "somewhat" less glycogen pericentrally in the livers of rats treated with TCE at 1,500 mg/kg than controls with less marked changes at lower doses restricted to fewer animals. They do not comment on changes in glycogen in mice. Dees and Travis (1993) reported TCE-induced changes to "include an increase in

eosinophilic cytoplasmic staining of hepatocytes located near central veins, accompanied by loss of cytoplasmic vacuolization." Since glycogen is removed using conventional tissue processing and staining techniques, an increase in glycogen deposition would be expected to increase vacuolization and thus, the report from Dees and Travis (1993) is consistent with less, not more, glycogen deposition. Neither study produced a quantitative analysis of glycogen deposition changes from TCE exposure. Although not explicitly discussing liver glycogen content or examining it quantitatively in mice, these studies suggest that TCE-induced liver weight increases did not appear to be due to glycogen deposition after 10 days of exposure, and any decreases in glycogen were not necessarily correlated with the magnitude of liver weight gain either.

For both rats and mice, the data from Elcombe et al. (1985) showed that tritiated thymidine incorporation in total liver DNA observed after TCE exposure did not correlate with mitotic index activity in hepatocytes with both Elcombe et al. (1985) and Dees and Travis (1993) reporting a small mitotic indexes and evidence of periportal hepatocellular hypertrophy from TCE exposure. Neither mitotic index or tritiated thymidine incorporation data support a correlation with TCE-induced liver weight increase in the mouse. If higher levels of hepatocyte replication had occurred earlier, such levels were not sustained by 10 days of TCE exposure. Both Elcombe et al. (1985) and Dees and Travis (1993) present data that represent "a snapshot in time," which do not show whether increased cell proliferation may have happened at an earlier time point and then subsided by 10 days. These data suggest that increased tritiated thymidine levels were targeted to mature hepatocytes and in areas of the liver where greater levels of polyploidization occur. Both Elcombe et al. (1985) and Dees and Travis (1993) show that tritiated thymidine incorporation in the liver was approximately twofold of controls between 250 and 1,000 mg/kg TCE, a result consistent with a doubling of DNA. Thus, given the normally quiescent state of the liver, the magnitude of this increase over control levels, even if a result of proliferation rather than polyploidization, would be confined to a very small population of cells in the liver after 10 days of TCE exposure.

Laughter et al. (2004) reported that there was an increase in DNA synthesis after aqueous gavage exposure to 500 and 1,000 mg/kg TCE given as three boluses a day for 3 weeks with BrdU given for the last week of treatment in mice. An examination of DNA synthesis in individual hepatocytes was reported to show that 1 and 4.5% of hepatocytes had undergone DNA synthesis in the last week of treatment for the 500 and 1,000 mg/kg doses, respectively. Both Elcombe et al. (1985) and Dees and Travis (1993) show TCE-induced changes for several parameters at the lowest level tested without toxicity and without evidence of regenerative hyperplasia or sustained hepatocellular proliferation.

In regards to susceptibility to liver cancer induction, the more susceptible ($B6C3F_1$) vs. less susceptible (Alderley Park/Swiss) strains of mice to TCE-induced liver tumors (Maltoni et al., 1988), the "less susceptible" strain was reported by Elcombe et al. (1985) to have a greater baseline level of liver weight/body weight ratio, a greater baseline level of thymidine

incorporation, and greater responses for those endpoints due to TCE exposure. However, both strains showed a hepatocarcinogenic response after TCE exposure, although there are limitations regarding determination of the exact magnitude of response for these experiments as previously discussed.

E.2.4.2. Summary of Results For Short-Term Effects of DCA and TCA: Comparisons With TCE

Short-term exposures from DCA and TCA have been studied either through gavage or in drinking water. Palatability became an issue at the highest level of DCA tested in drinking water experiments (5 g/L), which caused a significant reduction of drinking water intake in mice of 46–64% (Carter et al., 1995). Decreases in drinking water consumption have also been reported for a range of concentrations of DCA and TCA from 0.05 to 5.0 g/L, in both mice and rats, and with generally the higher concentrations producing the highest decrease in drinking water (DeAngelo et al., 1999; DeAngelo et al., 1997; Carter et al., 1995; Mather et al., 1990); (DeAngelo et al., 2008). However, results within studies (e.g., DeAngelo et al., 2008) and between studies have been reported to vary as to the extent of the reduction in drinking water from the presence of TCA or DCA. Some drinking water studies of DCA or TCA have not reported drinking water consumption. Therefore, although in general, DCA and TCA studies have do not include vehicle effects, such as those posed by corn oil, they have been affected by differences in drinking water consumption not only changing the dose received by the rodents and therefore, potentially the shape of the dose-response curve, but also the effects of dehydration are potentially added to any chemically-related reported effects.

Studies have attempted to determine short-term effects on DNA by TCE and its metabolites. Nelson and Bull (1988) administered TCE male to Sprague-Dawley rats and male B6C3F_1 mice and measured the rate of DNA unwinding under alkaline conditions 4 hours later. For rats, there was a significantly increased rate of unwinding at the two highest dose and for mice, there was a significantly increased level of DNA unwinding at a lower dose. In this same study, DCA was reported to be most potent in this assay with TCA being the lowest, while CH closely approximated the dose-response curve of TCE in the rat. In the mouse, the most potent metabolite in the assay was reported to be TCA, followed by DCA with CH considerably less potent. Nelson and Bull (1988) and Nelson et al. (1989) have reported increases in SSBs after DCA and TCA exposure. However, Styles et al. (1991) (for mice) and Chang et al. (1992) (for mice and rats) did not. Austin et al. (1996) report that the alkaline unwinding assay, a variant of the alkaline elution procedure, is noted for its variability and inconsistency depending on the techniques used while performing the procedure. In regard to oxidative damage as measured by TBARS for lipid peroxidation and 8-OHdG levels in DNA, increases appear to be small (<50% greater than control levels) and transient after DCA and TCA treatment in mice (see Section E.3.4.2.3) with TCE results confounded by vehicle or route of administration effects.

Although there is no comparative data for TCE, the study of Styles et al. (1991) is particularly useful for determining effects of TCA from 1 to 4 days of exposure in mice. Styles et al. (1991) reported no change in "hepatic" DNA uptake of tritiated thymidine up to 36 hours, a peak at 72 hours (~6-fold of control), and falling levels by 96 hours (~4-fold of controls) after 500 mg/kg TCA gavage exposure. Incorporation of tritiated thymidine observed for individual hepatocytes decreased between 24 and 36 hours, rose slowly back to control levels at 48 hours, significantly increased by 72 hours, and then decreased by 96 hours. Thus, increases in "hepatic" DNA tritiated thymidine uptake did not capture the decrease observed in individual hepatocytes at 36 hours. By either measure, the population of cells undergoing DNA synthesis was small, with the peak level being <1% of the hepatocyte population. Zonal distribution of labeled hepatocytes were decreased at 36 hours in all zones, appeared to be slightly greater in periportal than midzonal cells with centrilobular cells still below control levels by 48 hours, similarly elevated over controls in all zones by 72 hours, and to have returned to near control levels in the midzonal and centrilobular regions but with periportal areas still elevated by 96 hours. These results are consistent with all hepatocytes showing a decrease in DNA synthesis by 36 hours and then a wave of DNA synthesis to occur, starting at the periportal zone and progressing through the liver acinus that is decreased by 4 days after exposure.

Along with changes in liver weight, DNA synthesis, and glycogen accumulation, several studies of DCA and TCA have focused on the extent of peroxisome proliferation as measured by changes in peroxisome number, cytoplasmic volume and enzyme activity induction as potential "key events" occurring from shorter-term exposures that may be linked to chronic effects such as liver tumorigenicity. As noted above in Section E.2.4.1, TCE-induced liver weight gain has been reported to not be dependent on a functional PPARα receptor in female mice while as well as a significant portion of it not dependent on functional PPARα receptor in male mice. Also as noted, cyanide-insensitive PCO has also been reported to not be correlated with the volume or number of peroxisomes that are increased as a result of exposure to TCE or it metabolites (Nakajima et al., 2000; Nelson et al., 1989; Elcombe et al., 1985) and to be highly variable both as a baseline measure and in response to chemical exposures (e.g., variation of up to 6-fold between after WY-14,643 exposure in mice). Also as noted above, the vehicle used in many TCE gavage experiments, corn oil, has been reported to elevate PCO activity as well as catalase activity.

A number of short-term studies have examined the effects of TCA and DCA on liver weight increases and evidence of peroxisome proliferation and changes in DNA synthesis. In particular, two studies of DCA and TCA used a similar paradigm presented by Elcombe et al. (1985) and Dees and Travis (1993) for TCE effects in mice. Nelson et al. (1989) report findings from gavage doses of unbuffered TCA (500 mg/kg) and DCA (500 mg/kg) in male B6C3F$_1$ mice; Styles et al. (1991) also provide data on peroxisome proliferation using the same paradigm. Nelson et al. (1989) reported levels of PCO activity in mice administered 500 mg/kg DCA or TCA for 10 days with 250 mg/kg Clofibrate administration serving as a positive control. DCA

and TCA exposure were reported to not affect body weight, but both to significantly increase liver weight (1.63-fold of control for DCA and 1.30-fold of control for TCA treatments), and percent liver/body weight ratios (1.53-fold of control for DCA and 1.16-fold of control for DCA treatments). PCO activity was reported to be significantly increased by ~1.63-, 2.7-, and 5-fold of control for DCA, TCA, and Clofibrate treatments, respectively, and indicated that both DCA and TCA were weaker inducers of this activity than Clofibrate.

Results from randomly selected electron photomicrographs showed an increase in peroxisomes per unit area but gave a different pattern than PCO enzyme activity (i.e., 2.5- and 2.4-fold of control peroxisome volume for DCA and TCA, respectively). Evidence of gross hepatotoxicity was reported to not occur in vehicle or TCA-treated mice. Light microscopic sections were reported to show TCA and control hepatocytes to have the same intensity of PAS staining, but with slightly larger hepatocytes occurring in TCA-treated mice throughout the liver section with architecture and tissue pattern of the liver intact. For DCA, the histopathology was reported to be markedly different than control mice or TCA treated mice. DCA was reported to induce a marked increase in the size of hepatocytes throughout the liver with an approximately 1.4-fold of control diameter that was accompanied by increased PAS staining (indicative of glycogen deposition). All DCA-treated mice were reported to have multiple white streaks grossly visible on the surface of the liver corresponding with subcapsular foci of coagulative necrosis that were not encapsulated, varied in size, and accompanied by a slight inflammatory response characterized by neutrophil infiltration.

A quantitative comparison of effects from equivalent exposures of TCE, TCA, and DCA (500 mg/kg for 10 days in mice via corn oil gavage for TCE) shown in Table E-13 can be drawn between the Elcombe et al. (1985), Dees and Travis (1993), Styles et al. (1991), and Nelson et al. (1989) data for relationship to control values for percent liver/body weight, PCO, and qualitatively for glycogen deposition.

Table E-13. Comparison of liver effects from TCE, TCA, and DCA (10-day exposures in mice)

Model	Exposure	% Liver/body weight	Peroxisome volume	Peroxisome enzyme activity	Glycogen deposition
Nelson et al. (1989)[a]					
$B6C3F_1$ male	TCA	1.16-fold	2.4-fold	2.7-fold	No change
	DCA	1.53-fold	2.5-fold	1.63-fold	Increased
Styles et al. (1991)					
$B6C3F_1$ male	TCA	NR	1.9-fold	NR	NR
Elcombe et al. (1985)					
$B6C3F_1$ male	TCE	1.20-fold	8-fold	NR	NR
Alderley Park male (Swiss)	TCE	1.43-fold	4-fold	NR	NR
Dees and Travis (1993)					
$B6C3F_1$ male	TCE	1.05-fold[b]	NR	NR	NR
$B6C3F_1$ female	TCE	1.18-fold	NR	NR	NR

[a]Unbuffered. NR = not reported as no analysis was performed for this dose or the authors did not report this finding (i.e., did not note a change in glycogen in description of exposure-related changes).
[b]Statistically significant although small increase.

Although using a similar species, route of exposure, and dose, the comparison of responses for TCE and its metabolites shown above are in male mice and also are reflective of variability in strain, and variability and uncertainty of initial body weights. As described in more detail in Section E.2.2, initial age and body weight have an impact on TCE-related increases in liver weight. Male mice have been reported to have greater variability in response than female mice within and between studies and most of the comparative data for the 10-day 500 mg/kg doses of TCE or its metabolites were from studies in male mice. Corn oil, used as the vehicle for TCE gavage studies but not those of its metabolites, has been noted to specifically affect peroxisomal enzyme induction, body weight gain, and hepatic necrosis, specifically, in male mice (Merrick et al., 1989). Corn oil alone has also been reported to increase PCO activity in F344 rats and to potentiate the induction of PCO activity of TCA (DeAngelo et al., 1989). Thus, quantitative inferences regarding the magnitude of response in these studies are limited by a number of factors.

The variability in the magnitude of TCE-induced increases in percent liver/body weight across studies is readily apparent, but for TCE, TCA, and DCA, there is an increase in liver weight in mice at this dose after 10 days of exposure. The volume of the peroxisomal compartment in hepatocytes was reported to be more greatly increased from TCE-treatment by Elcombe et al. (1985) than for either TCA or DCA by Nelson et al. (1989) or Styles et al. (1991). However, the control values for the $B6C3F_1$ mice were half that of the other strain reported by Elcombe et al. (1985) and this parameter in general did not match the pattern of PCO activity values reported for TCA and DCA (Nelson et al., 1989). There is no PCO activity data at this

dose for TCE, but Elcombe et al. (1985) reported that the magnitude of TCE-induced increase in peroxisome volume was similar to that of PCO activity at the only dose where both were tested (1,000 mg/kg TCE).

However, Elcombe et al. (1985) reported that increased peroxisomal volumes in $B6C3F_1$ mice after 10 days of TCE treatment were not dose-related (i.e., there was little difference between 500, 1,000, and 1,500 mg/kg TCE exposures in the magnitude of TCE-induced increases in peroxisomal volume). The lack of dose-response for TCE-induced peroxisomal volume increases was not consistent with increases in percent liver/body weight that increased with increasing TCE exposure concentration. Also as noted above, PCO activity appears to be highly variable in untreated and treated rodents and to vary between experiments and between studies.

From the above comparison, it is clear that TCE, DCA, and TCA exposures were associated with increased liver weight in mice but a question arises as to what changes account for the liver weight increases. For TCE and TCA 500 mg/kg treatments, changes in glycogen were not reported in the general descriptions of histopathological changes (Dees and Travis, 1993; Styles et al., 1991; Elcombe et al., 1985) or were specifically described by the authors as being similar to controls (Nelson et al., 1989). However, for DCA, glycogen deposition was specifically noted to be increased with treatment, although no quantitative analyses were presented that could give information as to the nature of the dose-response (Nelson et al., 1989). Issues in regard to not only whether TCE and its metabolites each gives a similar response for a number of parameters, but also potential changes may be associated with carcinogenicity from long-term exposures can be examined by a comparison of the dose-response curves for these parameters from a range of exposure concentrations and durations of exposure. In addition, if glycogen accumulation results from DCA exposure, what proportion of DCA-induced liver weight increases result from such accumulation or other events that may be similar to those occurring with TCE exposure (see Section E.4.2.4)?

As noted in Section E.2.4.1, TCE-induced changes in liver weight appear to be proportional to the exposure concentration across route of administration, gender, and rodent species. As an indication of the potential contribution of TCE metabolites to this effect, a comparison of the shape of the dose-response curves for liver weight induction for TCE and its metabolites is informative. A number of studies of TCA and DCA in drinking water, conducted from 10 days to 4 weeks, have attempted to measure changes in liver weight induction, peroxisomal enzyme activity, and DNA synthesis predominantly in mice to provide insight into the mode(s) of action for liver cancer induction (DeAngelo et al., 2008; Parrish et al., 1996; Carter et al., 1995; Sanchez and Bull, 1990; DeAngelo et al., 1989).

Direct comparisons are harder to make between the drinking water studies of DCA and TCA and the gavage studies of TCE (Tables E-14, E-15, and E-16). Similar to 10-day gavage exposures to TCE, 14-day exposures to TCA or DCA via drinking water were reported to induce dose-related increases in liver weight in male $B6C3F_1$ mice (0.3, 1.0, and 2.0 g/L TCA or DCA)

with a greater increase in liver weight from DCA than TCA at 2 g/L and a difference in the shape of the dose-response curve (Sanchez and Bull, 1990). They reported a 1.08-, 1.31-, and 1.62-fold of control liver weight for DCA and a 1.15-, 1.22-, and 1.38-fold of control values for TCA at 0.3, 1.0, and 2.0 g/L concentrations, respectively (n = 12–14 mice). While the magnitude of difference between the exposures was ~6.7-fold between the lowest and highest dose, the differences between TCA exposure groups for change in percent of liver weight was ~2.5, but for DCA, the slope of the dose-response curve for liver weight increases appeared to be closer to the magnitude of difference in exposure concentrations between the groups (i.e., a difference of 7.7-fold between the highest and lowest dose for liver weight induction).

DeAngelo et al. (1989) reported that after 14 days of exposure to 5 or 2 g/L TCA in male mice, the magnitudes of the difference in the increase in exposure concentration (2.5-fold) was generally higher than the increase percent liver/body weight ratios at these doses (i.e., ~40% for the Swiss-Webster, C3H, and for one of the $B6C3F_1$ mouse experiments, and for the C57BL/6 mouse, there was no difference in liver weight induction between the 2 and 5 g/L TCA exposure groups). There was a range in the magnitude of percent liver/body weight ratio increases between the strains of mice with liver weight induction reported to range between 1.26- and 1.66-fold of control values for the four strains of mice at 5 g/L TCA and to range between 1.16- and 1.63-fold of control values at 2 g/L TCA. One strain, $B6C3F_1$, was chosen to compare responses between DCA and TCA. At 1, 2, and 5 g/L TCA or DCA, DCA was reported to induce a greater increase in liver weight that TCA (i.e., 1.55- vs. 1.39-fold of control percent liver/body weight ratio for 5.0 g/L DCA vs. TCA, respectively). At the 5 g/L exposures, DCA induced ~40% greater percent liver/body weight than TCA. Although as noted above, the majority of the data from this study in mice did not indicate that the magnitude of difference in exposure concentration was the same as that of liver weight induction for TCA, in the particular experiment that examined both DCA and TCA, the increase in percent liver/body weight ratios were similar to the magnitude of difference in dose between the 2 and 5 g/L exposure concentrations for both DCA and TCA (i.e., 2–2.5-fold increase in liver weight change corresponding to a 2.5-fold difference in exposure concentration).

Table E-14. Liver weight induction as percent liver/body weight fold-of-control in male B6C3F$_1$ mice from DCA or TCA drinking water studies

Concentration (g/L)	Duration of exposure				Mean for average of d 14–30
	14 or 15 d	20 or 21 d	25 d	28 or 30 d	
DCA					
0.1		1.02-fold			1.02-fold
0.3	1.08-fold				1.08-fold
0.5	1.12-fold	1.24-fold, 1.05-fold	1.16-fold	1.16-fold	1.15-fold
1.0	1.31-fold				1.31-fold
2.0	1.62-fold	1.46-fold, 2.01-fold	2.04-fold	1.99-fold, 1.42-fold	1.83-fold
5.0	1.67-fold				1.67-fold
TCA					
0.05				1.09-fold	1.09-fold
0.1		0.98-fold			0.98-fold
0.3	1.15-fold				1.15-fold
0.5		1.13-fold		1.16-fold	1.15-fold
1.0	1.23-fold, 1.08-fold				1.16-fold
2.0	1.38-fold, 1.16-fold, 1.26-fold	1.33-fold			1.30-fold
3.0				1.33-fold	1.33-fold
5.0	1.39-fold, 1.35-fold				1.37-fold

Table E-15. Liver weight induction as percent liver/body weight fold-of-control in male B6C3F$_1$ or Swiss mice from TCE gavage studies

Concentration (mg/kg-d)	10 d	28 d	42 d	Mean for average of d 10–42
B6C3F$_1$				
100	1.00-fold			1.00-fold
250	1.00-fold			1.00-fold
500	1.20-fold, 1.06-fold			1.13-fold
600		1.36-fold		1.36-fold
1,000	1.50-fold, 1.17-fold, 1.50-fold			1.39-fold
1,200		1.64-fold		1.64-fold
1,500	1.47-fold			1.47-fold
2,400		1.81-fold		1.81-fold
Swiss				
100			1.12-fold	1.12-fold
200			1.15-fold	1.15-fold
400			1.25-fold	1.25-fold
500	1.43-fold	1.32-fold		1.38-fold
800			1.36-fold	1.36-fold
1,000	1.56-fold	1.41-fold		1.49-fold
1,500	1.75-fold			1.75-fold
1,600			1.63-fold	1.63-fold
2,000		1.38-fold		1.38-fold
2,400		1.69-fold		1.69-fold

Carter et al. (1995) examined 0.5 and 5.0 g/L exposures to DCA in B6C3F$_1$ male mice and reported that percent liver/body weights were increased consistently from 0.5 g/L DCA treatment from 5 to 30 days of treatment (i.e., a range of 1.05–1.16-fold of control). For 5.0 g/L DCA exposure, the range of increase in percent liver/body weight was reported to be 1.37–2.04-fold of control for the same time period. At the 15 days of exposures, the percent liver/body weight ratios were 1.67- and 1.12-fold of control for 5.0 and 0.5 g/L DCA and at 30 days were 1.99- and 1.16-fold, respectively. The difference in magnitude of dose and percent liver/body weight increase is difficult to determine given that the 5 g/L dose of DCA reduced body weight and significantly reduced water consumption by ~50%. The differences in DCA-induced percent liver/body weights were ~6-fold for the 15-, 25-, and 30-day data between the 0.5 and 5 g/L DCA exposures rather than the 10-fold difference in exposure concentration in the drinking water.

Table E-16. B6C3F$_1$ and Swiss (data sets combined)

Concentration (mg/kg-d)	Mean for average of d 10–42
100	1.06-fold
200	1.15-fold
250	1.00-fold
400	1.25-fold
500	1.26-fold
600	1.36-fold
800	1.36-fold
1,000	1.49-fold
1,200	1.64-fold
1,500	1.61-fold
1,600	1.63-fold
2,000	1.38-fold
2,400	1.75-fold

Parrish et al. (1996) reported that for male B6C3F$_1$ mice exposed to TCA or DCA (0, 0.01, 0.5, and 2.0 g/L) for 3 or 10 weeks, the 4–5-fold magnitude of difference in doses resulted in increases in percent liver/body weight for the 21- and 71-day exposures that were greater for DCA than TCA. The percent liver/body weight ratio were 0.98-, 1.13-, and 1.33-fold of control levels at 0.1, 0.5, and 2.0 g/L TCA and for DCA were 1.02-, 1.24-, and 1.46-fold of control levels, respectively, after 21 days of exposure. Both TCA and DCA exposures at 0.1 g/L resulted in difference in percent liver/body weight change of ≤2%. For TCA, although there was a fourfold increase in magnitude between the 0.5 and 2.0 g/L TCA exposure concentrations, the magnitude of increase for percent liver/body weight increase was 2.5-fold between them at both 21 and 71 days of exposure. For DCA, the fourfold difference in dose between the 0.5 and 2.0 g/L DCA

exposure concentrations were reported to result in a ~2-fold increase in percent liver/body weight increase at 21 days and ~4.5-fold increase at 71 days.

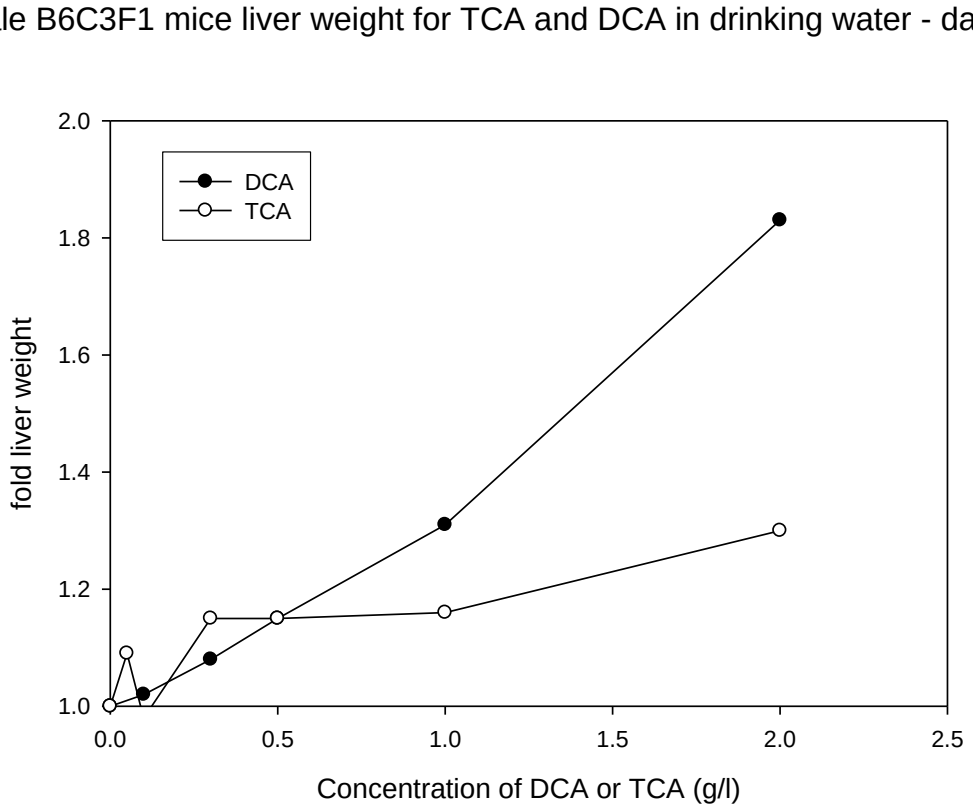
DeAngelo et al. (2008) studied three exposure concentrations of TCA in male $B6C3F_1$ mice, which were an order of magnitude apart, for 4 weeks of exposure. The percent liver/body weight ratios were 1.09-, 1.16-, and 1.35-fold of control levels, for 0.05, 0.5, and 5.0 g/L TCA exposures, respectively. The 10-fold differences in exposure concentration of TCA resulted in ~2-fold differences in percent liver/body weight increases. No dose-response inferences can be drawn from the 4-week study of DCA and TCA in $B6C3F_1$ male mice by Kato-Weinstein et al. (2001), but 2 g/L DCA and 3 g/L TCA in drinking water were reported to induce percent liver/body weights of 1.42- and 1.33-fold of control, respectively (n = 5).

The majority of short-term studies of DCA and TCA in mice have been conducted in the $B6C3F_1$ strain and in males. Studies conducted from 14 to 30 days show a consistent increase in percent liver/body weight induction by TCA or DCA. Analyses of this information regarding inferences for attribution and comparisons of dose-response have been published by Evans et al. (2009), Chiu et al., (2004), and Chiu (2011), and is discussed in Chapter 4 of the TCE assessment document and in Appendix A. A broader discussion of primarily issues and data related to Evans (2009) is contained below.

An examination of all of the data from Parrish et al. (1996), Sanchez and Bull (1990), Carter et al. (1995), Kato-Weinstein et al. (2001), and DeAngelo et al. (2008; 1989) from 14 to 30 days of exposure in male $B6C3F_1$ mice can give an approximation of the dose-response differences between DCA and TCA for liver weight induction as shown in Table E-14 and Figure E-1. Although the data for $B6C3F_1$ mice from Sanchez and Bull (1990) are reported as the fold of liver weight rather that percent liver/body weight increase, they are included in the comparison as both reflect increase in liver weight. Similar data can be assessed for TCE for comparative purposes. Short-duration studies (10–42 days) were selected because: (1) in chronic studies, liver weight increases are confounded by tumor burden; (2) multiple studies are available; (3) in this duration range, Kjellstrand et al. (1981a) reported that TCE-induced increases in liver weight plateau; and (4) TCA studies do not show significant duration-dependent differences in this duration range. These comparisons are presented in Table E-14.

DeAngelo et al. (1989) and Carter et al. (1995) used up to 5 g/L DCA and TCA in their experiments with Carter et al. (1995) noting a dramatic decrease in water consumption in the 5 g/L DCA treatment groups (46–64% reduction), which can affect body weight as well as dose received. DeAngelo et al. (1989) did not report drinking water consumption. The drinking water consumption was reported by DeAngelo et al. (2008) to be reduced by 11, 17, and 30% in the 0.05, 0.5, and 5 g/L TCA treated groups compared to 2 g/L sodium chloride control animals over 60 weeks. DeAngelo et al. (1999) reported mean drinking water consumption to be reduced by 26% in mice exposed to 3.5 g/L DCA over 100 weeks. Carter et al. (1995) reported that DCA at 5 g/L to decrease drinking water consumption by 64 and 46% but 0.5 g/L DCA to not affect

drinking water consumption. Thus, it appears that the 5 g/L concentrations of either DCA or TCA can significantly affect drinking water consumption as well as inducing reductions in body weight. Accordingly, an estimation of the shape of the dose-response curve for comparative purposes between DCA or TCA drinking water studies is best examined at concentrations at ≤2 g/L, especially for DCA.



Reproduced from Section 4.5.

Sources: (2008; Kato-Weinstein et al., 2001; Parrish et al., 1996; Carter et al., 1995; Sanchez and Bull, 1990; DeAngelo et al., 1989)).

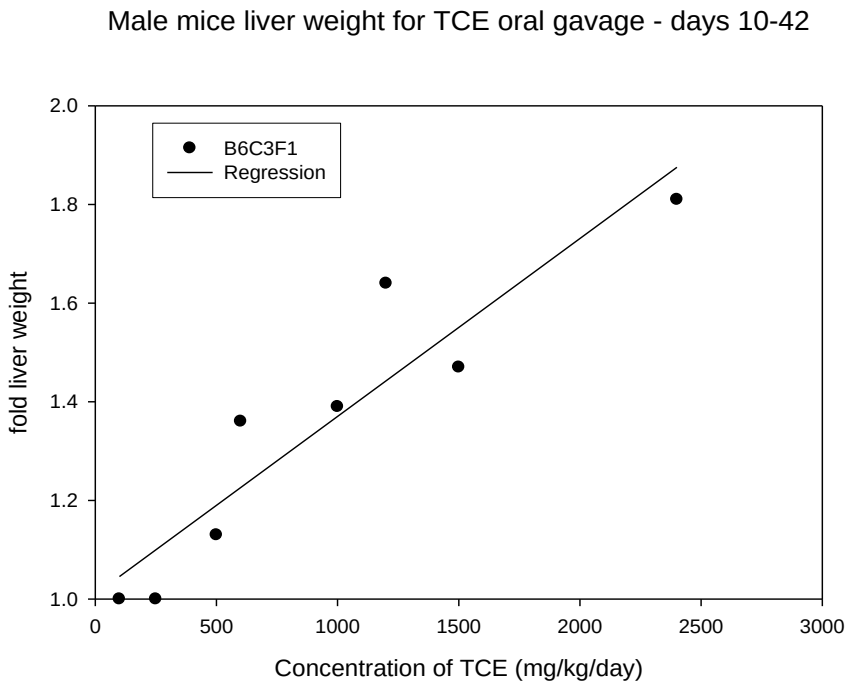
Figure E-1. Comparison of average fold-changes in relative liver weight to control and exposure concentrations of ≤2 g/L in drinking water for TCA and DCA in male B6C3F$_1$ mice for 14–30 days

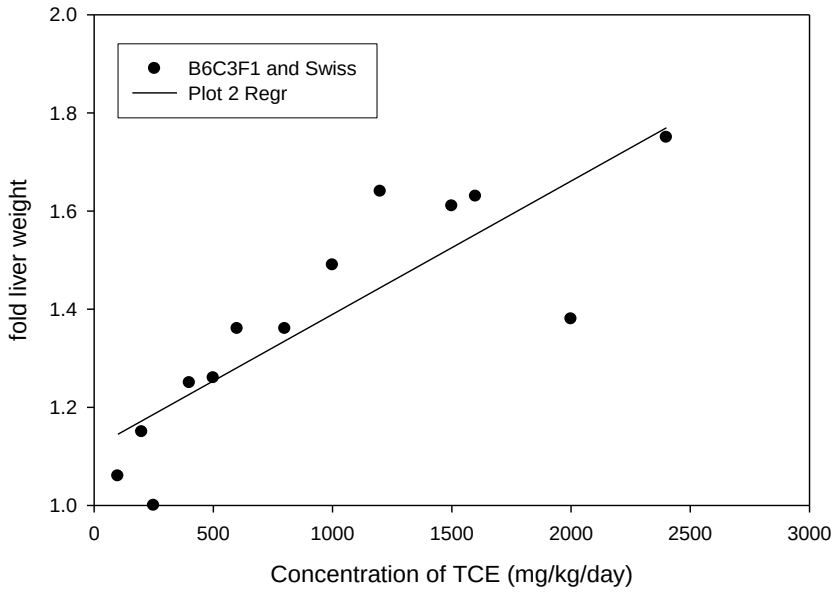
The dose-response curves for similar concentrations of DCA and TCA are presented in Figure E-1 for durations of exposure from 14 to 28 days in the male B6C3F$_1$ mouse, which was the most common sex and strain used. For this comparative analysis, an average is provided between two values for a given concentration and duration of exposure for comparison with other doses and time points. As noted in the discussion of individual experiments, there appears to be a linear correlation between dose in drinking water and liver weight induction up to 2 g/L of DCA. However, the shape of the dose-response curve for TCA appears to be quite different (i.e., lower concentrations of TCA inducing larger increase that does DCA but then the response reaching an

apparent plateau for TCA at higher doses while that of DCA continues to increase). As shown by DeAngelo et al. (2008), 10-fold differences in the magnitude of exposure concentration to TCA corresponded to approximately twofold differences in liver weight induction increases. In addition, TCA studies did not show significant duration-dependent difference in liver weight induction in this duration range as shown in Table E-14.

Of interest is the issue of how the dose-response curves for TCA and DCA compare to that of TCE in a similar model and dose range. Since TCA and DCA have strikingly different dose-response curves, which one, if either, best fits that of TCE and thus, can give insight as to which is causative agent for TCE's effects in the liver? In the case of the TCE database in the mouse two strains have been predominantly studied, Swiss and $B6C3F_1$, and both have been reported to get liver tumors in response to chronic TCE exposure.

Rather than administered in drinking water, oral TCE studies have been conducted via gavage and generally in corn oil for 5 days of exposure per week. The study by Goel et al. (1992) was conducted in ground-nut oil. Vehicle effects, the difference between daily and weekly exposures, the dependence of TCE effects in the liver on its metabolism to a variety of agents capable inducing effects in the liver, differences in response between strains, and the inherent increased variability in use of the male mouse model all add to increased difficulty in establishing the dose-response relationship for TCE across studies and for comparisons to the DCA and TCA database. Despite difference in exposure route, etc., a consistent pattern of dose-response emerges from combining the available TCE data. The effects of oral exposure to TCE from 10 to 42 days on liver weight induction is shown in Figure E-2 using the data of Elcombe et al. (1985), Dees and Travis (1993), Goel et al. (1992), Merrick et al. (1989), Goldsworthy and Popp (1987), and Buben and O'Flaherty (1985). More detailed discussion of the 4–6-week studies is presented in Section E.2.4.3 (e.g., for (Goel et al., 1992; Merrick et al., 1989; Buben and O'Flaherty, 1985)). For this comparative analysis, an average is provided between two values per concentration and duration of exposure for comparison with other doses and time points. As shown by the 10-day data in $B6C3F_1$ mice, there are significant differences in response between studies of male $B6C3F_1$ mice at the same dose of TCE. This variability is similar to findings from inhalation studies of TCE in male mice (Kjellstrand et al., 1983b).





Reproduced from Section 4.5.

Sources: (Dees and Travis, 1993; Merrick et al., 1989; Goldsworthy and Popp, 1987; Elcombe et al., 1985)).

Figure E-2. Comparisons of fold-changes in average relative liver weight and gavage dose of (top panel) male B6C3F$_1$ mice for 10–28 days of exposure and (bottom panel) in male B6C3F$_1$ and Swiss mice.

As shown in Figure E-2, oral TCE administration in male $B6C3F_1$ and Swiss mice appeared to induce a dose-related increase in percent liver/body weight that was generally proportional to the increase in magnitude of dose, though as expected, with more variability than observed for a similar exercise for DCA or TCA in drinking water. Common exposure concentrations between $B6C3F_1$ and Swiss mice were 100, 500, 1,000, 1,500 and 2,400 mg/kg-day TCE, which corresponded to a 5-, 2-, 1.5-, and 1.6-fold difference in the magnitude of dose. For the data from studies in $B6C3F_1$ mice, there was no increase reported at 100 mg/kg-day TCE but between 500 and 1,000, 1,000 and 1,500, and 1,500 and 2,400 mg/kg-day TCE, the magnitude of difference in doses matched that of the magnitude of increase in percent liver/body weight (i.e., a 2.6-, 1.4-, and 1.7-fold increase in liver weight was matched by a 2-, 1.5-, and 1.6-fold increase in TCE exposure concentration at these exposure intervals).

However, only a 10-day interval was available for doses between 100 and 500 mg/kg in $B6C3F_1$ mice and at the lower doses, a 10-day interval may have been too short for the increase in liver weight to have been fully expressed. The database for the Swiss mice, which has more data from 28 and 42 days of exposure, support this conclusion. At 28–42 days of exposure, there was a much greater increase in liver weight from TCE exposure in Swiss mice than the 10-day data in $B6C3F_1$ mice.

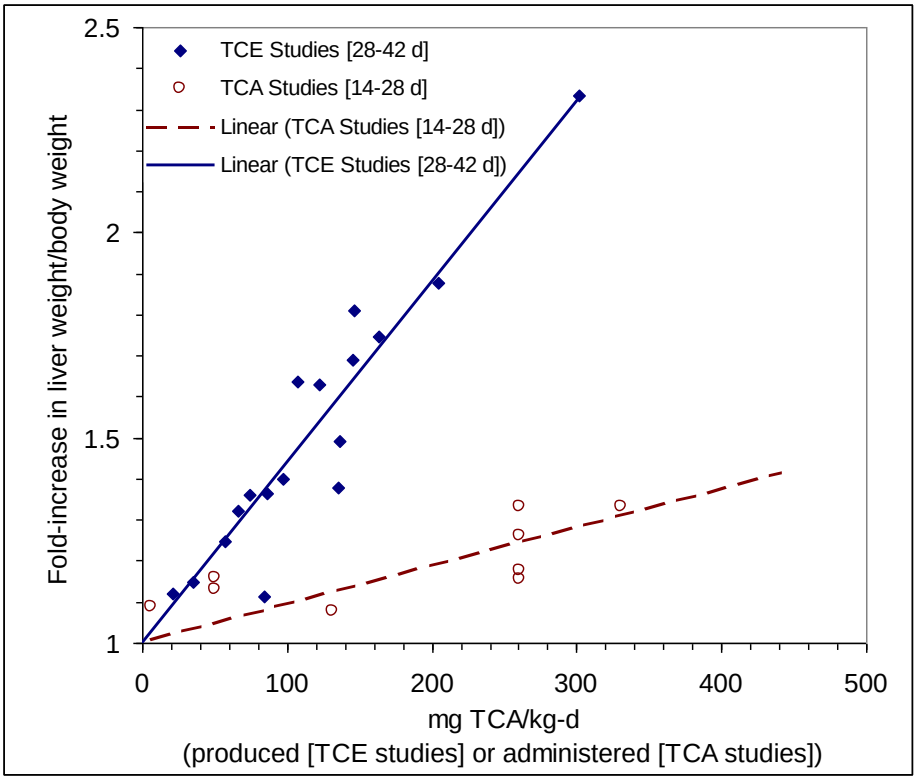
In Figure E-2, the 10-day data are included for comparative purpose for the $B6C3F_1$ data set and the Swiss and $B6C3F_1$ data sets combined. Both the combined TCE data and that for only $B6C3F_1$ mice shows a correlation with the magnitude of dose and magnitude of percent liver/body weight increase. The slope of the dose-response curves are both closer to that of DCA than TCA. The correlation coefficients for the linear regressions presented for the $B6C3F_1$ data are $R^2 = 0.861$ and for the combined data sets is $R^2 = 0.712$. Comparisons of the slopes of the dose-response curves indicate that TCA is not responsible for TCE-induced liver effects. In this regression, all data points were treated equally, although some came from several sets of data and others did not. Of note is that the 2,000 mg/kg TCE data point in the combined data set, which is much lower in liver weight response than the other data, is from one experiment (Goel et al., 1992), from six mice, at one time point (28 days), and one strain (Swiss). Deletion of these data point from the rest of the 23 used in the study results in a better fit to the data of the regression analysis.

A more direct comparison would be on the basis of dose rather than drinking water concentration. The estimations of internal dose of DCA or TCA from drinking water studies have been reported to vary with DeAngelo et al. (1989) calculated DCA drinking water concentrations of 1.0, 2.0, and 5.0 g/L to result in 90, 166, and 346 mg/kg-day, respectively, based on previous analyses in their laboratory. For TCA, 0.05, 0.5, 1.0, 2.0, and 5 g/L drinking water exposures were reported to result in 5.8 (range 3.6–8.0), 50 (range of 32.5–68), 131, 261, and 469 (range 364–602) mg/kg-day doses. The estimations of internal dose of DCA or TCA from drinking water studies, while varying considerably (DeAngelo et al., 2008; 1989), nonetheless suggest that

the doses of TCE used in the gavage experiments were much higher than those of DCA or TCA. However, only a fraction of ingested TCE is metabolized to DCA or TCA, as, in addition to oxidative metabolism, TCE is also cleared by GSH conjugation and by exhalation.

While DCA dosimetry is highly uncertain (see Sections 3.3 and 3.5), the mouse PBPK model, described in Section 3.5 was calibrated using extensive in vivo data on TCA blood, plasma, liver, and urinary excretion data from inhalation and gavage TCE exposures, and makes robust predictions of the rate of TCA production. If TCA were predominantly responsible for TCE-induced liver weight increases, then replacing administered TCE dose (e.g., mg TCE/kg/day) by the rate of TCA produced from TCE (mg TCA/kg/day) should lead to dose-response curves for increased liver weight consistent with those from directly administered TCA.

Figure E-3 shows this comparison using the PBPK model-based estimates of TCA production for four TCE studies from 28 to 42 days in the male NMRI, Swiss, and B6C3F$_1$ mice (Goel et al., 1992; Merrick et al., 1989; Buben and O'Flaherty, 1985; Kjellstrand et al., 1983a) and four oral TCA studies in B6C3F$_1$ male mice at ≤2 g/L drinking water exposures (DeAngelo et al., 2008; Kato-Weinstein et al., 2001; Parrish et al., 1996; DeAngelo et al., 1989) from 14 to 28 days of exposure. The selection of the 28–42 day data for TCE was intended to address the decreased opportunity for full expression of response at 10 days. PBPK modeling predictions of daily internal doses of TCA in terms of mg/kg-day via produced via TCE metabolism would be indeed lower than the TCE concentrations in terms of mg/kg-day given orally by gavage. The predicted internal dose of TCA from TCE exposure studies are of a comparable range to those predicted from TCA drinking water studies at exposure concentrations in which palatability has not been an issue for estimation of internal dose. Thus, although the TCE data are for higher exposure concentrations, they are predicted to produce comparable levels of TCA internal dose estimated from direct TCA administration in drinking water.



(Reproduced from Section 4.5.)

Abscissa for TCE studies consists of the median estimates of the internal dose of TCA predicted from metabolism of TCE using the PBPK model described in Section 3.5 of the TCE risk assessment. Lines show linear regression with intercept fixed at unity. All data were reported fold-change in mean liver weight/body weight ratios, except for Kjellstrand et al. (1983a), with were the fold-change in the ratio of mean liver weight to mean body weight. In addition, in Kjellstrand et al. (1983a), some systemic toxicity as evidence by decreased total body weight was reported in the highest-dose group.

Sources: Kjellstrand et al. (1983a); (Goel et al., 1992; Merrick et al., 1989; Buben and O'Flaherty, 1985); (DeAngelo et al., 2008; Kato-Weinstein et al., 2001; Parrish et al., 1996; DeAngelo et al., 1989).

Figure E-3. Comparison of fold-changes in relative liver weight for data sets in male B6C3F$_1$, Swiss, and NRMI mice between TCE studies [duration 28–42 days]) and studies of direct oral TCA administration to B6C3F$_1$ mice [duration 14–28 days]).

Figure E-3 clearly shows that for a given amount of TCA produced from TCE, but going through intermediate metabolic pathways, the liver weight increases are substantially greater than, and highly inconsistent with, that expected based on direct TCA administration. In particular, the response from direct TCA administration appears to "saturate" with increasing TCA dose at a level of about 1.4-fold, while the response from TCE administration continues to increase with

dose to 1.75-fold at the highest dose administered orally in Buben and O'Flaherty (1985) and over 2-fold in the inhalation study of Kjellstrand et al. (1983a). For this analysis, it is unlikely that strain differences can account for this inconsistency in the dose-response curves.

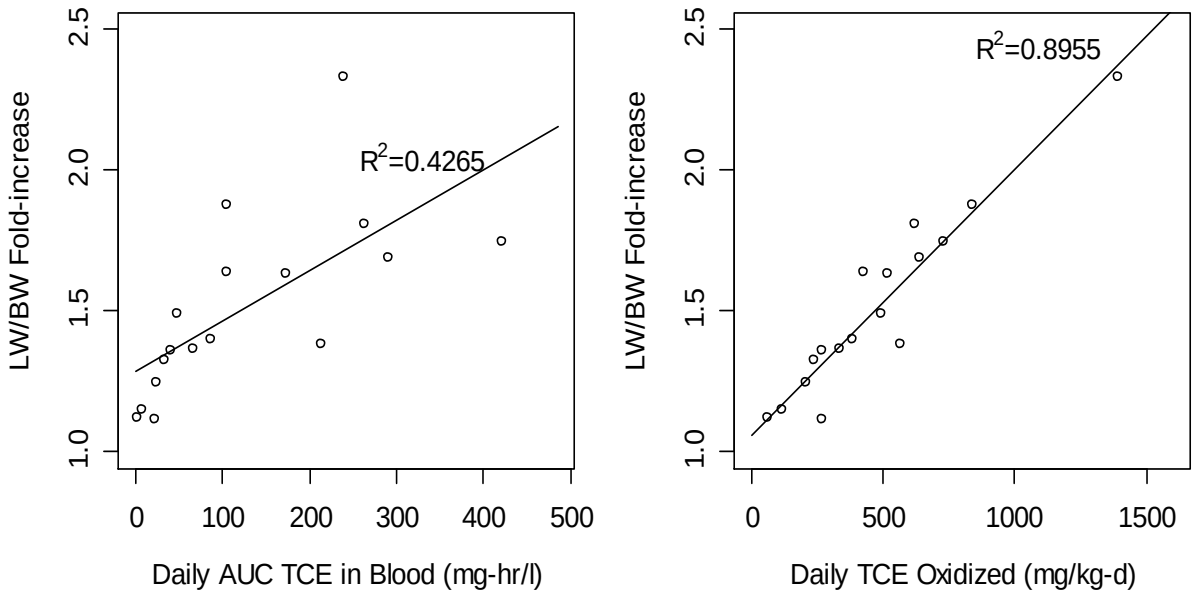
TCE-induced increases in liver weight appear to be generally similar between $B6C3F_1$ and Swiss male mice (see Table E-14) via oral exposure and between NMRI male and female mice after inhalation, although the NMRI strain appeared to be more prone to TCE-induced toxicity in male mice, and females appeared to have a smaller TCE-induced liver weight increase than other strains (Kjellstrand et al., 1983a). As noted previously, the difference in response between strains and between studies in the same strain for TCE liver weight increases can be highly variable. Little data exist to examine this issue for TCA studies, although DeAngelo et al. (1989) report a range of 1.16–1.63-fold of control percent liver/body weight increase after 14 days exposure at 2 g/L TCA in the Swiss-Webster, C3H, C57BL/6, and $B6C3F_1$ strains, with differences also noted between two studies of the $B6C3F_1$ mouse.

Furthermore, while as noted previously, oral studies appear to report a linear relationship between TCE exposure concentration and liver weight induction, the inclusion of inhalation studies on the basis of internal dose led to a highly consistent dose-response curve among TCE studies. Therefore, it is unlikely that differing routes of exposure can explain the inconsistencies in dose-response. The PBPK model predicted that matching average TCA production by TCE with the equivalent average dose from drinking water-administered TCA also led to an equivalent AUC of TCA in the liver.

Moreover, Dees and Travis (1993) administered 100–1,000 mg/kg-day TCA by gavage to male and female $B6C3F_1$ mice for 11 days, and did not observe increases in liver/body weight ratios >1.28-fold, no higher than those observed with drinking water exposures. Finally, the dose-response consistency between TCE inhalation and gavage studies argues against route of exposure significantly impacting liver weight increases. Thus, no level of TCA administration appears able account for the continuing increase in liver weights observed with TCE, quantitatively inconsistent with TCA being the predominant metabolite responsible for TCE-induced liver weight changes. Involvement of other metabolites, besides TCA, is implicated as the causes of TCE-induced liver effects.

Additional analyses do, however, support a role for oxidative metabolism in TCE-induced liver weight increases, and that the parent compound TCE is not the likely active moiety (as suggested previously by Buben and O'Flaherty, 1985). In particular, the same studies are shown in Figure E-4 using PBPK-model based predictions of the AUC of TCE in blood and total oxidative metabolism, which produces chloral, TCOH, DCA, and other metabolites in addition to TCA. The dose-response relationship between TCE blood levels and liver weight increase, while still having a significant trend, shows substantial scatter and a low R^2 of 0.43. On the other hand, using total oxidative metabolism as the dose-metric leads to substantially more consistency dose-response across studies, and a much tighter linear trend with an R^2 of 0.90 (see Figure E-4). A

similar consistency is observed using liver-only oxidative metabolism as the dose-metric, with R^2 of 0.86 (not shown). Thus, while the slope is similar between liver weight increase and TCE concentration in the blood and liver weight increase and rate of total oxidative metabolism, the data are a much better fit for total oxidative metabolism.



(Reproduced from Section 4.5).

Lines show linear regression. Use of liver oxidative metabolism as a dose-metric gives results qualitatively similar to (B), with $R^2 = 0.86$.

Sources: Kjellstrand et al. (1983a); (Goel et al., 1992; Merrick et al., 1989; Buben and O'Flaherty, 1985).

Figure E-4. Fold-changes in relative liver weight for data sets in male B6C3F$_1$, Swiss, and NRMI mice reported by TCE studies of duration 28–42 days using internal dose-metrics predicted by the PBPK model described in Section 3.5: (A) dose-metric is the median estimate of the daily AUC of TCE in blood, (B) dose-metric is the median estimate of the total daily rate of TCE oxidation.

As stated in many of the discussions of individual studies, there is a limited ability to detect a statistically significant change in liver weight change in experiments that use a relatively small number of animals. Many experiments have been conducted with 4–6 mice per dose group. The experiments of Buben and O'Flaherty used 12–14 mice per group, giving it a greater ability to detect a TCE-induced dose response. In some experiments, greater care was taken to document and age and weight match the control and treatment groups before the start of treatment. The approach taken above for the analyses of TCE, TCA, and DCA uses data across several data sets

and gives a more robust description of these dose-response curves, especially at lower exposure levels. For example, the data from DeAngelo et al. (2008) for TCA-induced percent liver/body weight ratio increases in male B6C3F$_1$ mice were only derived from five animals per treatment group after 4 weeks of exposure. The 0.05 and 0.5 g/L exposure concentrations were reported to give a 1.09- and 1.16-fold of control percent liver/body weight ratios, which were consistent with the increases noted in the cross-study database above. However, a power calculation shows that the type II error, which should be >50% and thus, greater than the chances of "flipping a coin," was only a 6 and 7% and therefore, the designed experiment could accept a false null hypothesis.

Although the qualitative similarity to the linear dose-response relationship between DCA and liver weight increases is suggestive of DCA being the predominant metabolite responsible for TCE liver weight increases, due to the highly uncertain dosimetry of DCA derived from TCE, this hypothesis cannot be tested on the basis of internal dose. Similarly, another TCE metabolite, CH, has also been reported to induce liver tumors in mice; however, there are no adequate comparative data to assess the nature of liver weight increases induced by this TCE metabolite (see Section E.2.5). Whether its formation in the liver after TCE exposure correlates with TCE-induced liver weight changes cannot be determined. Of note is the high variability in total oxidative metabolism reported in mice and humans of Section 3.3, which suggests that the correlation of total TCE oxidative metabolism with TCE-induced liver effects should not only lead to a high degree of variability in response in rodent bioassays, which is the case (see Section E.2.4.4), but also make detection of liver effects more difficult in human epidemiological studies.

The bioavailability of TCA has been assumed to be 100% in the analyses in Figure E-3. Further analyses are presented in Appendix A and in Chiu (2011) regarding the assertions by Sweeney, et al. (Sweeney et al., 2009) that previously unpublished kinetic data for mice exposed to TCA in drinking water indicates much lower absorption. The conclusions of Sweeney et al. (2009) were based on the TCE PBPK model of Hack et al. (2006) and not that of Evans et al. (2009) and Chiu et al. (2009). The analyses by Chiu (Chiu, 2011) show that while there is some decreased absorption of TCA at higher doses, it was not as low as that estimated by Sweeney et al. (2009) and as discussed in Appendix A, it may be more accurate to characterize the fractional absorption as an empirical parameter reflecting unaccounted-for biological processes as well as experimental variation. The Chiu (2011) re-analyses the data on TCE- and TCA-induced hepatomegaly, using the central estimates of the fractional absorption of TCA, showed that while reduced fractional absorption inferred from drinking water data reported by Sweeney et al. (2009) accounts for part of the difference in dose-responses between TCE- and TCA-induced hepatomegaly reported by Evans et al. (2009), it does not appear to be able to account for the entire difference. The inability of TCA to account for TCE-induced hepatomegaly was confirmed statistically by ANOVA and even with an assumption of reduced TCA bioavailability,

the available data are inconsistent with the toxicological hypothesis that TCA can fully account for TCE-induced hepatomegaly.

What mechanisms or events are leading to liver weight increases for DCA, TCA, and TCE can be examined by correlations between changes in glycogen content, hepatocyte volume, and evidence of polyploidization noted in short-term assays. Data have been reported regarding the nature of changes the TCE and its metabolites induce in the liver and are responsible for the reported increases in liver weight. Increased liver weight may result from increased size or hypertrophy of hepatocytes through changes in glycogen deposition, but also through increased polyploidization. Increased cell number may also contribute to increased liver weight. As noted in Section E.2.4.1, hepatocellular hypertrophy appeared to be related to TCE-induced liver weight changes after short-term exposures. However, neither glycogen deposition, DNA synthesis, nor increases in mitosis appear to be correlated with liver weight increases. In particular, DNA synthesis increases were similar from 250 to 1,000 mg/kg and peroxisomal volume was similar between 500 and 1,500 mg/kg TCE exposures after 10 days. Autoradiographs identified hepatocytes undergoing DNA synthesis in "mature" hepatocytes that were in areas where polyploidization typically takes place in the liver.

By 14 days of exposure, Sanchez and Bull (1990) reported that both dose-related TCA- and DCA-induced increases in liver weight were generally consistent with changing cell size increases, but were not correlated with patterns of change in hepatic DNA content, incorporation of tritiated thymidine in DNA extracts from whole liver, or incorporation of tritiated thymidine in hepatocytes. There are conflicting reports of DNA synthesis induction in individual hepatocytes for up to 14 days of DCA or TCA exposure and a lack of correlation with patterns observed for this endpoint and those of whole-liver thymidine incorporation. The inconsistency of whole-liver DNA tritiated thymidine incorporation with that reported for hepatocytes was noted by the Sanchez and Bull (1990) to be unexplained. Carter et al. (1995) also report a lack of correlation between hepatic DNA tritiated thymidine incorporation and labeling in individual hepatocytes in male mice. Carter et al. (1995) reported no increase in labeling of hepatocytes in comparison to controls for any DCA treatment group from 5 to 30 days of DCA exposure. Rather than increase hepatocyte labeling, DCA induced a decrease with no change reported from days 5 though 15 but significantly decreased levels between days 20 and 30 for 0.5 g/L that were similar to those observed for the 5 g/L exposures.

The most comparable time periods between TCE, TCA, and DCA results for whole-liver thymidine incorporation are the 10- and 14-day durations of exposure when peak tritiated thymidine incorporation into individual hepatocytes and whole liver for TCA and DCA have been reported to have already passed (Pereira, 1996; Carter et al., 1995; Styles et al., 1991; Sanchez and Bull, 1990). Whole-liver DNA synthesis was elevated over control levels by approximately twofold after from 250 to 1,000 mg/kg TCE exposure after 10 days of exposure but did not correlate with mitosis (Dees and Travis, 1993; Elcombe et al., 1985). After 3 weeks of exposure

to TCE, Laughter et al. (2004) reported that 1 and 4.5% of individual hepatocytes had undergone DNA synthesis in the last week of treatment for the 500 and 1,000 mg/kg TCE levels, respectively. More importantly, these data show that hepatocyte proliferation in TCE-exposed mice at 10 days of exposure or for DCA- or TCA-exposed mice for up to 14 days of exposure is confined to a very small population of cells in the liver.

In regard to cell size, although increased glycogen deposition with DCA exposure was noted by Sanchez and Bull (1990), lack of quantitative analyses of that accumulation in this study precludes comparison with DCA-induced liver weight gain. Although not presenting a quantitative analysis, Sanchez and Bull (1990) reported DCA-treated B6C3F_1 mice to have large amounts of PAS staining material and Swiss-Webster mice to have similar increase despite reporting differences of DCA-induced liver weight gain between the two strains. The lack of concordance of the DCA-induced magnitude of increase in liver weight with that of glycogen deposition is consistent with the findings for longer-term exposures to DCA reported by Kato-Weinstein et al. (2001) and Pereira et al. (2004a) in mice (see Section E.2.4.4). Carter et al. (1995) reported that in control mice, there was a large variation in apparent glycogen content and also did not perform a quantitative analysis of glycogen deposition. The variability of this parameter in untreated animals and the extraction of glycogen during normal tissue processing for light microscopy makes quantitative analyses for dose-response difficult unless specific methodologies are employed to quantitatively assess liver glycogen levels as was done by Kato-Weinstein et al. (2001) and Pereira et al. (2004a).

Although suggested by their data, polyploidization was not examined for DCA or TCA exposure in the study of Sanchez and Bull (1990). Carter et al. (1995) reported that hepatocytes from both 0.5 and 5 g/L DCA treatment groups were reported to have enlarged, presumably polyploidy nuclei with some hepatocyte nuclei labeled in the mid-zonal area. There were statistically significant changes in cellularity, nuclear size, and multinucleated cells during 30 days exposure to DCA. The percentage of mononucleated cells hepatocytes was reported to be similar between control and DCA treatment groups at 5- and 10-day exposures.

However, at 15 days and beyond, DCA treatments were reported to induce increases in mononucleated hepatocytes. At later time periods, there were also reports of DCA-induced increases nuclear area, consistent with increased polyploidization without mitosis. The consistent reporting of an increasing number of mononucleated cells between 15 and 30 days could be associated with clearance of mature hepatocytes as suggested by the report of DCA-induced loss of cell nuclei. The reported decrease in the numbers of binucleate cells in favor of mononucleated cells is not typical of any stage of normal liver growth (Brodsky and Uryvaeva, 1977). The linear dose-response in DCA-induced liver weight increase was not consistent with the increased numbers of mononucleate cells and increased nuclear area reported from day 20 onward by Carter et al. (1995). Specifically, the large differences in liver weight induction between the 0.5 g/L treatment group and the 5 g/L treatment groups at all times studied also did

not correlate with changes in nuclear size and percent of mononucleated cells. Thus, DCA-induced increases in liver weight were not a function of cellular proliferation, but probably included hypertrophy associated with polyploidization, increased glycogen deposition, and other factors.

In regard to necrosis, Elcombe et al. (1985) reported only small incidence of focal necrosis in 1,500 mg/kg TCE-exposed mice and no necrosis at exposures up to 1,000 mg/kg for 10 days as did Dees and Travis (1993). Sanchez and Bull (1990) report DCA-induced localized areas of coagulative necrosis both for B6C3F_1 and Swiss-Webster mice at higher exposure levels (1 or 2 g/L) by 14 days but not at the 0.3 g/L level or earlier time points. For TCA treatment, necrosis was reported to not be associated with TCA treatment for up to 2 g/L and up to 14 days of exposure. Carter et al. (1995) reported that mice given 0.5 g/L DCA for 15, 20, and 25 days had midzonal focal cells with less detectable or no cell membranes and loss of the coarse granularity of the cytoplasm, with some cells having apparent karyolysis, but for liver architecture to be normal.

As for apoptosis, both Elcombe et al. (1985) and Dees and Travis (1993) reported no changes in apoptosis other than increased apoptosis only at a treatment level of 1,000 mg/kg TCE. Rather than increases in apoptosis, peroxisome proliferators have been suggested to inhibit apoptosis as part of their carcinogenic mode of action (see Section E.3.4.1). However, the age and species studied appear to greatly affect background rates of apoptosis. Snyder et al. (1995) report that control mice were reported to exhibit apoptotic frequencies ranging from ~0.04 to 0.085%, that over the 30-day period of their study, the frequency rate of apoptosis declined, and suggest that this pattern is consistent with reports of the livers of young animals undergoing rapid changes in cell death and proliferation. They reported rat liver to have a greater the estimated frequency of spontaneous apoptosis (~0.1%) and therefore, greater than that of the mouse.

Carter et al. (1995) reported that after 25 days of 0.5 g/L DCA treatment apoptotic bodies were reported as well as fewer nuclei in the pericentral zone and larger nuclei in central and midzonal areas. This would indicate an increase in the apoptosis associated potential increases in polyploidization and cell maturation. However, Snyder et al. (1995) report that mice treated with 0.5 g/L DCA over a 30-day period had a similar trend as control mice of decreasing apoptosis with age. The percentage of apoptotic hepatocytes decreased in DCA-treated mice at the earliest time point studied and remained statistically significantly decreased from controls from 5 to 30 days of exposure. Although the rate of apoptosis was very low in controls, treatment with 0.5 g/L DCA reduced it further (~30–40% reduction) during the 30-day study period. The results of this study not only provide a baseline of apoptosis in the mouse liver, which is very low, but also to show the importance of taking into account the effects of age on such determinations. The significance of the DCA-induced reduction in

apoptosis reported in this study, from a level that is already inherently low in the mouse, to account for the mode of action for induction of DCA-induced liver cancer is difficult to discern.

Finally, short-term inhalation studies by Ramdhan et al. (2010) indicate that in wild type, PPARα-null, and humanized null mice, relatively high exposures to TCE induced increased liver size after 7 days of inhalation exposure. At the same highest concentration of TCE, although urinary TCA concentrations were lower in PPARα-null mice than wild type mice, the sum of urinary TCOH and TCA concentrations were the same, increases in percent liver/body weight were the same, and liver triglyceride content was much greater in the PPARα-null mice than wild type mice after TCE exposure. Hepatic steatosis was also greater as a baseline condition along with hepatic triglyceride content in the PPARα-null mice than wild type mice. These parameters were more elevated in humanized mice as a background dysregulation and even more elevated after treatment with TCE. Therefore, the nature of hepatomegally induced by TCE is complex and dependent on baseline lipid dysregulation states.

E.2.4.3. Summary of TCE Subchronic and Chronic Studies

The results of longer-term (Toraason et al., 1999; Channel et al., 1998; Parrish et al., 1996) studies of "oxidative stress" for TCE and its metabolites are discussed in Section E.3.4.2.3. Of note are the findings that the extent of increased enzyme activities associated with peroxisome proliferation do not appear to correlate with measures of oxidative stress after longer-term exposures (Parrish et al., 1996) and SSBs (Chang et al., 1992).

Similar to the reports of Melnick et al. (1987) in rats, Merrick et al. (1989) report that vehicle (aqueous or gavage) affects TCE-induced toxicity in mice. Vehicle type made a large difference in mortality, extent of liver necrosis, and liver weight gain in male and female $B6C3F_1$ mice after 4 weeks of exposure. The lowest dose used in this experiment was 600 mg/kg-day in males and 450 mg/kg-day in females. Administration of TCE via gavage using Emulphor resulted in mortality of all of the male mice and most of the female mice at a dose in corn oil that resulted in few deaths. However, use of Emulphor vehicle induced little, if any, focal necrosis in males at concentrations of TCE in corn oil gavage that caused significant focal necrosis, indicating vehicle effects.

As discussed in Section E.2.4.2, the extent of TCE-induced liver weight increases was consistent between 4 and 6 weeks of exposure and between 10-day and 4-week exposures at higher dose levels. In general, the reported elevations of enzymatic markers of liver toxicity and results for focal hepatocellular necrosis were not consistent and did not reflect TCE dose-responses observed for induction of liver weight increases (Merrick et al., 1989). Female mice given corn oil and male and female mice given TCE in Emulphor were reported to have "no to negligible necrosis," although they had increased liver weight from TCE exposure.

Using a different type of oil vehicle, Goel et al. (1992) exposed male Swiss mice to TCE in groundnut oil at concentrations ranging from 500 to 2,000 mg/kg for 4 weeks and reported no

changes in body weight up to 2,000 mg/kg. There was a 15% decrease at the highest dose and increased TCE-induced percent liver/body weight ratio. At a dose of 1,000 and 2,000 mg/kg, liver swelling, vacuolization, and widespread degenerative necrosis of hepatocytes was reported along with marked proliferation of "endothelial cells" but no quantitation regarding the extent or location of hepatocellular necrosis was reported, nor whether there was a dose-response relationship in these events. They reported a TCE-related dose-response in catalase and liver protein, but a decreased induction at the 2,000 mg/kg level where body weight had decreased.

Three studies were published by Kjellstrand and colleagues that examined effects of TCE inhalation primarily in mice using whole-body inhalation chambers (Kjellstrand et al., 1983a; Kjellstrand et al., 1983b; Kjellstrand et al., 1981a). Liver weight changes were used as the indication of TCE-induced effects. The quantitative results from these experiments had many limitations due to their experimental design including failure to determine body weight changes for individual animals and inability to determine the exact magnitude of TCE due to concurrent oral TCE ingestion from food and grooming behavior. An advantage of this route of exposure was that there were not confounding vehicle effects. The results from Kjellstrand et al. (1981a) were particularly limited by experimental design errors showed similar increases in liver weight gain in gerbils and rats exposed at 150 ppm TCE. For rats, Kjellstrand et al. (1981a) reported increases in liver/body weight ratios of 1.26- and 1.21-fold of control in male and female rat 30 days of continuous TCE inhalation exposure.

The unpublished report of Woolhiser et al. (2006) reports 1.05-, 1.07-, and 1.13-fold of control percent liver/body weight changes in 100, 300, and 1,000 ppm exposure groups that are exposed for 6 hours/day, 5 days/week for 4 weeks in groups of eight female Sprague-Dawley rats. At the two highest exposure levels, body weight was reduced by TCE exposure. The 150 ppm continuous exposure concentrations of Kjellstrand were analogous to 750 ppm exposures using the paradigm of Woolhiser et al. (2006) in terms of total daily dose. Therefore, the very limited inhalation database for rats does indicate TCE-related increases in liver weight.

The study of Kjellstrand et al. (1983b) employed a more successful experimental design that recorded liver weight changes in carefully matched control and treatment groups to determine TCE-treatment related effects on liver weight in seven strains of mice after 30 days of continuous inhalation exposure at 150 ppm TCE. Individual animal body weight changes were not recorded so that such an approach cannot take into account the effects of body weight changes and determine a relative percent liver/body weight ratio. The data presented in this report were for absolute liver weight changes between treated and nontreated groups with carefully matched average body weights at the initiation of exposure. A strength of the experimental design is its presentation of results between duplicate experiments and thus, its ability to show the differences in results between similar exposed groups that were conducted at different times. This information gives a measure of variability in response with time. Mouse strain groups that did not experience TCE-induced decreased body weight gain in comparison

to untreated groups (i.e., DBA and wild-type mice) represented the most accurate determination of TCE-induced liver weight changes given that systemic toxicity that affects body weight can also affect liver weight.

The C57BL, B6CBA, and NZB groups all had at least one group out of two of male mice with changes in final body weight due to TCE exposure. Only one group of NMRI mice were reported in this study and that group had TCE-induced decreases in final body weight. The A/sn group not only had both male groups with decreased final body weight after TCE exposure (along with differences between exposed and control groups at the initiation of exposure), but also a decrease in body weight in one of the female groups and thus, appears to be the strain with the greatest susceptibility to TCE-induced systemic toxicity. In strains of male mice in which there were no TCE-induced affects on final body weight (wild-type and DBA), the influence of gender on liver weight induction and variability of the response could be more readily assessed. In wild-type mice, there was a 1.76- and 1.80-fold of control liver weight in groups 1 and 2 for female mice, and for males, a 1.84- and 1.62-fold of control liver weight for groups 1 and 2, respectively. For DBA mice, there was a 1.87- and 1.88-fold of control liver weight in groups 1 and 2 for female mice, and for males, a 1.45- and 2.00-fold of control liver weight for groups 1 and 2, respectively. Of note, as described previously, the size of the liver is under strict control in relation to body size. An essential doubling of the size of the liver is a profound effect with the magnitude of liver weight size increase physiologically limited.

Overall, the consistency between groups of female mice of the same strain for TCE-induced liver weight gain, regardless of strain examined, was striking, as was the lack of body weight changes at TCE exposure levels that induced body weight changes in male mice. In the absence of body weight changes, the difference in TCE-response in female mice appeared to be reflective of strain and initial weight differences. Groups of female mice with higher body weights, regardless of strain, generally had higher increases in TCE-induced liver weight increases. For the C57BL and As/n strains, female mice starting weights were averaged 17.5 and 15.5 g, while the average liver weights were 1.63- and 1.64-fold of control after TCE exposure, respectively. For the B6CBA, wild-type, DBA, and NZB female groups, the starting body weights averaged 22.5, 21.0, 23.0, and 21.0 g, while the average liver weights were 1.70-, 1.78-, 1.88-, and 2.09-fold of control after TCE exposure, respectively. The NMRI group of female mice, did not follow this general pattern and had the highest initial body weight for the single group of 10 mice reported (i.e., 27 g) associated with 1.66-fold of control liver weight.

The results of Kjellstrand et al. (1983b) suggested that there was more variability between male mice than female mice in relation to TCE-induced liver weight gain. More strains exhibited TCE-induced body weight changes in male mice than female mice, suggesting increased susceptibility of male mice to TCE toxicity as well as more variability in response. Initial body weight also appeared to be a factor in the magnitude of TCE-induced liver weight induction rather than just strain. In general, the strains and groups within strain that had

TCE-induced body weight decreases had smaller TCE-induced increase in liver weight. Therefore, only examining liver weight in males as an indication of TCE treatment effects would not be an accurate predictor of strain sensitivity nor the magnitude or response at doses that also affect body weight. The results from this study show that comparison of the magnitude of TCE response, as measured by liver weight increases, should take into account strain, gender, initial body weight, and systemic toxicity. It shows a consistent pattern of increased liver weight in both male and female mice after TCE exposure of 150 ppm for 30 days.

Kjellstrand et al. (1983a) presented data in the NMRI strain of mice (a strain that appeared to be more prone to TCE-induced toxicity in male mice and a smaller TCE-induced increase in liver weight in female mice) after inhalation exposure of 37–300 ppm TCE. They used the same experimental paradigm as that reported in Kjellstrand et al. (1983b) except for exposure concentration.

For female mice exposed to concentrations of TCE ranging from 37 to 300 ppm TCE continuously for 30 days, only the 300 ppm group experienced a 16% decrease in body weight between control and exposed animals. Therefore, changes in TCE-induced liver weight increases were affected by changes in body weight only for that group. Initial body weights in the TCE-exposed female mice were similar in each of these groups (i.e., range of 29.2–31.6 g, or 8%), with the exception of the females exposed to 150 ppm TCE for 30 days (i.e., initial body weight of 27.3 g), reducing the effects of differences in initial body weight on TCE-induced liver weight induction. Exposure to TCE continuously for 30 days was reported to result in a linear dose-dependent increase in liver weight in female mice with 1.06-, 1.27-, 1.66-, and 2.14-fold of control liver weights reported at 37, 75, 150, and 300 ppm TCE, respectively.

In male mice, there were more factors affecting reported liver weight increases from TCE exposure. For male mice, both the 150 and 300 ppm exposed groups experienced a 10 and 18% decrease in final body weight after TCE exposure, respectively. The 37 and 75 ppm groups did not have decreased final body weight due to TCE exposure but varied by 12% in initial body weight. TCE-induced increases in liver weight were reported to be 1.15-, 1.50-, 1.69-, and 1.90-fold of control for 37, 75, 150, and 300 ppm TCE exposure in male mice, respectively. The flattening of the dose-response curve at the two highest doses is consistent with the effects of toxicity on final body weight.

Kjellstrand et al. (1983a) noted that liver mass increased and the changes in liver cell morphology were similar in TCE-exposed male and female mice. They report that after 150 ppm exposure for 30 days, liver cells were generally larger and often displayed a fine vacuolization of the cytoplasm, changes in nucleoli appearance. Kupffer cells of the sinusoid were reported to be increased in cellular and nuclear size. The intralobular connective tissue was infiltrated by inflammatory cells. Exposure to TCE in higher or lower concentrations during the 30 days was reported to produce a similar morphologic picture.

For mice that were exposed to 150 ppm TCE for 30 days and then examined 120 days after the cessation of exposure, liver weights were 1.09-fold of control for TCE-exposed female mice and the same as controls for TCE-exposed male mice. However, the livers were not the same as untreated liver in terms of histopathology. The authors reported that "after exposure to 150 ppm for 30 days, followed by 120 days of rehabilitation, the morphological picture was similar to that of the air-exposure controls except for changes in cellular and nuclear sizes." The authors did not present any quantitative data on the lesions they describe, especially in terms of dose-response, and most of the qualitative description is for the 150 ppm exposure level in which there are consistent reports of TCE induced body weight decreases in male mice.

Although stating that Kupffer cells were increased in cellular and nuclear size, no differential staining was applied to light microscopy sections and used to distinguish Kupffer from endothelial cells lining the hepatic sinusoid in this study. Without differential staining, such a determination is difficult at the light microscopic level and a question remains as to whether these are the same cells as described by Goel et al. (1992) as a proliferation of sinusoidal endothelial cells after exposures of 1,000 and 2,000 mg/kg-day TCE exposure for 28 days in male Swiss mice. As noted in Section E.2.4.2, the discrepancy in DNA synthesis measures between hepatocyte examinations of individual hepatocytes and whole liver measures in several reports of TCE metabolite exposure, is suggestive of increased DNA synthesis in the nonparenchymal cell compartment of the liver. Thus, nonparenchymal cell proliferation is suggested as an effect of subchronic TCE exposures in mice without concurrent focal necrosis via inhalation studies (Kjellstrand et al., 1983a) and with focal necrosis in the presence of TCE in a groundnut oil vehicle (Goel et al., 1992).

Although Kjellstrand et al. (1983a) did not discuss polyploidization, the changes in cell size and especially the continued change in cell size and nuclear staining characteristics after 120 days of cessation of exposure are consistent with changes in polyploidization induced by TCE that were suggested in studies from shorter durations of exposure (Dees and Travis, 1993; Elcombe et al., 1985) and of longer durations (e.g., Buben and O'Flaherty, 1985). Of note is that in the histological descriptions provided by Kjellstrand et al. (1983a), there was no mention of focal necrosis or apoptosis resulting from these exposures to TCE to mice. Vacuolization is reported and consistent with hepatotoxicity or lipid accumulation, which is lost during routine histological slide preparation. The lack of reported focal necrosis in mice exposed through inhalation is consistent with reports of gavage experiments of TCE in mice that do not use corn oil as the vehicle (Merrick et al., 1989).

Buben and O'Flaherty (1985) reported the effects of TCE via corn oil gavage after 6 weeks of exposure at concentrations ranging from 100 to 3,200 mg/kg-day. This study was conducted with older mice than those generally used in chronic exposure assays (male Swiss-Cox outbred mice between 3 and 5 months of age). Liver weight increases, decreases in liver G6P activity, increases in liver triglycerides, and increases in SGPT activity were examined as

parameters of liver toxicity. Few deaths were reported during the 6-week exposure period except at the highest dose and related to CNS depression. TCE exposure caused dose-related increases in percent liver/body weight with a dose as low as 100 mg/kg-day reported to cause a statistically significant increase (i.e., 112% of control).

The increases in liver size were attributed to hepatocyte hypertrophy, as revealed by histological examination and by a decrease in the liver DNA concentration, and although enlarged, were reported to appear normal. A dose-related trend toward triglyceride concentration was also noted. A dose-related decrease in glucose-6-phophatase activity was reported with similar small decreases (~10%) observed in the TCE exposed groups that did not reach statistical significance until the dose reached 800 mg/kg TCE exposure. SGPT activity was not observed to be increased in TCE-treated mice except at the two highest doses and even at the 2,400 mg/kg dose, half of the mice had normal values. The large variability in SGPT activity was indicative of heterogeneity of this response between mice at the higher exposure levels for this indicator of liver toxicity. Such variability of response in male mice is consistent with the work of Kjellstrand and colleagues. Thus, the results from Buben and O'Flaherty (1985) suggest that hepatomegaly is a robust response that was reported to be observed at the lowest dose tested, dose-related, and not accompanied by overt toxicity.

In terms of histopathology, Buben and O'Flaherty (1985) reported swollen hepatocytes with indistinct borders; their cytoplasm was clumped and a vesicular pattern was apparent and not simply due to edema in TCE-treated male mice. Karyorrhexis (the disintegration of the nucleus) was reported to be present in nearly all specimens from TCE-treated animals and suggestive of impending cell death. It was not present in controls, appeared at a low level at 400 mg/kg TCE exposure level, and appeared to be slightly higher at 1,600 mg/kg TCE exposure level. Central lobular necrosis was present only at the 1,600 mg/kg TCE exposure level and at a very low level. Buben and O'Flaherty (1985) report increased polyploidy in the central lobular region for both 400 and 1,600 mg/kg TCE and described it as hepatic cells having two or more nuclei or enlarged nuclei containing increased amounts of chromatin, but at the lowest level of severity or occurrence. Thus, the results of this study are consistent with those of shorter-term studies via gavage, which report hepatocellular hypertrophy in the centralobular region, increased liver weight induced at the lowest exposure level tested and at a level much lower than those inducing overt toxicity, and that TCE exposure is associated with changes in ploidy.

The NTP 13-week study of TCE gavage exposure in 10 F344/N rats (125–2,000 mg/kg [males] and 62.5–1,000 mg/kg [females]) and in B6C3F_1mice (375–6,000 mg/kg) reported that all rats survived the 13-week study. However, male rat receiving 2,000 mg/kg exhibited a 24% difference in final body weight. The study descriptions of pathology in rats and mice were not very detailed and included only mean liver weights. The rats had increased pulmonary

vasculitis at the highest concentration of TCE and viral titers were positive for Sendai virus. No liver effects were noted for them in the study.

For mice, liver weights (both absolute and percent liver/body weight) were reported to increase in a dose-related fashion with TCE exposure and to be increased by >10% in 750 mg/kg TCE-exposed males and ≥1,500 mg/kg TCE-exposed females. Hepatotoxicity was reported as centrilobular necrosis in 6/10 males and 1/10 females exposed to 6,000 mg/kg TCE and multifocal areas of calcifications scattered throughout 3,000 mg/kg TCE exposed male mice and only a single female 6,000 mg/kg dose, considered to be evidence of earlier hepatocellular necrosis. One female mouse exposed to 3,000 mg/kg TCE also had a hepatocellular adenoma, an extremely rare lesion in female mice of this age (20 weeks). At the lowest dose of exposure, there was a consistent decrease in liver weight in female and male mice after 13 weeks of TCE exposure.

Kawamoto et al. (1988b) exposed rats to 2 g/kg TCE subcutaneously for 15 weeks and reported TCE-induced increases in liver weight. They also reported increase in CYP, cytochrome b-5, and NADPH cytochrome c reductase. The difficulties in relating this route of exposure to more environmentally relevant ones is discussed in Section E.2.2.11.

For 2-year or lifetime studies of TCE exposure, a consistent hepatocarcinogenic response has been observed in mice of differing strains and genders and from differing routes of exposure. However, for rats, some studies have been confounded by mortality from gavage error or the toxicity of the dose of TCE administered. In some studies, a relative insensitive strain of rat has been used. However, in general, it appears that the mouse is more sensitive than the rat to TCE-induced liver cancer. Three studies give results the authors consider to be negative for TCE-induced liver cancer in mice, but have either design and/or reporting limitations, or are in strains and paradigms with apparent low ability for liver cancer induction or detection.

Fukuda et al. (1983) reported a 104-week inhalation bioassay in female Crj:CD-1 (ICR) mice and female Crj:CD (Sprague-Dawley) rats exposed to 0, 50, 150, and 450 ppm TCE (n = 50). There were no reported incidences of mice or rats with liver tumors for controls indicative of relatively insensitive strains used in the study for liver effects. While TCE was reported to induce a number of other tumors in mice and rats in this study, the incidence of liver tumors was <2% after TCE exposure. Of note is the report of cystic cholangioma reported in one group of rats.

Henschler et al. (1980) exposed NMRI mice and WIST random bred rats to 0, 100, and 500 ppm TCE for 18 months (n = 30). This study is limited by short duration of exposure, low number of animals, and low survival in rats. Control male mice were reported to have one HCC and one hepatocellular adenoma with the incidence rate unknown. In the 100 ppm TCE exposed group, two hepatocellular adenomas, and one mesenchymal liver tumor were reported. No liver tumors were reported at any dose of TCE in female mice or controls. For male rats, only one

hepatocellular adenomas at 100 ppm was reported. For female rats no liver tumors were reported in controls, but one adenoma and one cholangiocarcinoma was reported at 100 ppm TCE and at 500 ppm TCE, two cholangioadenomas, a relatively rare biliary tumor, was reported. The difference in survival in mice, did not affect the power to detect a response, as was the case for rats. However, the low number of animals studied, abbreviated exposure duration, and apparently low sensitivity of this paradigm (i.e., no background response in controls) suggests a study of limited ability to detect a TCE carcinogenic liver response. Of note is that both Fukuda et al. (1983) and Henschler et al. (1980) report rare biliary cell derived tumors in rats in relatively insensitive assays.

Van Duuren et al. (1979) exposed mice to 0.5 mg/mouse to TCE via gavage once a week in 0.1 mL trioctanion (n = 30). Inadequate design and reporting of this study limit that ability to use the results as an indicator of TCE carcinogenicity.

The NCI (1976) study of TCE was initiated in 1972 and involved the exposure of Osborne-Mendel rats and $B6C3F_1$ mice to varying concentrations of TCE. The animals were co-exposed to a number of other carcinogens as exhalation as multiples studies and control animals all shared the same laboratory space. Treatment duration was 78 weeks and animals received TCE via gavage in corn oil at two doses (n = 20 for controls, but n = 50 for treatment groups). For rats, the high dose was reported to result in significant mortality (i.e., 47/50 high-dose rats died before scheduled termination of the study). A low incidence of liver tumors was reported for controls and carbon tetrachloride positive controls in rats from this study. In $B6C3F_1$ mice, TCE was reported to increase incidence of HCCs in both doses and both genders of mice (~1,170 and 2,340 mg/kg for males and 870 and 1,740 mg/kg for female mice). HCC diagnosis was based on histologic appearance and metastasis to the lung. The tumors were described in detail and to be heterogeneous "as described in the literature" and similar in appearance to tumors generated by carbon tetrachloride. The description of liver tumors in this study and tendency to metastasize to the lung are similar to descriptions provided by Maltoni et al. (1986) for TCE-induced liver tumors in mice via inhalation exposure.

For male rats, noncancer pathology in the NCI (1976) study was reported to include increased fatty metamorphosis after TCE exposure and angiectasis or abnormally enlarged blood vessels. Angiectasis can be manifested by hyperproliferation of endothelial cells and dilatation of sinusoidal spaces. The authors conclude that due to mortality, "the test is inconclusive in rats." They note the insensitivity of the rat strain used from their data on the positive control of carbon tetrachloride exposure.

The NTP (1990) study of TCE exposure in male and female F344/N rats, and $B6C3F_1$ mice (500 and 1,000 mg/kg for rats and 1,000 mg/kg for mice) was limited in the ability to demonstrate a dose-response for hepatocarcinogenicity. There was also little reporting of non-neoplastic pathology or toxicity and no report of liver weight at termination of the study. However, by the end of a 2-year cancer bioassay, liver tumor induction can be a significant

factor in any changes in liver weight. No treatment-related increases in necrosis in the liver were observed in mice. A slight increase in the incidence of focal necrosis was noted for TCE-exposed male mice (8 vs. 2% in control) with a slight reduction in fatty metamorphosis in treated male mice (0 treated vs. 2 control animals). In female mice, there was a slight increase in focal inflammation (29 vs. 19% of animals) and no other changes. Therefore, this study did not show concurrent evidence of liver toxicity but did show TCE-induced neoplasia after 2 years of TCE exposure in mice. The administration of TCE was reported to cause earlier expression of tumors as the first animals with carcinomas were reported to have them 57 weeks for TCE-exposed animals and 75 weeks for control male mice.

The NTP (1990) study reported that TCE exposure was associated with increased incidence of HCC (tumors with markedly abnormal cytology and architecture) in male and female mice. Hepatocellular adenomas were described as circumscribed areas of distinctive hepatic parenchymal cells with a perimeter of normal appearing parenchyma in which there were areas that appeared to be undergoing compression from expansion of the tumor. Mitotic figures were sparse or absent but the tumors lacked typical lobular organization. HCCs had markedly abnormal cytology and architecture with abnormalities in cytology cited as including increased cell size, decreased cell size, cytoplasmic eosinophilia, cytoplasmic basophilia, cytoplasmic vacuolization, cytoplasmic hyaline bodies, and variations in nuclear appearance. Furthermore, in many instances, several or all of the abnormalities were present in different areas of the tumor and variations in architecture with some of the HCCs having areas of trabecular organization. Mitosis was variable in amount and location. Therefore, the phenotype of tumors reported from TCE exposure was heterogeneous in appearance between and within tumors.

For rats, the NTP (1990) study reported no treatment-related non-neoplastic liver lesions in males and a decrease in basophilic cytological change reported from TCE-exposure in females. The results for detecting a carcinogenic response in rats were considered to be equivocal because both groups receiving TCE showed significantly reduced survival compared to vehicle controls and because of a high rate (e.g., 20% of the animals in the high-dose group) of death by gavage error.

The NTP (1988) study of TCE exposure in four strains of rats to "diisopropylamine-stabilized TCE" was also considered inadequate for either comparing or assessing TCE-induced carcinogenesis in these strains of rats because of chemically induced toxicity, reduced survival, and incomplete documentation of experimental data. TCE gavage exposures of 0, 500, or 1,000 mg/kg-day (5 days/week, for 103 weeks) male and female rats was also marked by a large number of accidental deaths (e.g., for high-dose male Marshal rats, 25 animals were accidentally killed).

Results from a 13-week study were briefly mentioned in the report and indicated that exposure levels of 62.5–2,000 mg/kg TCE were not associated with decreased survival (with the

exception of three male August rats receiving 2,000 mg/kg TCE). Administration of the chemical for 13 weeks was not associated with histopathological changes.

In regard to evidence of liver toxicity, the 2-year study of TCE exposure reported no evidence of TCE-induced liver toxicity described as non-neoplastic changes ACI, August, Marshal, and Osborne-Mendel rats. Interestingly, for the control animals of these four strains, there was, in general, a low background level of focal necrosis in the liver of both genders. In summary, the negative results in this bioassay are confounded by the killing of a large portion of the animals accidently by experimental error but TCE-induced overt liver toxicity was not reported.

Maltoni et al. (1986) reported the results of several studies of TCE via inhalation and gavage in mice and rats. A large number of animals were used in the treatment groups but the focus of the study was detection of a neoplastic response with only a generalized description of tumor pathology phenotype given and limited reporting of non-neoplastic changes in the liver. Accidental death by gavage error was reported not to occur in this study. In regards to effects of TCE exposure on survival, "a nonsignificant excess in mortality" correlated to TCE treatment was observed only in female rats (treated by ingestion with the compound) and in male $B6C3F_1$ mice.

TCE-induced effects on body weight were reported to be absent in mice except for one experiment (BT 306 bis) in which a slight nondose correlated decrease was found in exposed animals. "Hepatoma" was the term used to describe all malignant tumors of hepatic cells, of different subhistotypes, and of various degrees of malignancy, and were reported to be unique or multiple and have different sizes (usually detected grossly at necropsy) from TCE exposure. In regard to phenotype, tumors were described as usual type observed in Swiss and $B6C3F_1$ mice, as well as in other mouse strains, either untreated or treated with hepatocarcinogens and to frequently have medullary (solid), trabecular, and pleomorphic (usually anaplastic) patterns. Swiss mice from this laboratory were reported to have a low incidence of hepatomas without treatment (1%). The relatively larger number of animals used in this bioassay (n = 90–100), in comparison to NTP standard assays, allows for a greater power to detect a response.

TCE exposure for 8 weeks via inhalation at 100 or 600 ppm may have been associated with a small increase in liver tumors in male mice in comparison to concurrent controls during the life span of the animals. In Swiss mice exposed to TCE via inhalation for 78 weeks, there a reported increase in hepatomas associated with TCE treatment that was dose-related in male, but not female, Swiss mice. In $B6C3F_1$ mice exposed via inhalation to TCE for 78 weeks, the results from one experiment indicated a greater increase in liver cancer in females than male mice, but in a second experiment in males, there was a TCE-exposure associated increase in hepatomas. Although the mice were supposed to be of the same strain, the background level of liver cancer was significantly different in male mice. The finding of differences in response in animals of the same strain but from differing sources has also been reported in other studies for

other endpoints (see Section E.3.1.2). However, for both groups of male B6C3F$_1$ mice, the background rate of liver tumors over the lifetime of the mice was <20%.

For rats, there were four liver angiosarcomas reported (one in a control male rat, one each in a TCE-exposed male and female at 600 ppm TCE for 8 weeks, and one in a female rat exposed to 600 ppm TCE for 104 weeks), but the specific results for incidences of hepatocellular "hepatomas" in treated and control rats were not given. Although Maltoni et al. (1986) concluded that the small number of these tumors was not treatment-related, the findings were brought forward because of the extreme rarity of this tumor in control Sprague-Dawley rats, untreated or treated with vehicle materials. In rats treated for 104 weeks, there was no report of a TCE treatment-related increase in liver cancer in rats. This study only presented data for positive findings, so it did not give the background or treatment-related findings in rats for liver tumors in this study. Thus, the extent of background tumors and sensitivity for this endpoint cannot be determined.

Of note is that the Sprague-Dawley strain used in this study was also noted in the Fukuda et al. (1983) study to be relatively insensitive for spontaneous liver cancer and to also be negative for TCE-induced hepatocellular liver cancer induction in rats. However, like the Fukuda et al. (1983) and Henschler et al. (1980) studies, which reported rare biliary tumors in insensitive strains of rat for hepatocellular tumors, Maltoni et al. (1986) reported a relatively rare tumor type, angiosarcoma, after TCE exposure in a relatively insensitive strain for "hepatomas." As noted above, many of the rat studies were limited by premature mortality due to gavage error or premature mortality (NTP, 1990, 1988; Henschler et al., 1980; NCI, 1976), which was reported not occur in Maltoni et al. (1986).

There were other reports of TCE carcinogenicity in mice from chronic exposures that were focused primarily on detection of liver tumors with limited reporting of tumor phenotype or non-neoplastic pathology. Herren-Freund et al. (1987) reported that male B6C3F$_1$ mice given 40 mg/L TCE in drinking water had increased tumor response after 61 weeks of exposure. However, concentrations of TCE fell by about half at this dose of TCE during the twice a week change in drinking water solution, so the actual dose of TCE the animals received was <40 mg/L. The percent liver/body weight was reported to be similar for control and TCE-exposed mice at the end of treatment. Despite difficulties in accurately establishing the dose received, an increase in adenomas per animal and an increase in the number of animals with HCCs were reported to be associated with TCE exposure after 61 weeks of exposure and without apparent hepatomegaly.

Anna et al. (1994) reported tumor incidences for male B6C3F$_1$ mice receiving 800 mg/kg-day TCE via gavage (5 days/week for 76 weeks). All TCE-treated mice were reported to be alive after 76 weeks of treatment. Although the control group contained a mixture of exposure durations (76–134 weeks) and concurrent controls had a very small number

of animals, TCE-treatment appeared to increase the number of animals with adenomas and the mean number of adenomas and carcinomas, but with no concurrent TCE-induced cytotoxicity.

E.2.4.4. Summary of Results for Subchronic and Chronic Effects of DCA and TCA: Comparisons With TCE

There are no similar studies for TCA and DCA conduced at 6 weeks and with the range of concentrations examined in Buben and O'Flaherty (1985) for TCE. In general, many studies of DCA and TCA have been conducted at few and high concentrations, with shortened durations of exposure, and varying and low numbers of animals to examine primarily a liver tumor response in mice. However, the analyses presented in Section E.2.4.2 gives comparisons of administered TCA and DCA dose-responses for liver weight increases for a number of studies in combination as well as comparing such dose-responses to that of TCE and its oxidative metabolism. As stated above, many subchronic studies of DCA and TCA have focused on elucidating a relationship between dose and hypothesized events that may be indicators of carcinogenic potential that have been described in chronic studies with a focus on indicators of peroxisome proliferation and DNA synthesis. Many chronic studies have focused on the nature of the DCA and TCA carcinogenic response in mouse liver through examination of the tumors induced.

Almost all of the chronic studies for DCA and TCA have been carried out in mice. As the database for examination of the ability of TCE to induce liver tumors in rats includes several studies that have been limited in ability determine a carcinogenic response in the liver, the database for DCA and TCA in rats is even more limited. For TCA, the only available study in rats (DeAngelo et al., 1997) has been frequently cited in the literature to indicate a lack of response in this species for TCA-induced liver tumors. Although reporting an apparent dose-related increase in multiplicity of adenomas and an increase in carcinomas over control at the highest dose, DeAngelo et al. (1997) use such a low number of animals per treatment group (n = 20–24) that the abilities of this study to determine a statistically significant increase in tumor response and to be able to determine that there was no treatment-related effect were limited. A power calculation of the study shows that the type II error, which should be >50%, was <8% probability for incidence and multiplicity of all tumors at all exposure TCA concentrations with the exception of the incidence of adenomas and adenomas and carcinomas for 0.5 g/L treatment group (58%) in which there was an increase in adenomas reported over control (15 vs. 4%) that was the same for adenomas and carcinomas combined. Therefore, the designed experiment could accept a false null hypothesis and erroneously conclude that there is no response due to TCA treatment. While suggesting a lower response than for mice for liver tumor induction, it is inconclusive for determination of whether TCA induces a carcinogenic response in the liver of rats.

For DCA, there are two reported long-term studies in rats (DeAngelo et al., 1996; Richmond et al., 1995) that appear to have reported the majority of their results from the same data set and which consequently were subject to similar design limitations and DCA-induced neurotoxicity in this species. DeAngelo et al. (1996) reported increased hepatocellular adenomas and carcinomas in male F344 rats exposed for 2 years. However, the data from exposure concentrations at a 5 g/L dose had to be discarded and the 2.5 g/L DCA dose had to be continuously lowered during the study due to neurotoxicity. There was a DCA-induced increased in adenomas and carcinomas combined reported for the 0.5 g/L DCA (24.1 vs. 4.4% adenomas and carcinomas combined in treated vs. controls) and an increase at a variable dose started at 2.5 g/L DCA and continuously lowered (28.6 vs. 3.0% adenomas and carcinomas combined in treated vs. controls). Only combined incidences of adenomas and carcinomas for the 0.5 g/L DCA exposure group were reported to be statistically significant by the authors, although the incidence of adenomas was 17.2 vs. 4% in treated vs. control rats.

Hepatocellular tumor multiplicity was reported to be increased in the 0.5 g/L DCA group (0.31 adenomas and carcinomas/animal in treated vs. 0.04 in control rats) but was reported by the authors to not be statistically significant. At the starting dose of 2.5 g/L, continuously lowered due to neurotoxicity, the increased multiplicity of HCCs was reported by the authors to be to be statistically significant (0.25 carcinomas/animals vs. 0.03 in control) as well as the multiplicity of combined adenomas and carcinomas (0.36 adenomas and carcinomas/animals vs. 0.03 in control rats).

Issues that affected the ability to determine the nature of the dose-response for this study include: (1) the use of a small number of animals (n = 23, n =21, and n = 23 at final sacrifice for the 2.0 g/L sodium chloride control, 0.05, and 0.5 g/L treatment groups) that limit the power of the study both to determine statistically significant responses and to determine that there are not treatment-related effects (i.e., power); (2) apparent addition of animals for tumor analysis not present at final sacrifice (i.e., 0.05 and 0.5 g/L treatment groups); and (3) most of all, the lack of a consistent dose for the 2.5 g/L DCA exposed animals.

Similar issues were present for the study of Richmond et al. (1995) that was conducted by the same authors as DeAngelo et al. (1996) and appeared to be from the same data set. The Richmond et al. (1995) data for the 2 g/L sodium chloride, 0.05 g/L DCA, and 0.5 g/L DCA exposure groups were the same data set reported by DeAngelo et al. (1996) for these groups. Additional data was reported for F344 rats administered and 2.5 g/L DCA that, due to hind-limb paralysis, were sacrificed 60 weeks (DeAngelo et al., 1996). Tumor multiplicity was not reported by the authors. There was a small difference in reports of the results between the two studies for the same data for the 0.5 g/L DCA group in which Richmond et al. (1995) reported a 21% incidence of adenomas and DeAngelo et al. (1996) reported a 17.2% incidence. The authors did not report any of the results of DCA-induced increases of adenomas and carcinomas to be statistically significant. The same issues discussed above for DeAngelo et al. (1996) apply

to this study. Similar to the DeAngelo study of TCA in rats (DeAngelo et al., 1997) the study of DCA exposure in rats reported by DeAngelo et al. (1996) and Richmond et al. (1995), the use of small numbers of rats limits the detection of treatment-related effects and the ability to determine whether there was no treatment related effects (Type II error), especially at the low concentrations of DCA exposure.

For mice, the data for both DCA and TCA is much more extensive and has shown that both DCA and TCA induced liver tumors in mice. Many of the studies are for relatively high concentrations of DCA or TCA, have been conducted for ≤1 year, and have focused on the nature of tumors induced to ascertain potential modes of action and to make inferences as to whether TCE-induced tumors in mice are similar. As shown previously in Section E.2.4.2, the dose-response curves for increased liver weight for TCE administration in male mice are more similar to those for DCA administration and TCE oxidative metabolism than for direct TCA administration. There are two studies in male B6C3F$_1$ mice that attempt to examine multiple concentrations of DCA and TCA for 2-year studies (DeAngelo et al., 2008; DeAngelo et al., 1999) at doses that do not induce cytotoxicity and attempt to relate them to subchronic changes and peroxisomal enzyme induction. However, the DeAngelo et al. (2008) study was carried out in B6C3F$_1$ mice that were of large size and prone to liver cancer and premature mortality, limiting its use for the determination of TCA-dose response in a 2-year bioassay. One study in female B6C3F$_1$ mice describes the dose-response for liver tumor induction at a range of DCA and TCA concentrations after 51 or 82 weeks (Pereira, 1996) with a focus on the type of tumor each compound produced.

DeAngelo et al. (1999) conducted a study of DCA exposure to determine a dose response for the hepatocarcinogenicity of DCA in male B6C3F$_1$ mice over a lifetime exposure and especially at concentrations that did not illicit cytotoxicity or were for abbreviated exposure durations. DeAngelo et al. (1999) used 0.05, 0.5, 1.0, 2.0, and 3.5 g/L exposure concentrations of DCA in their 100-week drinking water study. The number of animals at final sacrifice was generally low in the DCA treatment groups and variable (i.e., n = 50, n = 33, n = 24, n = 32, n = 14, and n = 8 for control, 0.05, 0.5, 1, 2.0, and 3.5 g/L DCA exposure groups). It was apparent that animals that died unscheduled deaths between weeks 79 and 100 were included in data reported for 100 weeks. Although the authors did not report how many animals were included in the 100-week results, it appeared that the number was no greater than 1 for the control, 0.05, and 0.5 exposure groups and varied between 3 and 7 for the higher DCA exposure groups.

The multiplicity or number of HCCs/animals was reported to be significantly increased over controls in a dose-related manner at all DCA treatments including 0.05 g/L DCA, and a NOEL reported not to be observed by the authors (i.e., 0.28, 0.58, 0.68, 1.29, 2.47, and 2.90 HCCs/animal for control, 0.05, 0.5, 1.0, 2.0, and 3.5 g/L DCA). Between the 0.5 and 3.5 g/L exposure concentrations of DCA, the magnitude of increase in multiplicity was similar

to the increases in magnitude in dose. The incidence of HCCs was reported to be increased at all doses as well, but not reported to be statistically significant at the 0.05 g/L exposure concentration. However, given that the number of mice examined for this response (n = 33), the power of the experiment at this dose was only 16.9% to be able to determine that there was not a treatment-related effect. The authors did not report the incidence or multiplicity of adenomas for the 0.05 g/L exposure group in the study and neither did they report the incidence or multiplicity of adenomas and carcinomas in combination. For the animals surviving from 79 to 100 weeks of exposure, the incidence and multiplicity of adenomas peaked at 1 g/L, while HCCs continued to increase at the higher doses. This would be expected where some portion of the adenomas would either regress or progress to carcinomas at the higher doses.

DeAngelo et al. (1999) reported that peroxisome proliferation was significantly increased at 3.5 g/L DCA only at 26 weeks, not correlated with tumor response, and not increased at either 0.05 or 0.5 g/L treatments. The authors concluded that DCA-induced carcinogenesis was not dependent on peroxisome proliferation or chemically sustained proliferation, as measured by DNA synthesis. DeAngelo et al. (1999) reported not only a dose-related increase in DCA-induced liver tumors, but also a decrease in time-to-tumor associated with DCA exposure at the lowest levels examined. In regards to cytotoxicity, there appeared to be a treatment-related, but not dose-related, increase in hepatocellular necrosis that did not involve most of the liver from 1 to 3.5 g/L DCA exposures for 26 weeks of exposure. By 52 weeks, this effect was diminished with no necrosis observed at the 0.5 g/L DCA treatment for any exposure period.

Hepatomegaly was reported to be absent by 100 weeks of exposure at the 0.05 and 0.5 g/L exposures, while there was an increase in tumor burden reported. However, slight hepatomegaly was present by 26 weeks in the 0.5 g/L group and decreased with time. Not only did the increase in multiplicity of HCCs increase proportionally with DCA exposure concentration after 79–100 weeks of exposure, but so did the increases in percent liver/body weight.

DeAngelo et al. (1999) presented a figure comparing the number of HCCs/animal at 100 weeks compared with the percent liver/body weight at 26 weeks that showed a linear correlation (r^2 = 0.9977), while peroxisome proliferation and DNA synthesis did not correlate with tumor induction profiles. The proportional increase in liver weight with DCA exposure was also reported for shorter durations of exposure as noted in Section E.2.4.2. The findings of the study illustrate the importance of examining multiple exposure levels at lower concentrations, at longer durations of exposure, and with an adequate number of animals to determine the nature of a carcinogenic response. Although Carter et al. (1995) suggested that there is evidence of DCA-induced cytotoxicity (e.g., loss of cell membranes and apparent apoptosis) at higher levels, the 0.5 g/L exposure concentration was shown by DeAngelo et al.

(1999) to increase hepatocellular tumors after 100 weeks of treatment without concurrent peroxisome proliferation or cytotoxicity in mice.

As noted in detail in Section E.2.3.2.13, DeAngelo et al. (2008) exposed male B6C3F$_1$ mice to neutralized TCA in drinking water to male B6C3F$_1$ mice in three studies. Rather than using five exposure levels that were generally twofold apart, as was done in DeAngelo et al. (1999) for DCA, DeAngelo et al. (2008) studied only three doses of TCA that were an order of magnitude apart, which limits the elucidation of the shape of the dose-response curve. In addition, DeAngelo et al. (2008) contained two studies, each conducted in a separate laboratories, for the 104-week data so that the two lower doses were studied in one study and the highest dose in another. The first study was conducted using 2 g/L sodium chloride, or 0.05, 0.5, or 5 g/L TCA in drinking water for 60 weeks (Study #1), while the other two studies were conducted for a period of 104 weeks (Study #2 with 2.5 g/L neutralized acetic acid or 4.5 g/L TCA exposure groups and Study #3 with deionized water, 0.05 and 0.5 g/L TCA exposure groups). In the studies reported in DeAngelo et al. (2008), a small number of animals has been used for the determination of a tumor response (~n = 30 at final necropsy), but for the data for liver weight or PCO activity at interim sacrifices, the number was even smaller (n = 5).

The percent liver/body weight changes at 4 weeks in Study #1 have been included in the analysis for all TCA data in Section E.2.4.2, and are consistent with that data. Although there was a 10-fold difference in TCA exposure concentration, there was a 9, 16, and 35% increase in liver weight over control for the 0.05, 0.5, and 5 g/L TCA exposures. PCO activity varied 2.7-fold as baseline controls, but the increase in PCO activity at 4 weeks was 1.3-, 2.4-, and 5.3-fold of control for the 0.05, 0.5, and 5 g/L TCA exposure groups in Study #1. The incidence data for adenomas observed at 60 weeks was 2.1-, 3.0-, and 5.4-fold of control values and the fold increases in multiplicity were similar after 0.05, 0.5, and 5.0 g/L TCA. Thus, in general, the dose-response for TCA-induced liver weight increases at 4 weeks was similar to the magnitude of induction of adenomas at 60 weeks. Such a result is more consistent with the ability of TCA to induce tumors and increases in liver weight at low doses with little change with increasing dose as shown by this study and the combined data for TCA liver weight induction by administered TCA presented in Section E.2.4.2.

While the 104-week data from Studies #2 and #3 could have been more valuable for determination of the dose-response, as it would have allowed enough time for full tumor expression, serious issues were apparent for Study #3, which was reported to have a 64% incidence rate of adenomas and carcinomas for controls, while that of Study #2 was 12%. As stated in Section E.2.3.2.13, the mice in Study #3 were of larger size than those of either Study #1 or #2 and the large background rate of tumors reported is consistent with mice of these size (Leakey et al., 2003a). However, the large background rate and increased mortality for these mice limit their use for determining the nature of the dose-response for TCA liver carcinogenicity.

Examination of the data for treatment groups shows that there was no difference in any of the results between the 0.5 g/L (Study #3) and 5 g/L (Study #2) TCA exposure groups (i.e., adenoma, carcinoma, and combinations of adenoma and carcinoma incidence and multiplicity) for 104 weeks of exposure. For these same exposure groups, but at 60 weeks of exposure (Study #1), there was a twofold increase in multiplicity for adenomas, and for adenomas and carcinomas combined between the 0.5 and 5.0 g/L TCA exposure groups. At the two lowest doses of 0.05 and 0.5 g/L TCA from Study #3 in the large-tumor prone mice, the differences in the incidences and multiplicities for all tumors were twofold at 104 weeks. These results are consistent with: the two highest exposure levels reaching a plateau of response after a long enough duration of exposure for full expression of the tumors (i.e., ~90% of animals having liver tumors at the 0.5 and 5 g/L exposures) with the additional tumors observed in a tumor-prone paradigm. Thus, without use of the 0.05 and 0.5 g/L TCA data from Study #3, only the 4.5 g/L TCA data from Study #2 can be used for determination of the TCA cancer response in a 2-year bioassay.

To put the 64% incidence data for carcinomas and adenomas reported in DeAngelo et al. (2008) for the control group of Study #3 in context, other studies cited in this review for male $B6C3F_1$ mice show a much lower incidence in liver tumors with: (1) NCI (1976) reporting a colony control level of 6.5% for vehicle and 7.1% incidence of HCCs for untreated male $B6C3F_1$ mice (n = 70–77) at 78 weeks; (2) Herren-Freund et al. (1987) reporting a 9% incidence of adenomas in control male $B6C3F_1$ mice with a multiplicity of 0.09 ± 0.06 and no carcinomas (n = 22) at 61 weeks; (3) NTP (1990) reporting an incidence of 14.6% adenomas and 16.6% carcinomas in male $B6C3F_1$ mice after 103 weeks (n = 48); and (4) Maltoni et al. (1986) reporting that $B6C3F_1$ male mice from the "NCI source" had a 1.1% incidence of "hepatoma" (carcinomas and adenomas) and those from "Charles River Co." had a 18.9% incidence of "hepatoma" during the entire lifetime of the mice (n = 90 per group).

The importance of examining an adequate number of control or treated animals before confidence can be placed in those results in illustrated by Anna et al. (1994), in which at 76 weeks, 3/10 control male $B6C3F_1$ mice that were untreated and 2/10 control animals given corn oil were reported to have adenomas, but from 76 to 134 weeks, 4/32 mice were reported to have adenomas (multiplicity of 0.13 ± 0.06) and 4/32 mice were reported to have carcinomas (multiplicity of 0.12 ± 0.06). Thus, the reported combined incidence of carcinomas and adenomas of 64% reported by DeAngelo et al. (2008) for the control mice of Study #3, is not only inconsistent and much higher than those reported in Studies #1 and #2, but also much higher than reported in a number of other studies of TCE.

Trying to determine a correspondence with either liver weight increases or increases in PCO activity after shorter periods of exposure will be depend on whether data reported in Study #3 in the 104-week studies can be used. DeAngelo et al. (2008) reported a regression analyses that compared "percent of hepatocellular neoplasia," indicated by tumor multiplicity, with TCA

dose, represented by estimations of the TCA dose in mg/kg-day, and with PCO activity for the 60- and 104-week data. Whether adenomas and carcinomas combined or individual tumor type were used in these analysis was not reported by the authors. Concerns arise also from comparing PCO activity at the end of the experiments, when there was already a significant tumor response, rather than at earlier time points. Such PCO data may not be useful as an indicator key event in tumorigenesis when tumors are already present.

In addition, regression analyses of these data are difficult to interpret because of the dose spacing of these experiments as the control and 5 g/L exposure levels will basically determine the shape of the dose-response curve. The 0.05 and 0.5 g/L exposure levels are close to the control value in comparison to the 5 g/L exposure level, the dose response appears to be linear between control and the 5.0 g/L value with the two lowest doses not affect changing the slope of the line (i.e., "leveraging" the regression). Thus, the value of these analyses is limited by: (1) use of data from Study #3 in a tumor-prone mouse that is not comparable to those used in Studies #1 and #2; (2) the appropriateness of using PCO values from later time points and the variability in PCO control values; (3) the uncertainty of the effects of palatability on the 5 g/L TCA results, which were reported in one study to reduce drinking water consumption; and (4) the dose-spacing of the experiment.

DeAngelo et al. (2008) attempted to identify a NOEL for tumorigenicity using tumor multiplicity data and estimated TCA dose. However, it is not an appropriate descriptor for these data, especially given that "statistical significance" of the tumor response is the determinant used by the authors to support the conclusions regarding a dose in which there is no TCA-induced effect. Due to issues related to the appropriateness of use of the concurrent control in Study #3, only the 60-week experiment (i.e., Study #1) is useful for the determination of tumor dose-response. However, there is no allowance for full expression of a tumor response at the 60-week time point. In addition, a power calculation of the 60-week study shows that the type II error, which should be >50% and thus, greater than the chances of "flipping a coin," was 41 and 71% for incidence and 7 and 15% for multiplicity of adenomas for the 0.05 and 0.5 g/L TCA exposure groups. For the combination of adenomas and carcinomas, the power calculation was 8 and 92% for incidence and 6 and 56% for multiplicity at 0.05 and 0.5 g/L TCA exposure. Therefore, the designed experiment could accept a false null hypothesis, especially in terms of tumor multiplicity, at the lower exposure doses and erroneously conclude that there is no response due to TCA treatment.

Pereira (1996) examined the tumor induction in female B6C3F_1 mice and demonstrated that foci, adenoma, and carcinoma development in mice are dependent on duration of exposure, (or period of observation in the case of controls) for full expression of a carcinogenic response. In control female mice, a 360- vs. 576-day observation period showed that at 360 days, no foci or carcinomas and only 2.5% of animals had adenomas, whereas by 576 days of observation, 11% had foci, 2% adenomas, and 2% had carcinomas. For DCA and TCA treatments, foci,

adenomas, and carcinoma incidence and multiplicity did not reach full expression until 82 weeks at the three doses employed (2.58 g/L DCA, 0.86 g/L DCA, 0.26 g/L DCA, 3.27 g/L TCA, 1.1.0 g/L TCA, and 0.33 g/L TCA). Although the numbers of animals were relatively low and variable at the two highest doses (18–28 mice), there were 50–53 mice studied at the lowest dose level and 90 animals studied in the control group.

The results of Pereira (1996) showed that not only were the incidences of mice with foci, adenoma, and carcinomas greatly increased with duration of exposure, but concentration also affected the nature and magnitude of the response in female mice. At 2.86, 0.86, and 0.26 g/L DCA exposures and controls, after 82 weeks, the incidence of adenomas in female B6C3F_1 mice was reported to be 84.2, 25.0, 6.0, and 2.2%, respectively, and carcinomas to be 26.3, 3.6, 0, and 2.2%, respectively. For the multiplicity or number of tumors/animal at these same exposure levels of DCA, the multiplicity was reported to be 5.58, 0.32, 0.06, and 0.02 adenomas/animal, and 0.37, 0.04, 0, and 0.02 carcinomas/animal. Thus, for DCA exposure in female mice, for ~3-fold increases in DCA exposure concentration, after 82 weeks of exposure, there was a similar magnitude of increase in adenomas incidence with much greater increases in multiplicity. For HCC induction, there was no increase in the incidence or multiplicity or carcinomas between the control and 0.33 g/L DCA dose.

At 3.27, 1.10, and 0.33 g/L TCA and controls, after 82 weeks, the incidence of adenomas in female B6C3F_1 mice was reported to be 38.9, 11.1, 7.6, and 2.2%, respectively, and carcinomas to be 27.8, 18.5, 0, and 2.2%, respectively. At these same exposure levels of TCA, the multiplicity was reported to be 0.61, 0.11, 0.08, and 0.02 adenomas/animal, and 0.39, 0.22, 0, and 0.02 carcinomas/animal, respectively. Thus, for TCA, the incidences of adenomas were lower at the two highest doses than DCA and the ~3-fold differences in dose between the two lowest doses only resulted in ~50% increase in incidences of adenomas. For incidence of carcinomas, the ~3-fold difference in dose between the two highest doses only resulted in ~50% increase in carcinoma incidence. A similar pattern was reported for multiplicity after TCA exposure. Foci were also examined and, in general, were similar to adenomas regarding incidence and multiplicity. Thus, the dose-response curve for tumor induction in female mice differed between DCA and TCA after 82 weeks of exposure with TCA having a much less steep dose-response curve than DCA. This is consistent with the pattern of liver weight increases reported for male B6C3F_1 mice in Section E.2.4.2.

DeAngelo et al. (1999) reported a linear increase in incidence and multiplicity of HCCs that was proportional to dose and as well as proportional to the magnitude of liver weight increase from subchronic exposure to DCA. However, the studies of DeAngelo et al. (2008) and Pereira (1996) are suggestive that TCA induced increase in tumor incidence are less proportional to increases in dose as are liver weight increases from subchronic exposure.

Given that TCE subchronic exposure also induced an increase in liver weight that was proportional to dose (i.e., similar to DCA but not TCA), it is of interest as to whether the dose-

response for TCE induced liver cancer in mice was similar. The database for TCE, while consistently showing a induction of liver tumors in mice, is very limited for making inferences regarding the shape of the dose-response curve. For many of these experiments, multiplicity was not given, only liver tumor incidence. NTP (1990), Bull et al. (2002), and Anna et al. (1994) conducted gavage experiments in which they only tested one dose of ~1,000 mg/kg-day TCE. NCI (1976) tested two doses that were adjusted during exposure to an average of 1,169 and 2,339 mg/kg-day in male mice with only twofold dose spacing in only two doses tested. Maltoni et al. (1988) conducted inhalation experiments in two sets of $B6C3F_1$ mice and one set of Swiss mice at three exposure concentrations that were threefold apart in magnitude between the low and mid-dose and twofold apart in magnitude between the mid- and high-dose. However, for one experiment in male $B6C3F_1$ mice, the mice fought and suffered premature mortality and for two the experiments in $B6C3F_1$ mice, although using the same strain, the mice were obtained from differing sources with very different background liver tumor levels.

For the Maltoni et al. (1988) study, a general descriptor of "hepatoma" was used for liver neoplasia rather than describing hepatocellular adenomas and carcinomas so that comparison of that data with those from other experiments is difficult. More importantly, while the number of adenomas and carcinomas may be the same between treatments or durations of exposure, the number of adenomas may decrease as the number of carcinomas increase during the course of tumor progression. Such information is lost by using only a hepatoma descriptor.

Maltoni et al. (1988) did not report an increase over control for 100 ppm TCE for the Swiss group and one of the $B6C3F_1$ groups and only a slight increase (1.12-fold) in the second $B6C3F_1$ group. At 300 ppm TCE exposure, the incidences of hepatoma were 2-fold of control values for the Swiss, 4-fold of control for group of $B6C3F_1$ mice, and 1.6-fold of control for the other group of $B6C3F_1$ mice. At 600 ppm TCE, the incidences of hepatoma were 3.3-fold of control for the Swiss group, 6.1-fold of control for one group of $B6C3F_1$ mice, and 1.2-fold for the other group of $B6C3F_1$ mice. Thus, for each group of TCE exposed mice in the Maltoni et al. (1988) inhalation study, the background levels of hepatomas and the shape of the dose-response curve for TCE-hepatoma induction were variable. However, an average of the increases, in terms of fold of control, between the three experiments gives a ~2.9-fold increase between the low- and mid-dose (100 and 300 ppm) and ~1.4-fold increase between the mid- and high-dose (300 and 600 ppm) groups.

Although such a comparison obviously has a high degree of uncertainty associated with it, it suggests that the magnitude of TCE-induced hepatoma increases over control is similar to the three- and twofold difference in the magnitude of exposure concentrations between these doses. Therefore, the increase in TCE-induced liver tumors would roughly be proportional to the magnitude of exposure dose. This result would be similar to the result for the concordance of the increases in liver weight and exposure concentration observed at 28–42-day exposures to

TCE (see Section E.2.4.2) using oral data from $B6C3F_1$ and Swiss mice, and inhalation data from NMRI mice.

The available inhalation data for TCE-induced liver weight dose-response is from one study in a strain derived from Swiss mice (Kjellstrand et al., 1983a) and was conducted in male and female mice with comparable doses of 75 and 300 ppm TCE. However, male mice of this strain exhibited decreased body weight at the 300 ppm level, which can affect percent liver/body weight increases. The magnitude of TCE-induced increases in liver weight between the 75 and 300 ppm exposures were ~1.80-fold for males (1.50 vs. 1.90-fold of control liver weights) and 4.2-fold for females (1.27- vs. 2.14-fold of control liver weight) in this strain.

Female mice were examined in one study each of Swiss and $B6C3F_1$ mice by Maltoni et al. (1988). Both the Swiss and $B6C3F_1$ mice studies reported increases in incidences of hepatomas over controls only at the 600 ppm TCE level in female mice, indicating less of a response than males. Similarly, the Kjellstrand et al. (1983a) data also showed less of a response in females compared to males in terms TCE induction of liver weight at the 37–150 ppm range of exposure in NMRI strain. While the data for TCE dose-response of liver tumor induction is very limited, it is suggestive of a correlation of TCE-induced increases in liver weight correlating liver tumor induction with a pattern that is dissimilar to that of TCA.

Of those experiments conducted at ~1,000 mg/kg-day gavage dose of TCE in male $B6C3F_1$ mice for at least 79 weeks (Bull et al., 2002; Anna et al., 1994; NTP, 1990; NCI, 1976), the control values were conducted in varying numbers of animals (some as low as n = 15, i.e., (Bull et al., 2002) and with varying results). The incidence of HCCs ranged from 1.2 to 16.7% (Anna et al., 1994; NTP, 1990; NCI, 1976) and the incidence of adenomas ranged from 1.2 to 14.6% (Anna et al., 1994; NTP, 1990) in control $B6C3F_1$ mice. After ~1,000 mg/kg-day TCE treatment, the incidence of carcinomas ranged from 19.4 to 62% (Bull et al., 2002; Anna et al., 1994; NTP, 1990; NCI, 1976) with three of the studies (Anna et al., 1994; NTP, 1990; NCI, 1976) reporting a range of incidences between 42.8 and 62.0%). The incidence of adenomas ranged from 28 to 66.7% (Bull et al., 2002; Anna et al., 1994; NTP, 1990). These data are illustrative of the variability between experiments to determine the magnitude and nature of the TCE response in the same gender (male), strain ($B6C3F_1$), time of exposure (3/4 studies were for 76–79 weeks and 1 was for 2 years duration), and roughly the same dose (800–1,163 mg/kg-day TCE).

Given that the TCE-induced liver response, as measured by liver weight increase, is highly correlated with total oxidative metabolism to a number of agents that are hepatoactive agents and hepatocarcinogens, the variability in response from TCE exposure would be expected to be greater than studies of exposure to a single metabolite such as TCA or DCA.

Caldwell et al. (2008b) and Caldwell and Keshava (2006) have commented on the limitations of experimental paradigms used to study liver tumor induction by TCE metabolites and show that 51-week exposure duration has consistently produced a tumor response for these

chemicals, but with greater lesion incidence and multiplicity at 82 weeks. As reported by DeAngelo et al. (1999) and Pereira (1996), full expression of tumor induction in the mouse does not occur until 78–100 weeks of DCA or TCA exposure, especially at lower concentrations. Thus, use of abbreviated exposure durations and concurrently high exposure concentrations limits the ability of such experiments to detect a treatment-related effect with the occurrence of additional toxicity not necessarily associated with tumor induction. Caldwell et al. (2008b) present a table that shows that the differences in the ability of the studies to detect treatment-related effects could also be attributed to a varying and low number of animals in some exposure groups and that because of the low numbers of animals tested at higher exposures, the power to detect a statistically significant change is very low and, in fact for many of the endpoints, is considerably less than "50% chance." Table E-17 from Caldwell et al. (2008b) illustrates the importance of experimental design and the limitations in many of the studies in the TCE metabolite database.

Table E-17. Power calculations[a] for experimental design described in text, using Pereira and colleagues (1996) as an example

Exposure concentration[b] in female B6C3F$_1$ mice	Number of animals	Power calculation for foci	Power calculation for adenomas	Power calculation for carcinomas
20.0 mmol/L NaCl (control) (82 wks)	90	Null hypothesis	Null hypothesis	Null hypothesis
2.58 g/L DCA (82 wks)	19	0.03	0.03	0.13
0.86 g/L DCA (82 wks)	28	0.74	0.20	0.91
0.26 g/L DCA (82 wks)	50	0.99	0.98	–
3.27 g/L TCA (82 wks)	18	0.15	0.09	0.14
1.10 g/L TCA (82 wks)	27	0.60	0.64	0.3
0.33 g/L TCA (82 wks)	53	0.93	0.91	–

[a]The power calculations represent the probability of rejecting the null hypothesis when, in fact, the alternate hypothesis is true for tumor multiplicity (i.e., the total number of lesions/number of animals). The higher the power number calculated, the more confidence we have in the null hypothesis. Assumptions made included: normal distribution for the fraction of tumors reported, null hypothesis represents what we expected the control tumor fraction to be, the probability of a Type I error was set to 0.05, and the alternate hypothesis was set to 4 times the null hypothesis value.
[b]Conversion of mmol/L to g/L from the original reports of Pereira (1996) and Pereira and Phelps (1996) is as follows: 20.0 mmol/L DCA = 2.58 g/L, 6.67 mmol/L DCA = 0.86 g/L, 2.0 mmol/L = 0.26 g/L, 20.0 mmol/L TCA = 3.27 g/L, 6.67 mmol/L TCA = 1.10 g/L, and 2.0 mmol/L TCA = 0.33 g/L.

Bull et al. (1990) examined male and female B6C3F$_1$ mice (age 37 days) exposed from 15 to 52 weeks to neutralized DCA and TCA (1 or 2 g/L) but tumor data were not suitable for dose response. They reported effects of DCA and TCA exposure on liver weight and percent liver/body changes that gave a pattern of hepatomegaly generally consistent with short-term exposure studies. Only 10 female mice were examined at 52 weeks, but the female mice were

reported to be as responsive as males at the exposure concentration tested. After 37 weeks of treatment and then a cessation of exposure for 15 weeks, liver weights and percent liver/body weight were reported to be elevated over controls, which Bull et al. (1990) partially attribute the remaining increases in liver weight to the continued presence of hyperplastic nodules in the liver.

Macroscopically, livers treated with DCA were reported to have multifocal areas of necrosis and frequent infiltration of lymphocytes on the surface and interior of the liver. For TCA-treated mice, similar necrotic lesions were reported but at such a low frequency that they were similar to controls. Marked cytomegaly was reported from exposure to either 1 or 2 g/L DCA throughout the liver. Cell size was reported to be increased from TCA and DCA treatment with DCA producing the greatest change. The 2 g/L TCA exposures were observed to have increased accumulations of lipofuscin but no quantitative analysis was done. Photographs of light microscopic sections, that were supposed to be representative of DCA- and TCA-treated livers at 2 g/L, showed such great hepatocellular hypertrophy from DCA treatment that sinusoids were obscured. Such a degree of cytomegaly could have resulted in reduction of blood flow and contributed to focal necrosis observed at this level of exposure.

As discussed in Sections E.3.2 and E.3.4.2.1, glycogen accumulation has been described to be present in foci in both humans and animals as a result from exposure to a wide variety of carcinogenic agents and predisposing conditions in animals and humans. Bull et al. (1990) reported that glycogen deposition was uniformly increased from 2 g/L DCA exposure with photographs of TCA exposure showing slightly less glycogen staining than controls. However, the abstract and statements in the paper suggest that there was increased PAS positive material from TCA treatment that has caused confusion in the literature in this regard. Kato-Weinstein et al. (2001) reported that in male B6C3F$_1$ mice exposed to DCA and TCA, the DCA treatment increased glycogen, and TCA decreased glycogen content of the liver by using both chemical measurement of glycogen in liver homogenates and by using ethanol-fixed sections stained with PAS, a procedure designed to minimize glycogen loss. Kato-Weinstein et al. (2001) reported that glycogen rich and poor cells were scattered without zonal distribution in male B6C3F$_1$ mice exposed to 2 g/L DCA for 8 weeks. For TCA treatments, they reported centrilobular decreases in glycogen and ~25% decreases in whole liver by 3 g/L TCA.

Kato-Weinstein et al. (2001) reported whole-liver glycogen to be increased ~1.50-fold of control (90 vs. 60 mg glycogen/g liver) by 2 g/L DCA after 8 weeks exposure in male B6C3F$_1$ mice with a maximal level of glycogen accumulation occurring after 4 weeks of DCA exposure. Pereira et al. (2004a) reported that after 8 weeks of exposure to 3.2 g/L DCA, liver glycogen content was 2.20-fold of control levels (155.7 vs. 52.4 mg glycogen/g liver) in female B6C3F$_1$ mice. Thus, the baseline level of glycogen content reported by (~60 mg/g) and the increase in glycogen after DCA exposure was consistent between Kato-Weinstein et al. (2001) and Pereira et al. (2004a). However, the increase in liver weight reported by Kato-Weinstein et al. (2001) of 1.60-fold of control percent liver/body weight cannot be accounted for by the 1.50-fold of

control glycogen content. Glycogen content only accounts for 5% of liver mass so that 50% increase in glycogen cannot account for the 60% increase liver mass induced by 2 g/L DCA exposure for 8 weeks reported by Kato-Weinstein (2001). Thus, DCA-induced increases in liver weight are occurring from other processes as well.

Carter et al. (2003) and DeAngelo et al. (1999) reported increased glycogen after DCA treatment at much lower doses after longer periods of exposure (100 weeks). Carter et al. (2003) reported increased glycogen at 0.5 g/L DCA and DeAngelo et al. (1999) reported increased glycogen at 0.03 g/L DCA in mice. However, there was no quantitation of that increase.

The issues involving identification of a mode of action through tumor phenotype analysis are discussed in detail below for the more general case of liver cancer as well as for specific hypothesized modes of action (see Sections E.3.1.4, E.3.1.8, E.3.2.1, and E.3.4.1.5). For TCE and its metabolites, c-Jun staining, H-rats mutation, tincture, and heterogeneity in dysplacity have been used to describe and differentiate liver tumors in the mouse.

Bull et al. (2002) reported 1,000 mg/kg TCE administered via gavage daily for 79 weeks in male $B6C3F_1$ mice to produce liver tumors and also reported deaths by gavage error (6/40 animals). The limitations of the experiment are discussed in Caldwell et al. (2008b). Specifically, for the DCA and TCA exposed animals, the experiment was limited by low statistical power, a relatively short duration of exposure, and uncertainty in reports of lesion prevalence and multiplicity due to inappropriate lesions grouping (i.e., grouping of hyperplastic nodules, adenomas, and carcinomas together as "tumors"), and incomplete histopathology determinations (i.e., random selection of gross lesions for histopathology examination).

For the TCE results, a high prevalence (23/36 $B6C3F_1$ male mice) of adenomas and HCC (7/36) was reported. For determinations of immunoreactivity to c-Jun, as a marker of differences in "tumor" phenotype, Bull et al. (2002) included all lesions in most of their treatment groups, decreasing the uncertainty of his findings. However, for immunoreactivity results hyperplastic nodules, adenomas, and carcinomas were grouped and thus, changes in c-Jun expression between the differing types of lesions were not determined.

Bull et al. (2002) reported lesion reactivity to c-Jun antibody to be dependent on the proportion of the DCA and TCA administered after 52 weeks of exposure. Given alone, DCA was reported to produce lesions in mouse liver for which approximately half displayed a diffuse immunoreactivity to a c-Jun antibody, half did not, and none exhibited a mixture of the two. After TCA exposure alone, no lesions were reported to be stained with this antibody. When given in various combinations, DCA and TCA co-exposure induced a few lesions that were only c-Jun+, many that were only c-Jun-, and a number with a mixed phenotype whose frequency increased with the dose of DCA. For TCE exposure of 79 weeks, TCE-induced lesions were reported to also have a mixture of phenotypes (42% c-Jun+, 34% c-Jun-, and 24% mixed) and to be most consistent with those resulting from DCA and TCA co-exposure but not either metabolite alone.

Stauber and Bull (1997) exposed male B6C3F$_1$ mice (7 weeks old at the start of treatment) to 2.0 g/L neutralized DCA or TCA in drinking water for 38 or 50 weeks, respectively, and then exposed (n = 12) to 0, 0.02, 0.1, 0.5, 1.0, and 2.0 g/L DCA or TCA for an additional 2 weeks. Foci and tumors were combined in reported results as "lesions" and prevalence rates were not reported. The DCA-induced larger "lesions" were reported to be more "uniformly reactive to c-Jun and c-Fos" but many nuclei within the lesions displaying little reactivity to c-Jun. Stauber and Bull (1997) stated that while most DCA-induced "lesions" were homogeneously immunoreactive to c-Jun and C-Fos (28/41 lesions), the rest were stained heterogeneously. For TCA-induced lesions, the authors reported no difference in staining between "lesions" and normal hepatocytes in TCA-treated animals. These results are slightly different that those reported by Bull et al. (2002) for DCA, who report c-Jun positive and negative foci in DCA-induced liver tumors but no mixed lesions. Because "lesions" comprised of foci and tumors, different stages of progression reported in these results. The duration of exposures also differed between DCA and TCA treatment groups that can affect phenotype. The shorter duration of exposure can also prevent full expression of the tumor response.

Stauber et al. (1998) presented a comparison of in vitro results with "tumors" from Stauber and Bull (1997) and note that 97.5% of DCA-induced "tumors" were c-Jun+, while none of the TCA-induced "tumors" were c-Jun+. However, the concentrations used to give tumors in vivo for comparison with in vitro results were not reported. This appears to differ from the heterogeneity of result for c-Jun staining reported by Bull et al. (2002) and Stauber and Bull (1997). There was no comparison of c-Jun phenotype for spontaneous tumors with the authors stating that because of such short time, no control tumors results were given. However, the results of Bull et al. (2002) and Stauber and Bull (1997), do show TCA-induced lesions to be uniformly c-Jun negative and thus, the phenotypic marker was able to show that TCE-induced tumors were more like those induced by DCA than TCA.

The premise that DCA induced c-Jun positive lesions and TCA induced c-Jun negative lesions in mouse liver was used as the rationale to study induction of "transformed" hepatocytes by DCA and TCE treatment in vitro. Stauber et al. (1998) isolated primary hepatocytes from 5–8-week-old male B6C3F$_1$ mice (n = 3) and subsequently cultured them in the presence of DCA or TCA. In a separate experiment, 0.5 g/L DCA was given to mice as pretreatment for 2 weeks prior to isolation. The authors assumed that the anchorage-independent growth of these hepatocytes was an indication of an "initiated cell." DCA and TCA solutions were neutralized before use.

After 10 days in culture with DCA or TCA (0, 0.2, 0.5, and 2.0 mM), concentrations of ≥0.5 mM DCA and TCA both induced an increase in the number of colonies that was statistically significant, increased with dose with DCA, and slightly greater for DCA. In a time-course experiment, the number of colonies from DCA treatment in vitro peaked by 10 days and did not change through days 15–25 at the highest dose and, at lower concentrations of DCA,

increased time in culture induced similar peak levels of colony formation by days 20–25 as that reached by 10 days at the higher dose. Therefore, the number of colonies formed was independent of dose if the cells were treated long enough in vitro.

However, not only did treatment with DCA or TCA induce anchorage-independent growth, but untreated hepatocytes also formed larger numbers of colonies with time, although at a lower rate than those treated with DCA. The level reached by untreated cells in tissue culture at 20 days was similar to the level induced by 10 days of exposure to 0.5 mM DCA. The time course of TCA exposure was not tested to see if it had a similar effect with time as did DCA. The colonies observed at 10 days were tested for c-Jun expression with the authors noting that "colonies promoted by DCA were primarily c-Jun positive in contrast to TCA promoted colonies that were predominantly c-Jun negative." Of the colonies that arose spontaneously from tissue culture conditions, 10/13 (76.9%) were reported to be c-Jun+, those treated with DCA 28/34 (82.3%) were c-Jun+, and those treated with TCA 5/22 (22.7%) were c-Jun+. Thus, these data show heterogeneity in cell in colonies, but with more c-Jun+ colonies occurring by tissue culture conditions alone and in the presence of DCA, rather than in the presence of TCA.

The authors reported that with time (24, 48, 72, and 96 hours) of culture conditioning the number of c-Jun+ colonies was increased in untreated controls. The authors reported that DCA treatment delayed the increase in c-Jun+ expression induced by tissue culture conditions alone in untreated controls, while TCA treatment was reported to not affect the increasing c-Jun+ expression that increased with time in tissue culture. These results seems paradoxical given that DCA induced a higher number of colonies at 10 days of tissue culture than TCA and that most of the colonies were c-Jun positive. The number of colonies was greater for pretreatment with DCA, but the magnitude of difference over the control level was the same after DCA treatment in vitro with and without pretreatment. As to the relationship of c-Jun staining and peroxisome proliferators as a class, as pointed out by Caldwell and Keshava (2006), although Bull et al. (2004) have suggested that the negative expression of c-Jun in TCA-induced tumors may be consistent with a characteristic phenotype shown in general by peroxisome proliferators as a class, there is no supporting evidence of this.

An approach to determine the potential modes of action of DCA and TCA through examination of the types of tumors each "induced" or "selected" was to examine H-ras activation (Bull et al., 2002; Ferreira-Gonzalez et al., 1995; Anna et al., 1994; Nelson et al., 1990). This approach has also been used to try to establish an H-ras activation pattern for "genotoxic" and "nongenotoxic" liver carcinogens compounds and to make inferences concerning peroxisome proliferator-induced liver tumors.

However, as noted by Stanley et al. (1994), the genetic background of the mice used and the dose of carcinogen may affect the number of activated H-ras containing tumors that develop. In addition, the stage of progression of "lesions" (i.e., foci vs. adenomas vs. carcinomas) also has been linked the observance of H-ras mutations.

Fox et al. (1990) note that tumors induced by phenobarbital (0.05% drinking water (H_2O), 1 year), chloroform (200 mg/kg corn oil gavage, 2 times weekly for 1 year), or Ciprofibrate (0.0125% diet, 2 years) had a much lower frequency of H-ras gene activation than those that arose spontaneously (2-year bioassays of control animals) or induced with the "genotoxic" carcinogen benzidine-2 hydrochloric acid (HCl; 120 ppm, drinking H_2O, 1 year) in mice. In that study, the term "tumor" was not specifically defined, but a correlation between the incidence of H-ras gene activation and development of either a hepatocellular adenoma or HCC was reported to be made with no statistically significant difference between the frequency of H-ras gene activation in the hepatocellular adenomas and carcinomas. Histopathological examination of the spontaneous tumors, tumors induced with benzidine-2HCL, phenobarbital, and chloroform was not reported to reveal any significant changes in morphology or staining characteristics.

Spontaneous tumors were reported to have 64% point mutation in codon 61 (n = 50 tumors examined) with a similar response for benzidine of 59% (n = 22 tumors examined), whereas for phenobarbital, the mutation rate was 7% (n = 15 tumors examined), chloroform 21% (n = 24 tumors examined), and Ciprofibrate 21% (n = 39 tumors examined). The Ciprofibrate-induced tumors were reported to be more eosinophilic as were the surrounding normal hepatocytes.

Hegi et al. (1993) tested Ciprofibrate-induced tumors in the NIH3T3 cotransfection-nude mouse tumorigenicity assay, which the authors stated is capable of detecting a variety of activated proto-oncogenes. The tumors examined (Ciprofibrate-induced or spontaneously arising) were taken from the Fox et al. (1990) study, screened previously, and found to be negative for H-ras activation. With the limited number of samples examined, Hegi et al. (1993) concluded that ras proto-oncogene activation or activation of other proto-oncogenes using the nude mouse assay were not frequent events in Ciprofibrate-induced tumors and that spontaneous tumors were not promoted with it. Using the more sensitive methods, the H-ras activation rate was reported to be raised from 21 to 31% for Ciprofibrate-induced tumors and from 64 to 66% for spontaneous tumors.

Stanley et al. (1994) studied the effect of methylclofenapate (MCP) (25 mg/kg for up to 2 years), a peroxisome proliferator, in $B6C3F_1$ (relatively sensitive) and C57BL/10J (relatively resistant) mice for H-ras codon 61 point mutations in MCP-induced liver tumors (hepatocellular adenomas and carcinomas). In the $B6C3F_1$ mice, the number of tumors with codon 61 mutations was 11/46 and for C57BL/10J mice 4/31. Unlike the findings of Fox et al. (1990), Stanley et al. (1994) reported an increase in the frequency of mutation in carcinomas, which was reported to be twice that of adenomas in both strains of mice, indicating that stage of progression was related to the number of mutations in those tumors, although most tumors induced by MCP did not have this mutation.

In terms of liver tumor phenotype, Anna et al. (1994) reported that the H-ras codon 61 mutation frequency was not statistically different in liver tumors from DCA and TCE-treated

mice from a highly variable number of tumors examined. In regard to mutation spectra in H-ras oncogenes in control or spontaneous tumors, the patterns were slightly different but mostly similar to that of DCA-induced tumors (0.5% in drinking water). From their concurrent controls they reported that H-ras codon 61 mutations in 17% (n = 6) of adenomas and 100% (n = 5) of carcinomas. For historical controls (published and unpublished), they reported mutations in 73% (n = 33) of adenomas and mutations in 70% (n = 30) of carcinomas. For tumors from TCE treated animals, they reported mutations in 35% (n = 40) of adenomas and 69% (n = 36) of carcinomas, while for DCA-treated animals, they reported mutations in 54% (n = 24) of adenomas and in 68% (n = 40) of carcinomas. Anna et al. (1994) reported more mutations in TCE-induced carcinomas than adenomas.

The study of Ferreira-Gonzalez et al. (1995) in male B6C3F_1 mice has the advantage of comparison of tumor phenotype at the same stage of progression (HCC), for allowance of the full expression of a tumor response (i.e., 104 weeks), and an adequate number of spontaneous control lesions for comparison with DCA or TCA treatments. However, tumor phenotype at an endstage of tumor progression reflects of tumor progression and not earlier stages of the disease process. In spontaneous liver carcinomas, 58% were reported to show mutations in H-61 as compared with 50% of tumor from 3.5 g/L DCA-treated mice and 45% of tumors from 4.5.g/L TCA-treated mice. Thus, there was a heterogeneous response for this phenotypic marker for the spontaneous, DCA-, and TCA-treatment induced HCCs and not a pattern of reduced H-ras mutation reported for a number of peroxisome proliferators.

A number of peroxisome proliferators have been reported to have a much smaller mutation frequency than spontaneous tumors (e.g., 13–24% H-ras codon 61 mutations after Methylclofenopate depending on mouse strain, Stanley et al. (1994)): 21–31% for Ciprofibrate-induced tumors and 64–66% for spontaneous tumors, Fox et al. (1990) and Hegi et al. (1993).

Bull (2000) suggested that "the report by Anna et al. (1994) indicated that TCE-induced tumors possessed a different mutation spectra in codon 61 of the H-ras oncogene than those observed in spontaneous tumors of control mice." Bull (2000) stated that "results of this type have been interpreted as suggesting that a chemical is acting by a mutagenic mechanism" but went on to suggest that it is not possible to a priori rule out a role for selection in this process and that differences in mutation frequency and spectra in this gene provide some insight into the relative contribution of different metabolites to TCE-induced liver tumors. Bull (2000) noted that data from Anna et al. (1994), Ferreira-Gonzalez et al. (1995), and Maronpot et al. (1995a) indicated that mutation frequency in DCA-induced tumors did not differ significantly from that observed in spontaneous tumors. Bull (2000) also noted that the mutation spectra found in DCA-induced tumors has a striking similarity to that observed in TCE-induced tumors, and DCA-induced tumors were significantly different than that of TCA-induced liver tumors.

Bull et al. (2002) reported that mutation frequency spectra for the H-ras codon 61 in mouse liver "tumors" induced by TCE (n = 37 tumors examined) to be significantly different

than that for TCA (n = 41 tumors examined), with DCA-treated mice tumors giving an intermediate result (n = 64 tumors examined). In this experiment, TCA-induced "tumors" were reported to have more mutations in codon 61 (44%) than those from TCE (21%) and DCA (33%). This frequency of mutation in the H-ras codon 61 for TCA is the opposite pattern as that observed for a number of peroxisome proliferators in which the number of mutations at H-ras 61 in tumors has been reported to be much lower than spontaneously arising tumors (see Section E.3.4.1.5). Bull et al. (2002) noted that the mutation frequency for all TCE, TCA, or DCA tumors was lower in this experiment than for spontaneous tumors reported in other studies (they had too few spontaneous tumors to analyze in this study), but that this study utilized lower doses and was of shorter duration than that of Ferreira-Gonzalez et al. (1995). These are concerns in addition to the effects of lesion grouping in which a lower stage of progression is grouped with more advanced stages. In a limited subset of tumors that were both sequenced and characterized histologically, only 8 of 34 (24%) TCE-induced adenomas but 9/15 (60%) of TCE-induced carcinomas were reported to have mutated H-ras at codon 61, which the authors suggest is evidence that this mutation is a late event.

Thus, in terms of H-ras mutation, the phenotype of TCE-induced tumors appears to be more like DCA-induced tumors (which are consistent with spontaneous tumors), or those resulting from a co-exposure to both DCA and TCA (Bull et al., 2002), than from those induced by TCA. As noted above, Bull et al. (2002) reported the mutation frequency spectra for the H-ras codon 61 in mouse liver tumors induced by TCE to be significantly different than that for TCA, with DCA-treated mice tumors giving an intermediate result and for TCA-induced tumors to have a H-ras profile that is the opposite than those of a number of other peroxisome proliferators. More importantly, these data suggest that using measures, other than dysplasticity and tincture, mouse liver tumors induced by TCE are heterogeneous in phenotype.

With regard to tincture, Stauber and Bull (1997) reported the for male $B6C3F_1$ mice, DCA-induced "lesions" contained a number of smaller lesions that were heterogeneous and more eosinophilic with larger "lesions" tending to less numerous and more basophilic. For TCA results using this paradigm, the "lesions" were reported to be less numerous, more basophilic, and larger than those induced by DCA.

Carter et al. (2003) used tissues from the DeAngelo et al. (1999) study and examined the heterogeneity of the DCA-induced lesions and the type and phenotype of preneoplastic and neoplastic lesions pooled across all time points. Carter et al. (2003) examined the phenotype of liver tumors induced by DCA in male $B6C3F_1$ mice and the shape of the dose-response curve for insight into its mode of action. They reported a dose-response of histopathologic changes (all classes of premalignant lesions and carcinomas) occurring in the livers of mice from 0.05 to 3.5 g/L DCA for 26–100 weeks and suggest that foci and adenomas demonstrated neoplastic progression with time at lower doses than observed DCA genotoxicity. Preneoplastic lesions

were identified as eosinophilic, basophilic, and/or clear cell (grouped with clear cell and mixed cell) and dysplastic.

Altered foci were 50% eosinophilic with about 30% basophilic. As foci became larger and evolved into carcinomas, they became increasingly basophilic. The pattern held true throughout the exposure range. There was also a dose and length of exposure related increase in atypical nuclei in "noninvolved" liver. Glycogen deposition was also reported to be dose-dependent with periportal accumulation at the 0.5 g/L exposure level. Carter et al. (2003) suggested that size and evolution into a more malignant state are associated with increasing basophilia, a conclusion consistent with those of Bannasch (1996) and that there is a greater periportal location of lesions suggestive as the location from which they arose.

Consistent with the results of DeAngelo et al. (1999), Carter et al. (2003) reported that DCA (0.05–3.5 g/L) increased the number of lesions per animal relative to animals receiving distilled water, shortened the time to development of all classes of hepatic lesions, and that the phenotype of the lesions was similar to those spontaneously arising in controls. Along with basophilic and eosinophilic lesions or foci, Carter et al. (2003) concluded that DCA-induced tumors also arose from isolated, highly dysplastic hepatocytes in male B6C3F_1 mice chronically exposed to DCA, suggesting another direct neoplastic conversion pathway other than through eosinophilic or basophilic foci.

Rather than male B6C3F_1 mice, Pereira (1996) studied the dose-response relationship for the carcinogenic activity of DCA and TCA and characterized their lesions (foci, adenomas, and carcinomas) by tincture in females (the generally less sensitive gender). Like the studies of TCE by Maltoni et al. (1986), female mice were also reported to have increased liver tumors after TCA and DCA exposures. Pereira (1996) pooled lesions for phenotype analyses so the effect of duration of exposure could not be determined, nor could adenomas be separated from carcinomas for "tumors."

However, as the concentration of DCA was decreased, the number of foci was reported by Pereira (1996) to be decreased but the phenotype of the foci to go from primarily eosinophilic foci (i.e., ~95% eosinophilic at 2.58 g/L DCA) to basophilic foci (~57% eosinophilic at 0.26 g/L). For TCA, the number of foci was reported to ~40 basophilic and ~60 eosinophilic regardless of dose. Spontaneously occurring foci were more basophilic by a ratio of 7/3. Pereira (1996) described the foci of altered hepatocytes and tumors induced by DCA in female B6C3F_1 mice to be eosinophilic at higher exposure levels but at lower or intermittent exposures to be half eosinophilic and half basophilic. Regardless of exposure level, half of the TCA-induced foci were reported to be half eosinophilic and half basophilic with tumors 75% basophilic. In control female mice, the limited numbers of lesions were mostly basophilic, with most of the rest being eosinophilic with the exception of a few mixed tumors. The limitations of descriptions tincture and especially for inferences regarding peroxisome proliferator from the description of "basophilia" is discussed in Section E.3.4.1.5.

The results appear to differ between male and female $B6C3F_1$ mice in regard to tincture for DCA and TCA at differing doses. What is apparent is that the tincture of the lesions is dependent on the stage of tumor progression, agent (DCA or TCA), gender, and dose. Also what is apparent from these studies is the both DCA and TCA are heterogeneous in their tinctoral characteristics as well as phenotypic markers such as mutation spectra or expression of c-Jun.

The descriptions of TCE-induced tumors in mice reported by the NCI, NTP, and Maltoni et al. studies are also consistent with phenotypic heterogeneity as well as consistency with spontaneous tumor morphology (see Section E.3.4.1.5). As noted in Section E.3.1, HCCs observed in humans are also heterogeneous. For mice, Maltoni et al. (1986) described malignant tumors of hepatic cells to be of different subhistotypes, and of various degrees of malignancy and were reported to be unique or multiple, and have different sizes (usually detected grossly at necropsy) from TCE exposure. In regard to phenotype, tumors were described as usual type observed in Swiss and $B6C3F_1$ mice, as well as in other mouse strains, either untreated or treated with hepatocarcinogens and to frequently have medullary (solid), trabecular, and pleomorphic (usually anaplastic) patterns.

For the NCI (1976) study, the mouse liver tumors were described in detail and to be heterogeneous "as described in the literature" and similar in appearance to tumors generated by carbon tetrachloride. The description of liver tumors in this study and tendency to metastasize to the lung are similar to descriptions provided by Maltoni et al. (1986) for TCE-induced liver tumors in mice via inhalation exposure.

The NTP (1990) study reported TCE exposure to be associated with increased incidence of HCC (tumors with markedly abnormal cytology and architecture) in male and female mice. Hepatocellular adenomas were described as circumscribed areas of distinctive hepatic parenchymal cells with a perimeter of normal appearing parenchyma in which there were areas that appeared to be undergoing compression from expansion of the tumor. Mitotic figures were sparse or absent but the tumors lacked typical lobular organization. HCCs were reported to have markedly abnormal cytology and architecture with abnormalities in cytology cited as including increased cell size, decreased cell size, cytoplasmic eosinophilia, cytoplasmic basophilia, cytoplasmic vacuolization, cytoplasmic hyaline bodies, and variations in nuclear appearance. Furthermore, in many instances, several or all of the abnormalities were reported to be present in different areas of the tumor and variations in architecture with some of the HCCs having areas of trabecular organization. Mitosis was variable in amount and location. Therefore, the phenotype of tumors reported from TCE exposure was heterogeneous in appearance between and within tumors from all three of these studies.

Caldwell and Keshava (2006) reported:

> that Bannasch (2001) and Bannasch et al. (2001) describe the early phenotypes of preneoplastic foci induced by many oncogenic agents (DNA-reactive chemicals,

radiation, viruses, transgenic oncogenes and local hyperinsulinism) as insulinomimetic. These foci and tumors have been described by tincture as eosinophilic and basophilic and to be heterogeneous.

The tumors derived from them after TCE exposure are consistent with the description for the main tumor lines of development described by Bannasch et al. (2001) (see Section E.3.4.1.5). Thus, the response of liver to DCA (glycogenosis with emergence of glycogen poor tumors) is similar to the progression of preneoplastic foci to tumors induced from a variety of agents and conditions associated with increased cancer risk.

Furthermore, Caldwell and Keshava (2006) noted that Bull et al. (2002) reported expression of insulin receptor (IR) to be elevated in tumors of control mice or mice treated with TCE, TCA, and DCA but not in nontumor areas, suggesting that this effect is not specific to DCA.

There is a body of literature that has focused on the effects of TCE and its metabolites after rats or mice have been exposed to "mutagenic" agents to "initiate" hepatocarcinogenesis and this is discussed in Section E.4.2, below. TCE and its metabolites were reported to affect tumor incidence, multiplicity, and phenotype when given to mice as a co-exposure with a variety of "initiating" agents and with other carcinogens. Pereira and Phelps (1996) reported that MNU alone induced basophilic foci and adenomas. MNU and low concentrations of DCA or TCA in female mice were reported to induce heterogeneous for foci and tumor with a higher concentration of DCA inducing more eosinophilic and a higher concentration of TCA inducing more tumors that were basophilic. Pereira et al. (2001) reported that not only dose, but also gender affected phenotype in mice that had already been exposed to MNU and were then exposed to DCA. As for other phenotypic markers, Lantendresse and Pereira (1997) reported that exposure to MNU and TCA or DCA induced tumors that had some commonalities and were heterogeneous, but for female mice, were overall different between DCA and TCA as co-exposures with MNU.

Stop experiments, which attempt to ascertain whether progression differences exist between TCA and DCA, have used higher concentrations at much lower durations of exposure. A question arises as to whether the differences in results occurred because animals in which treatment was suspended were not allowed to have full expression of response rather than "progression" as well as the effects of using large doses.

After 37 weeks of treatment and then a cessation of exposure for 15 weeks, Bull et al. (1990) reported that liver weight and percent liver/body weight still was statistically significantly elevated after DCA or TCA treatment. The authors partially attribute the remaining increases in liver weight to the continued presence of hyperplastic nodules in the liver. In terms of liver tumor induction, the authors stated that "statistical analysis of tumor incidence employed a general linear model ANOVA with contrasts for linearity and deviations from linearity to

determine if results from groups in which treatments were discontinued after 37 weeks were lower than would have been predicted by the total dose consumed."

The multiplicity of tumors observed in male mice exposed to DCA or TCA at 37 weeks and then sacrificed at 52 weeks were reported by the authors to have a response in animals that received DCA very close to that which would be predicted from the total dose consumed by these animals. The response to TCA was reported by the authors to deviate significantly ($p = 0.022$) from the linear model predicted by the total dose consumed. Multiplicity of lesions per mouse and not incidence was used as the measure. Most importantly, the data used to predict the dose response for "lesions" used a different methodology at 52 weeks than those at 37 weeks. Not only were not all animal's lesions examined, but foci, adenomas, and carcinomas were combined into one measure. Therefore, foci, of which a certain percentage have been commonly shown to spontaneously regress with time, were included in the calculation of total "lesions."

Pereira and Phelps (1996) note that in MNU-treated mice that were then treated with DCA, the yield of altered hepatocytes decreases as the tumor yields increase between 31 and 51 weeks of exposure, suggesting progression of foci to adenomas. Initiated and noninitiated control mice were reported to also have fewer foci/mouse with time. Because of differences in methodology and the lack of discernment between foci, adenomas, and carcinomas for many of the mice exposed for 52 weeks, it is difficult to compare differences in composition of the "lesions" after cessation of exposure in the Bull et al. (1990) study.

For TCA treatment, the number of animals examined for determination of which "lesions" were foci, adenomas, and carcinomas was 11/19 mice with "lesions" at 52 weeks, while all 4 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were examined. For DCA treatment, the number of animals examined was only 10/23 mice with "lesions" at 52 weeks, while all 7 mice with lesions after 37 weeks of exposure and 15 weeks of cessation were examined. Most importantly, when lesions were examined microscopically, they did not all turn out to be preneoplastic or neoplastic. Two lesions appeared "to be histologically normal" and one necrotic. Not only were a smaller number of animals examined for the cessation exposure than continuous exposure, but only the 2 g/L exposure levels of DCA and TCA were studied for cessation. The number of animals bearing "lesions" after 37 weeks and then 15 cessation weeks was 7/11 (64%), while the number of animals bearing lesions at 52 weeks was 23/24 (96%) after 2 g/L DCA exposure. For TCA, the number of animals bearing lesions at 37 weeks and then 15 weeks cessation was 4/11 (35%), while the number of animals bearing lesions at 52 weeks was 19/24 (80%). While suggesting that cessation of exposure diminished the number of "lesions," conclusions regarding the identity and progression of those lesion with continuous vs. noncontinuous DCA and TCA treatment are tenuous.

E.2.5. Studies of CH

Given that total oxidative metabolism appears to be highly correlated with TCE-induced increases in liver weight in the mouse rather than merely the presence of TCA, other metabolites are of interest as potential agents mediating the effects observed for TCE. Recently, Caldwell and Keshava (2006) provided a synopsis of the results of more recent studies involving CH. A large fraction of TCE oxidative metabolism appears to go through CH, with subsequent metabolism to TCA and TCOH (Chiu et al., 2006b). Merdink et al. (2008) demonstrated that CH administered to humans can be extremely variable and complex in its pharmacokinetic behavior with a peak plasma concentration of CH in plasma 40–50 times higher than observed at the same time interval for other subjects. Studies of CH toxicity in rodents are consistent, with the general presumption that oxidative metabolites are important for TCE-induced liver tumors, but whether CH and its metabolites are sufficient to explain all of the TCE liver tumorigenesis remains unclear, particularly because of uncertainties regarding how DCA may be formed (Chiu et al., 2006b). Studies of CH may enable a comparison between toxicity of TCE and CH and may help elucidate its role in TCE effects. As with other TCE metabolites, the majority of the studies have focused on the mouse liver tumor response. For rats, while the limited data suggest that there is less of a response than mice to CH, those studies are limited in power or reporting.

Daniel et al. (1992) exposed adult male B6C3F_1 (C57B1/6jC male mice bred to C3Heb/Fej female mice) 28-day-old mice to CH, 2-chloroacetaldehyde, or DCA in two different phases (I and II) with initial weights ranging from 9.4 to 13.6 g. The test compounds were buffered and administered in drinking water for 30 and 60 weeks (n = 5 for interim sacrifice), and for 104 weeks (n = 40). The concentration of CH was 1 g/L and the concentration of DCA was 0.5 g/L; the estimated doses of DCA were 85, 93, and 166 mg/kg-day for the DCA group I, DCA group II, and CH exposed group, respectively. Microscopic examination of tissues was conducted for all tissues for five animals of the CH groups with liver, kidneys, testes, and spleen, in addition to all gross lesions, reported to be examined microscopically in all of the 104-week survivors.

The initial body weight for drinking water controls was reported to be 12.99 ± 3.04 g for group I (n = 23) and 10.48 ± 1.70 for group II (n = 10). For DCA-treated animals, initial body weights were 13.44 ± 2.57 g for group I (n = 23) and 9.65 ± 2.72 g for group II (n = 10). For the CH-treated group, the initial body weights were reported to be 10.42 ± 2.49 g (n = 40). It is not clear from the report what control group best matched, if any, the CH group. Thus, the mean initial body weights of the groups as well as the number of animals varied considerably in each group (i.e., ~40% difference in mean body weights at the beginning of the study).

The number of animals surviving until the termination of the experiment was 10, 10, 16, 8, and 24 for the control group I, control group II, DCA group I, DCA group II, and CH groups, respectively. An increase in absolute and relative liver weight was reported to be observed at 30 weeks for DCA and CH groups and at 60 weeks for CH but data were not shown in the study.

At 104 weeks, the data for the surviving control groups were combined as was the data for the two DCA treatment groups. Of note was that for CH treated survivors (n = 24), water consumption was significantly reduced in comparison to controls. Absolute liver weight was reported to be 2.09 ± 0.6, 3.17 ± 1.3, and 2.87 ± 1.1 g for control, DCA, and CH treatment groups, respectively. The % liver to body weight was reported to be similarly elevated (1.57-fold of control for DCA and 1.41-fold of control for CH) at 104 weeks.

At 104 weeks, the treatment-related liver lesions in histological sections were reported to be most prominently hepatocytomegaly and vacuolization in DCA-treated animals. Cytomegaly was also reported to be in 5, 92, and 79% of control, DCA, and CH treatment groups, respectively. Cytomegaly in CH-treated mice was described as minimal and associated with an increased number of basophilic granules (rough endoplasmic reticulum). Hepatocellular necrosis and chronic active inflammation were reported to be mildly increased in both prevalence and severity in all treated groups. The histological findings, from interim sacrifices (n = 5), were considered by the authors to be unremarkable and were not reported.

Liver tumors were increased by DCA and CH treatment. The percent incidence of liver carcinomas and adenomas combined in the surviving animals was 15, 75, and 71% in control, DCA, and CH treated mice, respectively. In the CH-treated group, the incidence of HCC was 46%. The number of tumors/animals was also significantly increased with CH treatment. Most importantly, morphologically, the authors noted that there did not appear to be any discernable differences in the visual appearance of the DCA- and CH-induced tumors.

George et al. (2000) exposed male B6C3F$_1$ mice and male F344/N rats to CH in drinking water for 2 years (up to 162.6 mg/kg-day). Target drinking water concentrations were 0, 0.05, 0.5, and 2 g/L CH in rats and 0, 0.05, 0.5, and 1.0 g/L CH in mice. Groups of animals (n = 6/group) were sacrificed at 13 (rats only), 26, 52, and 78 weeks following the initiation of dosing with terminal sacrifices at week 104. A complete pathological examination was performed on five rats and mice from the high-dose group, with examination primarily of gross lesions except for liver, kidney, spleen, and testes. BrdU incorporation was measured in the interim sacrifice groups in rats and mice with PCO examined at 26 weeks in mice. In rats, the number of animals surviving >78 weeks and examined for hepatocellular proliferative lesions was 42, 44, 44, and 42 for the control, 7.4, 37.4, and 163.6 mg/kg-day CH treatment groups, respectively. Only 32, 36, 35, and 32 animals were examined at the final sacrifice time.

Only the lowest treatment group had increased liver tumors, which were marginally significantly increased by treatment. The percent of animals with hepatocellular adenomas and carcinomas was reported to be 2.4, 14.3, 2.3 and 6.8% in male rats. In mice, preneoplastic foci and adenomas were reported to be increased in the livers of all CH treatment groups (13.5–146.6 mg/kg-day) at 104 weeks. The incidences of adenomas were reported to be statistically increased at all dose levels, the incidences of carcinomas significantly increased at the highest dose, and time-to-tumor decreased in all CH-treatment groups. The percent incidence of

hepatocellular adenomas was reported to be 21.4, 43.5, 51.3, and 50% in control, 13.5, 65.0, and 146.6 mg/kg-day treatment groups, respectively. The percent incidence of HCCs was reported to be 54.8, 54.3, 59.0, and 84.4% in these same groups. The resulting percent incidence of hepatocellular adenomas and carcinomas was reported to be 64.3, 78.3, 79.5, and 90.6%.

The number of mice surviving >78 weeks was reported to be 42, 46, 39, and 32 and the number surviving to final sacrifice was 34, 42, 31, and 25 for control, 13.5, 65.0, and 146.56 mg/kg-day, respectively. CH exposure was reported to not alter serum chemistry, hepatocyte proliferation (i.e., DNA synthesis), or hepatic PCO activity (an enzyme associated with PPARα agonism) in rats and mice at any of the time periods monitored (all interim sacrifice periods for BrdU incorporation, 52 or 78 weeks for serum enzymes, and 26 weeks for PCO) with the exception of 0.58 g/L CH at 26 weeks slightly increasing hepatocyte labeling (~2–3-fold increase over controls) in rats and mice, but the percent labeling still represented ≤3% of hepatocytes.

With regard to other carcinogenic endpoints, only five animals were examined at the high dose, thereby limiting the study's power to determine an effect. Control mice were reported to have a high spontaneous carcinoma rate (54%), thereby limiting the ability to detect a treatment-related response. No descriptions of the foci or tumor phenotype were given. However, of note is the lack of induction of PCO response with CH at 26 weeks of administration in either rats or mice.

Leakey et al. (2003b) studied the effects of CH exposure (0, 25, 50, and 100 mg/kg, 5 days/week for 104–105 weeks via gavage) in male B6C3F_1 mice with dietary control used to manipulate body growth (n = 48 for 2-year study and n = 12 for the 15-month interim study). Dietary control was reported to decrease background liver tumor rates (incidence of 15–20%) and was reported to be associated with decreased variation in liver-to-body weight ratios, thereby potentially increasing assay sensitivity. In dietary-controlled groups and groups fed ad libitum, liver adenomas and carcinomas (combined) were reported to be increased with CH treatment. With dietary restriction, there was a more discernable CH tumor-response with overall tumor incidence reduced, and time-to-tumor increased by dietary control in comparison to ad-libitum-fed mice. Incidences of hepatocellular adenoma and carcinoma overall rates were reported to be 33, 52, 49, and 46% for control, 25, 50, and 100 mg/kg ad-libitum-fed mice, respectively. For dietary-controlled mice, the incidence rates were reported to be 22.9, 22.9, 29.2, and 37.5% for controls, 25, 50, and 100 mg/kg CH, respectively. Body weights were matched and carefully controlled in this study.

After 2 years of CH treatment, the heart weights of ad-libitum-fed male mice administered 100 mg/kg CH were reported to be significantly less and kidney weights of the 50 and 100 mg/kg were less than vehicle controls. No other significant organ weight changes due to CH treatment were reported to be observed in either diet group except for liver. The liver weights of CH treated groups for by dietary groups were reported to be increased at 2 years and

the absolute liver weights of dosed groups to be generally increased at 15 months, with percent liver/body weight ratios increased in CH treated dietary-controlled mice at 15 months. There was 1.0-, 0.87-, and 1.08-fold of control percent liver/body weight for ad-libitum-fed mice exposed to 25, 50, and 100 mg/kg CH, respectively. For dietary-controlled mice, there was 1.05-, 1.08-, and 1.11-fold of control percent liver/body weight for the same dose groups at 15 months. Thus, there was no corresponding dose-response for percent liver/body weight in the ad-libitum-fed mice, which were reported to show a much larger variation in liver-to-body-weight ratios (i.e., the SD and SEs were 2–17-fold lower in dietary-controlled groups than for ad-libitum-fed groups).

Liver weight increases at 15 months did not correlate with 2-year tumor incidences with this group. However, for dietary-controlled groups, the increase in percent liver/body weights at 15 months were generally correlated with increases in liver tumors at 2 years.

The incidences of peripheral or focal fatty change were reported to be increased in all CH-treated groups of ad-libitum-fed mice at 15 months (approximately half the animals showed these changes for all dose groups, with no apparent dose-response). Of the enzymes associated with PPARα agonism (total CYP, CYP2B isoform, CYP4A, or lauric acid β-hydroxylase activity), only CYP4A and lauric acid β-hydroxylase activity were significantly increased at 15 months of exposure in the dietary-restricted group administered 100 mg/kg CH, with no other groups reported showing a statistically significant increased response (n = 12/group). Although not statistically significant, the 100 mg/kg CH exposure group of ad-libitum-fed mice also had an increase in CYP4A and lauric acid β-hydroxylase activity.

The authors reported that the increase in magnitude of CYP4A and lauric acid β-hydroxylase activity at 100 mg/kg CH at 15 months in dietary controlled mice correlated with the increase incidence of mice with tumors. However, there was no correlation of tumor incidence and the increased enzyme activity associated with peroxisome proliferation in the ad-libitum-fed mice. No descriptions of liver pathology were given other than incidence of mice with fatty liver changes. Hepatic malondialdehyde concentration in ad-libitum-fed and dietary controlled mice did not change with CH exposure at 15 months, but the dietary-controlled groups were all approximately half that of the ad-libitum-fed mice. Thus, while overall increased tumors observed in the ad libitum diet correlated with increased malondialdehyde concentration, there was no association between CH dose and malondialdehyde induction for either diet.

Induction of peroxisome-associated enzyme activities was also reported for shorter times of CH exposure. Seng et al. (2003) described CH toxicokinetics in mice at doses up to 1,000 mg/kg-day for 2 weeks with dietary control and caloric restriction slightly reducing acute toxicity. Lauric acid β-hydroxylase and PCO activities were reported to be induced only at doses >100 mg/kg in all groups, with dietary-restricted mice showing the greatest induction. Differences in serum levels of TCA, the major metabolite remaining 24 hours after dosing, were reported not to correlate with hepatic lauric acid β-hydroxylase activities across groups.

Leuschner and Beuscher (1998) examined the carcinogenic effects of CH in male and female Sprague-Dawley rats (69–79 g, 25–29 days old at initiation of the experiment) administered 0, 15, 45, and 135 mg/kg CH in unbuffered drinking water 7 days/week (n = 50/group) for 124 weeks in males and 128 weeks in females. Two control groups were noted in the methods section without explanation as to why they were conducted as two groups.

The mean survival for males was similar in treated and control groups, with 20, 24, 20, 24, and 20% of Control I, Control II, 15, 45, and 135 mg/kg CH-treated groups, respectively, surviving until the end of the study. For female rats, the percent survival was 12, 30, 24, 28, and 16% for of Control I, Control II, 15, 45, and 135 mg/kg CH-treated groups, respectively. The authors reported no substance-related influence on organ weights and no macroscopic evidence of tumors or lesions in male or female rats treated with CH for 124 or 128 weeks. However, no data were presented on the incidence of tumors using this paradigm, especially background rates.

The authors reported a statistically significant increase in the incidence of hepatocellular hypertrophy in male rats at the 135 mg/kg dose (14/50 animals vs. 4/50 and 7/50 in Controls I and II). For female rats, the incidence of hepatocellular hypertrophy was reported to be 10/50 rats (Control I) and 16/50 (Control II) rats with 18/50, 13/50, and 12/50 female rats having hepatocellular hypertrophy after 15, 45, and 135 mg/kg CH, respectively. The lack or reporting in regard to final body weights, histology, and especially background and treatment group data for tumor incidences, limit the interpretation of this study. Whether this paradigm was sensitive for induction of liver cancer cannot be determined.

From the CH studies in mice, there is an apparent increase in liver adenomas and carcinomas induced by CH treatment by either drinking water or gavage with all available studies performed in male $B6C3F_1$ mice. However, the background levels of hepatocellular adenomas and carcinomas in the mice in George et al. (2000) and body weight data from this study show that it is from a tumor-prone mouse model.

Comparisons with concurrent studies of mice exposed to DCA revealed that while both CH and DCA induced hepatomegaly and cytomegaly, DCA-induced cytomegaly was accompanied by vacuolization, while that of CH was associated with increased number of basophilic granules (rough endoplasmic reticulum), which would suggest separate effects. However, the morphology of the CH-induced tumors was reported to be similar between DCA- and CH-induced tumors (Daniel et al., 1992).

Using a similar paradigm (2-year study of $B6C3F_1$ male mice), DeAngelo et al. (1999) and Carter et al. (2003) described DCA-induced tumors to be heterogeneous. This is the same description given for TCE-induced tumors in the studies by NTP, NCI, and Maltoni et al. and to be a common description for tumors caused by a variety of carcinogenic agents. Similar to the studies cited above for CH, DeAngelo et al. (1999) reported that PCO levels were only elevated at 26 weeks at 3.5 g/L DCA and had returned to control levels by 52 weeks. Similar to CH, no

increased tritiated thymidine was reported for DCA at 26 and 52 weeks, with only twofold of control values reported at 0.05 g/L at 4 weeks.

Leakey et al. (2003b) reported that ad-libitum-fed male mice exhibited a similar degree of increase in the incidence of peripheral or focal fatty change at 15 months for all CH doses; however, enzymes associated with peroxisome proliferation were not similarly altered at all CH doses. While dietary restriction seemed to have decreased background levels of tumors and increased time-to-tumor, CH-gave a clear dose-response in dietary restricted animals. However, while the overall level of tumor induction was reduced, there was a greater induction of PPARα enzymes by CH. Induction of liver tumors by CH observed in ad-libitum-fed mice were not correlated with PPARα induction, with dietary restriction alone appearing to have greater levels of lauric acid ω-hydrolase activity in control mice at 15 months. Seng et al. (2003) report that lauric acid β-hydroxylase and PCO were induced only at exposure levels >100 mg/kg CH, again with dietary restricted groups showing the greatest induction. Such data argues against the role of peroxisome proliferation in CH-liver tumor induction in mice.

E.2.6. Serum Bile Acid Assays

Serum bile acids (SBA) have been suggested as a sensitive indicator of hepatotoxicity to a variety of halogenated solvents with an advantage of increased sensitivity and specificity over conventional liver enzyme tests that primarily reflect the acute perturbation of hepatocyte membrane integrity and "cell leakage" rather than liver functional capacity (i.e., uptake, metabolism, storage, and excretion functions of the liver) (Neghab et al., 1997; Bai et al., 1992b). While some studies have reported negative results, a number of studies have reported elevated SBA in organic solvent-exposed workers in the absence of any alterations in normal liver function tests. These variations in results have been suggested to arise from failure of some methods to detect some of the more significantly elevated SBA and the short-lived and reversible nature of the effect (Neghab et al., 1997).

Neghab et al. (1997) have reported that occupational exposure to 1,1,2-trichloro-1,2,2-trifluoroethane and TCE has resulted in elevated SBA and that several studies have reported elevated SBA in experimental animals to chlorinated solvents such as carbon tetrachloride, chloroform, hexachlorobutadiene, tetrachloroethylene, 1,1,1-trichloroethane, and TCE at levels that do not induce hepatotoxicity (Hamdan and Stacey, 1993; Bai et al., 1992b; Wang and Stacey, 1990). Toluene, a nonhalogenated solvent, has also been reported to increase SBA in the absence of changes in other hepatobiliary functions (Neghab and Stacey, 1997). Thus, disturbance in SBA appears to be a generalized effect of exposure to chlorinated solvents and nonchlorinated solvents and not specific to TCE exposure.

Neghab et al. (1997) reported that 8-hour TWA exposures to TCE of 8.9 ppm, measured in the breathing zone using a charcoal tube personal sampler for the whole mean duration of exposure of 3.4 years, do not result in significant changes in albumin, bilirubin, ALP, ALT,

5'-nucleosidase, γ-glutamyltransferase, but do have significantly increased total serum bile acids. Not only were total bile acids significantly increased in these TCE-exposed workers compared to controls (approximately twofold of control), but specifically, deoxycholic acid and subtotal of free bile acids were increased. Neghab et al. (1997) did not show the data, but also reported that "despite the apparent overall low level of exposure, there was a very good correlations (r = 0.94) between the degree of increase in serum concentration of total bile acids and level of TCE." Neghab et al. (1997) noted that while a sensitive indicator or exposure to such solvents in asymptomatic workers, there is no indication that actual liver injury occurs in conjunction with SAB increases.

Wang and Stacey (1990) administered TCE in corn oil via i.p. injection to male Sprague-Dawley rats (300–500 g) at concentrations of 0.01, 0.1, 1, 5, and 10 mmol/kg on 3 consecutive days (n = 4, 5, or 6) with liver enzymes and SBA examined 4 hours after the last TCE treatment. At these doses, there were no differences between treated and control animals in regard to ALP and SDH concentrations, and an elevation of ALT was noted only at the highest dose. However, there was generally a reported dose-related increase in cholic acid, chenodeoxycholic acid, deoxycholic acid, taurocholic acid, and tauroursodeoxycholic acid, with cholic acid and taurochlolic acid increased at the lowest dose. The authors reported that "examination of liver sections under light microscopy yielded no consistent effects that could be ascribed to trichloroethylene."

In the same study, rats were also exposed to TCE via inhalation (n = 4) at 200 ppm for 28 days, and 1,000 ppm for 6 hours/day. Using this paradigm, cholic acid and taurocholic acid were significantly elevated at the 200 ppm level, (~10- and ~5-fold of control, respectively) with very large SEs. At the 1,000 ppm level (6 hours/day), cholic acid and taurocholic acid were elevated to approximately twofold of control but neither was statistically significant. The large variability in responses between rats and the low number of rats tested in this paradigm limit its ability to determine quantitative differences between groups. Nevertheless, without the complications associated with i.p. exposure (see Section E.2.2.1), inhalation exposure of TCE at a relative low exposure level was also associated with increased SBA levels. The authors stated that "no increases in alanine amino transferase levels were observed in the rats exposed to trichloroethylene via inhalation." No histopathology results were reported for rats exposed via inhalation.

As stated by Wang and Stacey (1990), "intraperitoneal injection is not particularly relevant to humans," which was the rationale given for the inhalation exposure experiments in the study. They point out that intestinal interactions require consideration because a major determinant of SBA is that their absorption from the gut and intestinal flora may play a role in bile acid metabolism. They also noted that grooming done by the experimental rats would probably result in low exposure via ingestion of TCE as well. However, Wang and Stacey (1990) reported consistent results in terms of TCE-induced changes in SBA at relatively low

concentrations by either inhalation or i.p. routes of exposure that were not associated with other measures of toxicity.

Hamdan and Stacey (1993) administered TCE in corn oil (1 mmol/kg) in male Sprague-Dawley rats (300–400 g) and followed the time-course of SBA elevation, TCE concentration, and TCOH in the blood at 2, 4, 8, and 16 hours after dosing (n = 4, 5, or 6 per group). Liver and blood concentration of TCE were reported to peak at 4 hours, while those of TCOH peaked at 8 hours after dosing. TCE levels were not detectable by 16 hours in either blood or liver, while those of TCOH were still elevated. Elevations of SBA were reported to parallel those of TCE, with cholic acid and taurochloate acid reported to show the highest levels of bile acids. The dose given was based on that reported by Wang and Stacey (1990) to give no hepatotoxicity but an increase in SBA. The authors stated that liver injury parameters were checked and found unaffected by TCE exposure but do not show the data. Thus, it was TCE concentration and not that of its metabolite that was most closely related to changes in SBA and after a single exposure, the effect was reversible.

In an in vitro study by Bai and Stacey (1993), TCE was studied in isolated rat hepatocytes with TCE reported to cause a dose-related suppression of initial rates of cholic acid and taurocholic acid but with no significant effects on enzyme leakage or intracellular calcium contents, further supporting a role for the parent compound in this effect. The authors noted that the changes in SBA result from interference with a physiological process rather "than an event associated with significant pathological consequences."

E.3. STATE OF SCIENCE OF LIVER CANCER MODES OF ACTION

The experimental evidence in mice shows that TCE and its metabolites induce foci, hepatocellular adenomas, and carcinomas that are heterogeneous in nature as indicated by phenotypic differences in tincture, mutational markers, or gene expression markers. The tumors induced by TCE are reflective of phenotypes that are either similar to those induced by mixtures of DCA and TCA exposure, or more like those induced by DCA. These tumors have been described to be similar also to those arising spontaneously in mice or from chemically induced hepatocarcinogenesis and to arise from preneoplastic foci, and in the case of DCA, single dysplastic hepatocytes as well as foci. HCC observed in humans also has been described to be heterogeneous and to be associated with formation of preneoplastic nodules. Although several conditions have been associated with increased risk of liver cancer in humans, the mechanism of HCC is unknown at this time. A great deal of attention has been focused on predicting which cellular targets (e.g., "stem-cell" or mature hepatocyte) are associated with HCC as well as on phenotypic markers in HCC that can provide insight not only into mode of action and origin of tumor, but also for prediction of clinical course. Examination of pathways and epigenetic changes associated with cancer and the relationship of these changes to liver cancer are also discussed below.

The field of cancer research has been transformed by the recent discoveries of epigenetic changes and their role in cancer and chronic disease states. The following discussion describes not only these advances, but also the issues involved with the technologies that have emerged to describe them (see Section E.3.1.2). Exposure to TCE and its metabolites, like many others, induces a heterogeneous response, even in a relatively homogeneous genetic paradigm as the experimental laboratory rodent model. The importance of phenotypic anchoring is a major issue in the study of any modes of action using these new technologies of gene expression pattern. Although a large amount of information is now available using microarray technologies and transgenic mouse models, specifically for TCE and in study of suggested modes of action for TCE and its metabolites, use of these approaches has limitations that need to be considered in the interpretation of data and conclusions derived from such data, especially quantitative conclusions.

For TCE and its metabolites, the extent of acute to subchronic induction of hepatomegaly correlated with hepatocellular carcinogenicity, although each had differing factors contributing to that hepatomegaly from periportal glycogen deposition to hepatocellular hypertrophy and increased polyploidy. The extent of transient DNA synthesis, peroxisome proliferation, or cytotoxicity was not correlated with carcinogenicity. Hepatomegaly is also a predictor of carcinogenicity for a number of other compounds in mice and rats. Allen et al. (2004) examined the NTP database (87 compounds for rat and 83 for mice) and tried to correlate specific hepatocellular pathology in prechronic studies with carcinogenic endpoints in the chronic 2-year assays. The best single predictor of liver cancer in mice was hepatocellular hypertrophy. Hepatocellular cytomegaly and hepatocyte necrosis also contributed, although the numbers of positive findings were less than hypertrophy.

With regard to genotoxicity studies, there was no evidence of a correlation between mouse liver tumor chemicals and *Salmonella* or micronucleus assay outcome. None of the prechronic liver lesions examined were correlated with either *Salmonella* or Micronucleus assays. In rats, no single prechronic liver lesions (when considered individually) was a strong predictor of liver cancer in rats. The most predictive lesions was hepatocellular hypertrophy. There was not a significant correlation between liver tumors/toxicity and the two mutagenicity measures.

Although the lack of correlation with the mutagenicity assays could be interpreted as rodent assays predominantly identifying nongenotoxic liver carcinogens, this conclusion could be questioned because it is solely dependent on *Salmonella* mutagenicity and additional genotoxic endpoints could conceivably shift the association between liver cancer and genotoxicity towards a more positive correlation. As to questions of the usefulness of the mouse bioassay, the two mutagenicity assays did not correlate with rat results either and an important indicator for carcinogenicity would be lost.

Examination of tumor phenotype from TCE, DCA, and TCA exposures in mice shows a large heterogeneity, which is also consistent with the heterogeneity observed in human HCC (see Section E.3.1.8). The heterogeneity of tumor phenotype has been correlated with survival outcome and tumor aggressiveness in humans and in transgenic mouse models that share some of the same perturbations in gene pathway expression (see Sections E.3.1.8 and E.3.2.1, below). An examination of common pathway disturbances that may be common to all cancers and those of liver tumors shows that there are pathways in common, but that there is greater heterogeneity in disturbance of hepatic pathways in cancer that may make is useful as a marker of disturbances indicative of different targets of carcinogenicity depending on the cellular context and target. Thus, although primate and human liver may not be as susceptible to HCC as the rodent liver, the pathways leading to HCC in rodents and humans appear to be similar and heterogeneous, with some indicative of other susceptible cellular targets for neoplasia in a differing context.

E.3.1. State of Science for Cancer and Specifically Human Liver Cancer

E.3.1.1. Epigenetics and Disease States (Transgenerational Effects, Effects of Aging, and Background Changes)

Wood et al. (2007) published their work on "genomic landscapes" of human breast and colorectal cancers that significantly forwards the understanding of "key events" involved with induction of cancer. They state that there are ~80 DNA mutations that alter amino acid in a typical cancer, but that examination of the overall distribution of these mutations in different cancers of the same type leads to a new view of cancer genome landscapes: they are composed of a handful of commonly mutated genes "mountains" but are dominated by a much larger number of infrequently mutated gene "hills."

> Statistical analyses suggested that most of the ~ 80 mutation in an individual tumor were harmless and that <15 were likely to be responsible for driving the initiation, progression, or maintenance of the tumor…Historically the focus of cancer research has been on the gene mountains, in part because they were the only alterations that could be identified with available technologies. However, our data show that vast majority of mutations in cancers do not occur in such mountains. This new view of cancer is consistent with the idea that a large number of mutations, each associated with a small fitness advantage, drive tumor progression. It is the "hills" and not the "mountains" that dominate the cancer genomic landscape.

The large number of "hills" actually reflects alterations in a much smaller number of cell signaling pathways. Indeed, pathways rather than individual genes appear to govern the course of tumorigenesis.

> It is becoming increasingly clear that pathways rather than individual genes govern the course of tumorigenesis. Mutations in any of several genes of a single pathway can thereby cause equivalent increases in net cell proliferation.…This new view of cancer is consistent with the idea that a large number of mutations, each associated with a small fitness advantage, drive tumor progression.

Thus, when pathways are altered, the same phenotype can arise from alterations in any of several genes.

Consistent with the arguments put forth by Wood et al. (2007) for mutations in cancer is the additional insight into pathway alterations by epigenomic mechanisms, which can act similarly as mutation. Weidman et al. (2007) report that:

> cell phenotype is not only dependent on its genotype but also on its unique epigenotype, which is shaped by developmental history and environmental exposures. The human and mouse genome projects identified approximately 15,500 and 29,000 CpG islands, respectively. Hypermethylation of CpG-rich regions of gene promoters inhibit expression by blocking the initiation of transcription. DNA methylation is also involved in the allelic inactivation of imprinted genes, the silencing of genes on the inactive X chromosome, and the reduction of expression of transposable elements. Because epigenomic modifications are copied after DNA synthesis by DNMT1, they are inherited during somatic cell replication…Inherited and spontaneous or environmentally induced epigenetic alterations are increasingly being recognized as early molecular events in cancer formation. Furthermore, such epigenetic alterations are potentially more adverse than nucleotide mutations because their effects on regional chromatin structure can spread, thereby affecting multiple genetic loci. Although tumor suppressor gene silencing by DNA methylation occurs frequently in cancer, genome-wide hypomethylation is one of the earliest events to occur in the genesis of cancer. Demethylation of the genome can lead to the reactivation of transposable elements, thereby altering the transcription of adjacent genes, the activation of oncogenes such as H-Ras, and biallelic expression of imprinted loci (e.g., loss of IGF2 imprinting).

Thus, epigenetic modification may be worse than mutation in terms of cancer induction.

Dolinoy et al. (2007) report on the role of environmental exposures on the epigenome, especially during critical periods of development and their role in adult disease susceptibility. They report that:

> aberrant epigenetic gene regulation has been proposed as a mechanism of action for nongenotoxic carcinogenesis, imprinting disorders, and complex disorders including Alzheimer's disease, schizophrenia, asthma, and autism. Epigenetic modifications are inherited not only during mitosis but also can be transmitted transgenerationally (Anway et al., 2005; Rakyan et al., 2003; Rakyan et al., 2002)). The influence on environmental factors on epigenetic gene regulation may also persist transgenerationally despite lack of continued exposure in second,

> third, and fourth generations (Anway et al., 2005). Therefore if the genome is compared to the hardware in a computer, the epigenome is the software that directs the computer's operation…The epigenome is particularly susceptible to deregulation during gestation, neonatal development, puberty and old age. Nevertheless, it is most vulnerable to environmental factors during embryogenesis because DNA synthetic rate is high, and the elaborate DNA methylation pattern and chromatin structure required for normal tissue development is established during early development…83 imprinted genes have been identified in mice and humans with 29 or about one third being imprinted in both species. Since imprinted genes are functionally haploid, they are denied the protection from recessive mutations that diploidy would normally afford. Imprinted genes that have been linked to carcinogenesis include IGF2 (bladder, lung, ovarian and others), IGF2R (breast, colon, lung, and others), and Neuronatin (pediatric leukemia).

Bjornsson et al. (2008) recently reported that not only were there time-dependent changes in global DNA methylation within the same individuals in two separate populations in widely separated geographic locations, but also these changes showed familial clustering in both increased and decreased methylation. These results were suggested not only to support the relationship of age-related loss of normal epigenetic patterns as a mechanism for late onset of common human diseases, but also that losses and gains of DNA methylation observed over time in different individuals could contribute to disease with the example provided of cancer, which is associated with both hypomethylation and hypermethylation through activation of oncogenes and silencing of tumor suppressor genes. The study also showed considerable interindividual age variation, with differences accruing over time within individuals that would be missed by studies that employed group averaging.

The review by Reamone-Buettner and Borlak (2007) provide insight into the role of noncoding RNAs in diseases such as cancer. They report that:

> a large number of noncoding RNAs (ncRNAs) play important role in regulating gene expressions, and advances in the identification and function of eukaryotic ncRNAs, e.g., microRNAs and their function in chromatin organization, gene expression, disease etiology have been recently reviewed. The regulatory pathways mediated by small RNAs are usually collectively referred to as RNA interference (RNAi) or RNA-mediated silencing. RNAi can be triggered by small double-stranded RNA (dsRNA) either introduced exogenously into cells as small interfering siRNAs or that have been produced endogenously from small non-coding RNAs known as microRNAs (miRNAs). The dsRNAs are characteristically cleaved by the ribonuclease III-enzyme Dicer into 21- to 23 nt duplexes and the resulting fragments base-pair with complementary mRNA to target cleavage or to repress translation…Two mechanisms exist of miRNA-mediated gene regulation, degradation of the target mRNA, and translational repression. Whether one or the other of these mechanisms is used depends on the degree of the complementary between the miRNA and target mRNA. For a near perfect match, the Argonaute protein in the RNA-induced silencing complex

(RISC) cleaves the mRNA target, which is destined for subsequent degradation by ribonucleases. In the situation of a less degree of complimentarity, commonly occurring in humans, the translational repression mechanism is used to control gene expression. However, the exact mechanism for translational inhibition is unclear.

The varying degrees in complimentarity would help explain the large number of genes that could be affected by miRNA and pleiotropic response.

The review by Feinberg et al. (2006) specifically addresses the epigenetic progenitor origin of human cancer. They conclude that epigenetic alterations are ubiquitous and serve as surrogate alterations for genetic change (oncogene activation, tumor-suppressor-gene silencing), by mimicking the effect of genetic change. They report that:

> Advances in characterizing epigenetic alterations in cancer include global alterations, such as hypomethylation of DNA and hypoacetylation of chromatin, as well as gene-specific hypomethylation and hypermethylation. Global DNA hypomethylation leads to chromosomal instability and increased tumour frequency, which has been shown *in vitro* and *in vivo* in mouse models, as well as gene-specific oncogene activation, such as R-ras in gastric cancer, and cyclin D2 and maspin in pancreatic cancer. In addition, the silencing of tumour-suppressor genes is associated with promoter DNA hypermethylation and chromatin hypoacetylation, which affect divergent genes such as retinoblastoma 1 (RB1), p16 (also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), von Hippel-Lindau tumor suppressor (VHL), and MutL protein homologue (MLH1).
>
> Genetic mechanisms are not the only path to gene disruption in cancer. Pathological epigenetic changes - non-sequence-based alteration that are inherited through cell division - are increasingly being considered as alternatives to mutations and chromosomal alterations in disrupting gene function. These include global DNA hypomethylation, hypermethylation and hypomethylation of specific genes, chromatin alterations and loss of imprinting. All of these can lead to aberrant activation of growth-promoting genes and aberrant silencing of tumour-suppressor genes.
>
> Most CG dinucleotides are methylated on cytosine residues in vertebrate genomes. CG methylation is heritable, because after DNA replication the DNA methyltransferase 1, DNMT1, methylates unmethylated CG on the base-paired strand. CG dinucleotides within promoters within promoters tend to be protected from methylation. Although individual genes vary in hypomethylation, all tumours have shown global reduction of DNA methylation. This is a striking feature of neoplasia.
>
> In addition to global hypomethylation, promoters of individual genes show increased DNA methylation levels. Hypermethylation of tumour-suppressor genes can be tumour-type specific. An increasing number of genes are found to be normally methylated at promoters but hypomethylated and activated in the corresponding tumours. These include R-RAs in gastric cancer, melanoma

antigen family A, 1(MAGE1) in melanoma, maspin in gastric cancer, S100A4 in colon cancer, and various genes in pancreatic cancer.

Our genetic material is complexed with proteins in the form of histones in a one-to-one weight ratio. Core histones H2A, H2B, H3 and H4 form nucleosome particles that package 147 bp of DNA, and the linker histone H1 packages more DNA between core particles, forming chromatin. It is chromatin and not just DNA, that is the substrate for all processes that affect genes and chromosomes. In recent years, it has become increasingly evident that chromatin, like DNA methylation, can impart memory to genetic activity. There are dozens of post-translational histone modifications. Studies in many model systems have shown that particular histone modifications are enriched at sites of active chromatin (histone H3 and H4 hyperacetylation, lysing at 4 and H3 (H3-K4) dimethylation and trimethylation, and H3-K79 methylation) and others are enriched at sites of silent chromatin (H3-K9 and H3-K27 methylation). These and other histone modifications survive mitosis and have been implicated in chromatin memory.

Overproduction of key histone methyltransferases that catalyze the methylation of either H3-K4 or H3-K27 residues are frequent events in neoplasia. Global reductions in monoacetylated H4-K16 and trimethylated H4-K20 are general features of cancer cells.

Genomic imprinting is parent-of –origin-specific gene silencing. It results from a germ-line mark that causes reduced or absent expression of a specific allele of a gene in somatic cells of the offspring. Imprinting is a feature of all mammals affecting genes that regulate cell growth, behaviour, signaling, cell cycle and transport; moreover, imprinting is necessary for normal development. Imprinting is important in neoplasia because both gynogenotes (embryos derived only from the maternal genetic complement) and androgenotes (embryos derived only from the paternal genetic complement) form tumours – ovarian teratomas, and hydtidiform moles/ choriocarcinomas, respectively. Loss of imprinting (LOI) refers to activation of the normally silenced allele, or silencing of the normally active allele, of an imprinted gene. LOI of the insulin-like growth factor 2 gene (IGF2) accounts for half of Wilms tumours in children. LOI of IGF2 is also a common epigenetic variant in adults and is associated with a fivefold increased frequency of colorectal neoplasia. LOI of IGF2 might cause cancer by increasing the progenitor cell population in the kidney in Wilm's tumor and in the gastrointestinal tract in colorectal cancer.

Feinberg et al. (2006) propose that epigenetic changes can provide mechanistic unity to understanding cancer, can occur earlier and set the stage for genetic alterations, and have been linked to the pluripotent precursor cells from which cancers arise. "To integrate the idea of these early epigenetic events, we propose that cancer arises in three steps; an epigenetic disruption of progenitor cells, an initiating mutation and genetic and epigenetic plasticity."

The first step involves an epigenetic disruption of progenitor cells in a given organ or system, which leads to a polyclonal precursor population of neoplasia-

ready cells. These cells represent a main target of environmental, genetic and age-dependent exposure that largely accounts for the long latency period of cancer. Epigenetic disruption might perturb the normal balance between undifferentiated progenitor cells and differentiated committed cells within a given anatomical compartment, either in number or in their capacity for aberrant differentiation, which provides a common mechanism of neoplasia.

All tumours show global changes in DNA methylation, and DNA methylation is clonally inherited through cell division. Because the conventional genetic changes in cancer are also clonal, global hypomethylation would have to occur universally, at the same moment as the mutational changes, which seems unlikely. This suggests that global DNA hypomethylation (and global reductions of specific histone modifications) precedes genetic change in cancer. Similarly, hypermethylation of tumour-suppressor genes has been observed in the normal tissue of patients in which the same gene is hypermethylated in the tumour tissue. Recent data demonstrate LOI of IGF2 throughout the normal colonic epithelium of patients who have LOI-associated colorectal cancer. LOI is associated with increased risk of intestinal cancers in both humans and mice. A specific change in the epithelium is seen in mice that are engineered to have biallelic expression of IGF2 – a shift in the proportion of progenitor to differentiated cells throughout the epithelium; a similar abnormality was observed in humans with LOI of IGF2.

The proposed existence of the epigenetically disrupted progenitors of cancer implies that the earliest stages in neoplastic progression occur even before what a pathologist would recognize as a benign pre-neoplastic lesion. Such alterations are inherently polyclonal. This is in contrast with the widely accepted model of cancer as a monoclonal disorder that arises from an initiating mutation- a model that was proposed and accepted when little was known about epigenetic phenomena in cancer.

Thus, Feinberg et al. (2006) provide a hypothesis for the latency period of cancer and suggest that epigenetic changes predate mutational ones in cancer. Tissues that look phenotypically "normal" may harbor epigenetic changes and predispositions toward neoplasia. In regard to what cells may be targets or epigenetic changes that can be "progenitor cells" in the case of cancer, Feinberg et al. (2006) define such cell having "capacity for self-renewal and pluripotency—over their tendency toward limited replicative potential and differentiation." Within the liver, there are multiple cell types that would fit such a definition, including those who are considered "mature" (see Section E.3.1.4). Feinberg et al. (2006) also note that epigenetic states can be continuously modified to become heterogeneous at all states of the neoplastic process.

Telomere erosion results in chromosome shortening and uncapped ends that begin to fuse and the resulting dicentric chromosomes break at anaphase. DNA palindromes have recently been found to form at high levels in cancer cells. Like telomere erosion, DNA palindrome formation can lead to genetic instability by

initiating bridge-breakage-fusion cycles. However, it is not known how or exactly when palindromes form, although they appear early in cancer progression. Epigenetic instability can also promote cancer through pleiotropic alterations in the expression of genes that modify chromatin.

Epigenetic changes are reversible but the changes can initiate irreversible genetic changes. Permanent epigenetic changes can have an epigenetic basis. On a background of cancer-associated epigenetic instability, the effects of mutations in oncogenes and tumour –suppressor genes might be exacerbated. Therefore the risk of developing malignancy would be much higher for a given mutations event if it occurred on the background of epigenetic disruption.

The environmental dependence of cancer fits an epigenetic model generally for human disease – the environment might influence disease onset not simply through mutational mechanisms but in epigenetically modifying genes that are targets for either germline or acquired mutation; that is, by allowing genetic variates to be expressed. Little is known about epigenetic predispositions to cancer, but a recent twin study indicates that, similar to cancer risk, global epigenetic changes show striking increase with age.

Environmental insults might affect the expression of tumour-progenitor genes, leading to both genetic and epigenetic alterations. Liver regeneration after tissue injury leads to widespread hypomethylation and hypermethylation of individual genes; both of these epigenetic changes occur in cancer.

In regard to the implications of epigenomic changes and human susceptibility to toxic insult, the review by Szyf (2007) provided additional insights.

The basic supposition in the field has been that the interindividual variations in response to xenobiotic are defined by genetic differences and that the main hazard anticipated at the genomic level from xenobiotic is mutagenesis or physical damage to DNA. In accordance with this basic hypothesis, the main focus of attention in pharmacogenetics has been on identifying polymorphisms in genes encoding drug metabolizing enzymes and receptors. New xenobiotics were traditionally tested for their genotoxic effects. However, it is becoming clear that epigenetic programming plays an equally important role in generating interindividual phenotypic differences, which could affect drug response. Moreover, the emerging notion of the dynamic nature of the epigenome and its responsibility to multiple cellular signaling pathways suggest that it is potentially vulnerable to the effects of xenobiotics not only during critical period in development but also later in life as well. Thus, non-genotoxic agents might affect gene function through epigenetic mechanisms in a stable and long-term fashion with consequences, which might be indistinguishable from the effects of physical damage to the DNA. Epigenetic programming has the potential to persist and even being transgenerationally transmitted (Anway et al., 2005) and this possibility creates a special challenge for toxicological assessment of safety of xenobiotics. Any analysis of interindividual phenotype diversity should therefore take into account epigenetic variations in addition to genetic sequence

> polymorphisms. Whereas, a germ-line polymorphism is a static property of an individual and might be mapped in any tissue at any point in life, epigenetic differences must be examined at different time points and at diverse cell types.

Karpinets and Foy (2005) proposed that epigenetic alterations precede mutations and that succeeding mutations are not random, but in response to specific types of epigenetic changes the environment has encouraged. This mechanism was also suggested as to explain both the delayed effects of toxicant exposure and the bystander effect of radiation on tumor development, which are inconsistent with the accepted mechanism of direct DNA damage.

> In a study of ionizing radiation, non-irradiated cells acquired mutagenesis through direct contact with cells whose nuclei had previously been irradiated with alpha-particles (Zhou et al., 2003). Molecular mechanisms underlying these experimental findings are not known but it is believed that it may be a consequence of bystander interactions involving intercellular signaling and production of cytokines (Lorimore et al., 2003).

Caldwell and Keshava (2006) reported that:

> aberrant DNA methylation has emerged in recent years as a common hallmark of all types of cancers with hypermethylation of the promoter region of specific tumor suppressor genes and DNA repair genes leading to their silencing (an effect similar to their mutation), and genomic hypomethylation (Pereira et al., 2004a; Ballestar and Esteller, 2002; Berger and Daxenbichler, 2002; Rhee et al., 2002; Herman et al., 1998). Whether DNA methylation is a consequence or cause of cancer is a long-standing issue(Ballestar and Esteller, 2002). Fraga et. al. (2005; 2004) report global loss of monoacetylation and trimethylation of histone H4 as common a hallmark of human tumor cells but suggest genomone-wide loss of 5-methylcytosine (associated with the acquisition of a transformed phenotype) does not exist as a static predefined value throughout the process of carcinogenesis but as a dynamic parameter (i.e., decreases are seen early and become more marked in later stages).

E.3.1.2. Emerging Technologies, DNA and siRNA, miRNA Microarrays—Promise and Limitations for Modes of Action

Currently, new approaches are emerging for the study of changes in gene expression and protein production induced by chemical exposure that could be related to their toxicity and serve as an anchor for determining similar patterns between rodent models and human diseases or risks of chemically-induced health impacts. Such approaches have the promise to extend the definitions of "genotoxic" and "nongenotoxic" effects, which with the advent of epigenomic study have become obsolete as they assume that only alteration of the DNA sequence is important in cancer induction and progression. However, not only is phenotypic anchoring an issue in regard to the differing cell types, regions, and lobes of the liver (see Section E.1.2), it is

also an issue for overall variability of response between animals and is critical for interpretation of microarray and other genomic database approaches.

As shown in the discussions of TCE effects in animal models, TCE treatment resulted in a large variability in response between what are supposed to be relatively homogeneous genetically similar animals, and there was an apparent difference in response between studies using the same paradigm. It is important that as varying microarray approaches and analyses of TCE toxicity or of potential modes of action are published, the issue of phenotypic anchoring at the cellular to animal level is addressed. Several studies of TCE microarray results and those of PPARα agonists have been reported in the literature in an attempt to discern modes of action. Issues related to conduct of these experiments and interpretation of their results are listed below.

Perhaps one of the most important studies of this issue has been reported by Baker et al. (2004). The ILSI HESI formed a hepatotoxicity working group to evaluate and compare biological and gene expression responses in rats exposed to well-studied hepatotoxins (Clofibrate and methapyrilene), using standard experimental protocol and to address the following issues: (1) how comparable the biological and gene expression data are from different laboratories running identical in vivo studies; (2) how reproducible the data are generated across laboratories using the same microarray platform; (3) how data compare using different microarray platforms; (4) how data compare using RNA from pooled and individual animals; and (5) whether the gene expression changes demonstrate time- and dose-dependent responses that correlate with known biological markers of toxicity (Baker et al., 2004).

The rat model studied was the male Sprague-Dawley rat (57 or 60–66 days of age) exposed to 250 or 25 mg/kg-day Clofibrate for 1, 3, or 7 days. Two separate in vivo studies were conducted: one at Abbott Laboratories and one at GlaxoSmithKline (GSK, in United Kingdom). There was a difference in biological response between the two laboratories. The high dose (250 mg/kg-day) group at day 3 had a 15% increase in liver weight relative to body weight in the GSK study, compared with a 3% liver weight increase in the Abbott study. At 7 days, there was a 31% liver weight increase in the GSK study and a 15% increase in the Abbott study. Observed changes in clinical chemistry parameters also indicated differences in the biological response of the in vivo study concordant with difference in liver weight. A significant reduction in total cholesterol levels was seen in the GSK study at the high dose for all time points. However, the Abbott study demonstrated a significant reduction only at one dose and time point. The incidence of mitotic figures also differed between the labs. In both studies, there was a 2–3 times greater Acyl-CoA enzyme (ACOX) activity at the high dose but no difference from control in the low dose. Again, the GSK lab gave greater response. For microarrays, GSK and ULR pooled samples from each treatment group of four animals. U.S. EPA did some of the microarray analyses as well as GSK and ULR (GSK in United Kingdom). It is apparent that although the changes in genes were demonstrated by both laboratories, there were quantitative differences in the fold change values observed between the two sites.

The U.S. EPA analyzed gene expression in individual RNA samples obtained from day 7 high- and low-dose animals that had been treated at Abbot. GSK (United States) and ULR analyzed gene expression in pooled RNA from day 7 high- and low-dose animals treated at GSK (United Kingdom). Gene expression data from individual animal samples indicated that 7 genes were significantly upregulated (maximum of 7.2-fold) and 12 genes were down regulated (maximum of 4.3-fold decrease) in the high-dose group. The low-dose group generated only one statistically significant gene expression change, namely heat shock protein 70 (HSP70). In comparison, expression changes in the 7-day pooled high-dose samples analyzed by GSK (United States) ranged from 43.3-fold to a 3.5-fold decrease. Changes in these same samples analyzed by ULR ranged from a 4.9-fold increase to a 4.3-fold decrease. As an example, the microarray fold change at 7-day 250 mg/kg-day Clofibrate showed a 3.8-fold increase for U.S. EPA individual animals sampled, a 2.2-fold increase for pooled samples by ULR, and a 20.3-fold increase in pooled samples by GSK (United States) for CYP4A1 (Baker et al., 2004). Thus, these results show a very large difference not only between treatment groups, but also between pooled and nonpooled data and between labs analyzing the same RNA.

Not only was there a difference in DNA microarray results but, also a comparison of gene expression data from day 7 high-dose samples obtained using quantitative realtime PCR vs. data generated using cDNA microarrays has shown a quantitative difference but qualitative similar patterns. Although both methods of quantitative real time PCR on the pooled sample showed the PPARα gene to be downregulated, the GSK (United States) pooled sample microarray analysis indicated upregulation; the URL pooled and U.S. EPA individual microarray analyses showed no change. The microarray for PPARα at 7-day 250 mg/kg-day Clofibrate showed no change for individual animals (U.S. EPA), no change for pooled samples (ULR), and upregulation of 1.8-fold value for pooled samples for GSK (United States). The quantitative real time PCR on the pooled sample using Taqman gave a 4.5-fold downregulation and using SYBR Green gave a 1.2-fold downregulation of PPARα.

Baker et al. (2004) reported that the pooling of samples for microarray analysis has been used in the past to defray the cost of microarray experiments, reduce the effect of biological variation, and in some cases, overcome availability of limiting amounts of tissues. Unfortunately, this approach essentially produced a sample size (n) of one animal. Repeated microarray experiments with such pooled RNA produces technical replicates as opposed to true biological replicates, and thus, does not allow calculation of biologically significant changes in gene expression between different dose groups or time points. Another possible consequence of pooling is to mask individual gene changes and leave open the possibility of introducing error due to individual outlier responses.

Woods et al. (2007b) note that:

> because toxicogenomics is a relatively novel technology, there are a number of limitations that must be resolved before array data is widely accepted. Microarray studies have been touted as being highly sensitive for detecting toxic responses at much earlier time points and/or lower doses than histopathology, clinical chemistry or other traditional toxicological assays can detect. However, based on the nature of the assay, measurements of extreme levels of gene expression – low or high –are thought to be unreliable. Also the reproducibility of microarray experiments has raised concerns. "Batch effects" based on the day, user, and laboratory environment have been observed in array datasets. To address these concerns, confirmation of microarray-derived gene expression profiles is typically performed using quantitative real time polymerase chain reaction (RT-PCR) or Northern blot analysis.

In addition to the issues raised above, Waxman and Wurmbach (2007) raise issues regarding how quantitative real time PCR experiments are conducted. They state that cancer development affects almost all pathways and genes including the "housekeeping" genes, which are involved in the cell's common basic functions (e.g., glyceraldehyde-3-phosphate dehydrogenase [GADPH], beta actin [ACTB], TATA-binding protein, ribosomal proteins, and many more). However, "many of these genes are often used to normalize quantitative real-time RT-PCR (qPCR) data to account for experimental differences, such as differences in RNA quantity and quality, the overall transcriptional activity and differences in cDNA synthesis. GADPH and ACTB are most commonly used for normalization, including studies of cancer." Waxman and Wurmbach (2007) suggest that despite the fact that it has been shown that these genes are differentially expressed in cancers, including colorectal-, prostate-, and bladder-cancer, some qPCR studies on HCC used GAPDH or ACTB for normalization. Since many investigations on cancer include multiple comparisons, and analyze different stages of the disease, such as normal tissue, preneoplasm, and consecutive stages of cancer, "it crucial to find an appropriate gene for normalization" whose expression is constant throughout all disease stages and not response to treatment.

For liver cancers associated with exposure to hepatitis C virus (HCV), Waxman and Wurmbach (2007) reported that differing states, including preneoplastic lesions (cirrhosis and dysplasia) and consecutive stages of HCC, had differential expression of "housekeeping" genes and that using them for normalization had an effect on the fold change of qPCR data and on the general direction (up or down) of differentially expressed genes. For example, GAPDH was strongly upregulated in advanced and very advanced stages of HCC (in some samples up to sevenfold) and ACTB was upregulated two- to threefold in many advanced and very advanced tumor samples. Waxman and Wurmbach (2007) concluded that:

> microarray data are known to be highly variable. Due to its higher dynamic range qPCR is thought to be more accurate and therefore is often used to corroborate microarray results. Mostly, general direction (up and down-regulation) and rank

> order of the fold-changes are similar, but the levels of the fold changes of microarray experiments differ compared to qPCR data and show a marked tendency of being smaller. This effect is more pronounced as the fold change is very high.

In relation to use of gene expression and indicators of cancer causation, Volgelstein and Kinzler (2004) made important points regarding their use:

> Levels of gene expression are unreliable indicators of causation because disturbance of any network invariably leads to a multitude of such changes only peripherally related to the phenotype. Without better ways to determine whether an unmutated but interesting candidate gene has a causal role in neoplasia, cancer researchers will likely be spending precious time working on genes only peripherally related to the disease they wish to study.

This is an important caveat for gene expression studies for mode of action that are "snapshots in time" without phenotypic anchoring and even more applicable to experimental paradigms where there is ongoing necrosis or toxicity in addition to gene changes that may or may not be associated with neoplasia.

For an endpoint that is not as complex as neoplasia, there are issues regarding uses of microarray data. In regard to the determination of acute liver toxicity caused by one of the most studied hepatotoxins, acetaminophen, and its correlation with microarray data, Beyer et al. (2007) also have reported the results of a landmark study examining issues regarding use of this approach.

> The biology of liver and other tissues in normal and disease states increasingly is being probed using global approaches such as microarray transcriptional profiling. Acceptance of this technology is based principally on a satisfactory level of reproducibility of data among laboratories and across platforms. The issue of reproducibility and reliability of genomics data obtained from similar (standardized) biological experiments performed in different laboratories is crucial to the generation and utility of large databases of microarray results. While several recent studies uncovered important limitation of expression profiling of chemical injury to cells and tissues (Beekman et al., 2006; Baker et al., 2004; Ulrich et al., 2004), determining the effects of intralaboratory variables on the reproducibility, validity, and general applicability of the results that are generated by different laboratories and deposited into publicly available databases remains a gap…The National Institutes of Environmental Health Sciences (NIEHS) established the Toxicogenomics Research Consortium to apply the collective and specialized expertise from academic institutions to address issues in integrating gene expression profiling, bioinformatics, and general toxicology. Key elements include developing standardized practices for gene expression studies and conducting systematic assessments of the reproducibility of traditional toxicity endpoints and microarray data within and among laboratories. To this end the consortium selected the classical hepatotoxicant acetaminophen (APAP)

> for its proof of concept experiments. Despite more than 30 years of research on APAP, we are far from a complete understanding of the mechanisms of liver injury, risk factors, and molecular markers that predict clinical outcome after poisoning. APAP–induced hepatotoxicity was performed at seven geographically dispersed Centers. Parallel studies with N-acetyl-m-aminophenol (AMAP), the non-hepatotoxic isomer of APAP, provided a method to isolate transcripts associated with hepatotoxicity (Beyer et al., 2007).

Beyer et al. (2007) identified potential sources of interlaboratory variability when microarray analyses were conducted by one laboratory on RNA samples generated in different laboratories but using the same experimental paradigm and source of animals. Toxic injury by APAP showed variability across Centers and between animals (e.g., percent liver affected by necrosis [<20–80% at one time period and 0–60% at another], control animal serum ALT [threefold difference], and in GSH depletion [<5–>60%] between centers). There was concordance between APAP toxicity as measured in individual animals (rather than expressed as just a mean with SE) and transcriptional response. Of course, the variability between gene platforms and processing of the microarrays had been reduced by using the same facility to do all of the microarray analyses. However, the results show that phenotypic anchoring of gene expression data are required for biologically meaningful meta-analysis of genomic experiments.

Woods et al. (2007b) noted that:

> improvements should continue to be made on statistical analysis and presentation of microarray data such that it is easy to interpret. Prior to the current advances in bioinformatics, the most common way of reporting results of microarray studies involved listing differentially expressed genes, with little information about the statistical significance or biological pathways with which the genes are associated.

However, there are issues with the use of "Classifiers" or predictive genomic computer programs based on genes showing altered expression in association with the observed toxicities.

> Although these metrics built on different machine learning algorithms could be useful in estimating the severity of potential toxicities induced by compounds, the applications of these classifiers in understanding the mechanisms of drug-induced toxicity are not straightforward. In particular this approach is unlikely to distinguish the upstream causal genes from the downstream responsive genes among all the genes associated with an induced toxicity. Without knowledge of the causal sufficiency order, designing experiments to test predicted toxicity in animal models remains difficult" (Dai et al., 2007).

Ulrich (2003) stated the limitation of microarray analysis to study nuclear receptors (e.g., PPARα).

> Nuclear receptors comprise a large group of ligand-activated transcription factors that control much of cellular metabolism. Toxicogenomics is the study of the structure and output of the entire genome as it related and responds to adverse xenobiotic exposure. Traditionally, the genes regulated by nuclear receptors in cells exposed to toxins have been explored at the mRNA and protein levels using northern and western blotting techniques. Though effective when studying the expression of individual genes, these approaches do not enable the understanding of the myriad of genes regulated by individual receptors or of the crosstalk between receptors…Discovery of the multiple genes regulated by each receptor type has thus been driven by technological advances in gene expressional analysis, most commonly including differential display, RT-PCR and DNA microarrays., and in the development or receptor transgenic and knockout animal models. There is much cross talk between receptors and many agonists interact with multiple receptors. Off target effects cannot be predicted by target specificity. Though RCR can affect transcription directly, much of its effects are exerted through heterodimeric binging with other nuclear receptors (PXR, CAR, PPARα, PPARγ, FXR, LXR, TR) (Ulrich, 2003).

Another tool recent developed is gene silencing by introduction of siRNA. Dai et al. (2007) noted issues involved in the siRNA to change gene expression for exploration of mode of action etc., to include the potential of off-target effects, incomplete knockdown, and nontargeting of splice variants by the selected siRNA sequence. Using knockdown of PPARα in mice, Dai et al. (2007) report "PPARα knockdown was variable between mice ranging from ~80% knockdown to little or no knockdown and that differing siRNAs gave different patterns of gene expression with some grouped with PPARα -/- null mice but others grouped with expression patterns of mice injected with control siRNA or Ringers buffer alone and showing no PPARα knockdown." Dai et al. (2007) concluded that it is possible that it is the change in PPARα levels that is important for perturbing expression of genes modulated by PPARα rather than the absolute levels of PPARα.

Not only is the finding of variability in knockdowns by siRNA technologies important, but the finding that level of PPAR is not necessarily correlated with function and that it could be the change and not absolute level that matters in modulation in gene expression by PPARα is of importance as well. How an animal responds to decreased PPARα function may also depend on its gender. Dai et al. (2007) observed more dramatic phenotypes in female vs. male mice treated with siRNA. Costet et al. (1998) have reported sexually dimorphic phenotypes including obesity and increased serum triglyceride levels in females, and steatosis and increased hepatic triglyceride levels in male PPARα-null mice. Ramdhan et al. (2010) provided extensive date regarding lipid dysregulation in male PPARα-null mice and humanized mice.

In regard to the emerging science and preliminary reports of the effects of microRNA as oncogenes and tumor suppressors and of possible importance to hypothesized modes of action for liver cancer, the same caveats as described for DNA microarray analyses all apply, along with additional uncertainties. miRNAs repress their targeted mRNAs by complementary base

pairing and induction of the RNA interference pathway. Zhang et al. (2007) reported Northern blot detection of gene expression at the mRNA level and its correlation with miRNA expression in cancer cells as well as realtime PCR. These PCR-based analyses quantify miRNA precursors and not the active mature miRNAs. However, they reported that the relationship between pri-miRNA and mature miRNA expression has not been thoroughly addressed and is critical in order to use real time PCR analysis to study the function of miRNAs in cancers. They go on to state that:

> although Northern Blotting is a widely used method for miRNA analysis, it has some limitations, such as unequal hybridization efficiency of individual probes and difficulty in detecting multiple miRNAs simultaneously. For cancer studies, it is important to be able to compare the expression pattern of all known miRNAs between cancer cells and normal cells. Thus, it is better to have methods which detect all miRNA expression at a single time…Although Northern blot analysis, real-time PCR, and miRNA microarray can detect the expression of certain miRNAs and determine which miRNAs may be associated with cancer formation, it is difficult to determine whether or not miRNAs play a unique role in cancers. Also these techniques cannot directly determine the correlation between mRNA expression levels and whether the up-regulation or down-regulation of certain miRNAs is the cause of cancer or a downstream effect of the disease…Many miRNA genes have been found that are significantly overexpressed in different cancers. All of them appear to function as oncogenes; however, only a few of them have been well characterized.

Zhang et al. (2007) suggested that bioinformatic studies indicate that numerous genes are the targets of miR-17-92: >600 for miR-19a and miR-20, two members of the miR-17-92 cluster.

Cho (2007) stated that:

> though more than 530 miRNAs have been identified in human, much remains to be understood about their precise cellular function and role in the development of diseases…Although each miRNA can control hundreds of target genes, it remains a great challenge to identify the accurate miRNA targets for cancer research.

Thus, miRNAs have multiple targets so, like other transcription factors, may have pleotropic effects that are cell, timing, and context specific.

Vogelstein and Kinzler (2004) stated "in the last decade many important gene responsible for the genesis of various cancers have been discovered." Most importantly, they and others suggest that pathways rather than individual gene expression should be the focus of study. As a specific example, Volgelstein and Kinzler noted:

> another example of the reason for focusing on pathways rather than individual genes has been provided by studies of TP53 tumor-suppressor gene. The p53 protein is a transcription factor that normally inhibits cell growth and stimulates

> cell death when induced by cellular stress. The most common way to disrupt the p53 pathway is through a point mutation that inactivates its capacity to bind specifically to its cognate recognition sequence. However, there are several other ways to achieve the same effects, including amplification of the MDM2 gene and infection with DNA tumor viruses whose products bind to p53 and functionally inactivate it.

In regard to cellular anchoring for gene expression or pathway alterations associated with cancer and the importance of "context" of gene expression changes, Vogelstein and Kinzler (2004) gave several examples.

> In solid tumors the important of the interactions between stroma and epithelium is becoming increasingly recognized (e.g., the importance of the endothelial cell)…One might expect that a specific mutation of a widely expressed gene would have identical or at least similar effects in different mammalian cell types. But this is not in general what is observed. Different effects of the same mutation are not only found in distinct cell types; difference can even be observed in the same cell types, depending on when the mutation occurred during the tumorigenic process. The RAS gene mutations provide informative examples of these complexities. *KRAS2 g*ene mutation in normal pancreatic duct cells seem to initiate the neoplastic process, eventually leading to the development of pancreatic cancer. The same mutations occurring in normal colonic or ovarian epithelial cells lead to self-limiting hyperplastic or borderline lesions that do not progress to malignancy. In many human and experimental cancers, *RAS* genes seem to function as oncogenes. But *RAS* genes can function as suppressor genes under other circumstances, inhibiting tumorigenesis after administration of carcinogens to mice. These and similar observation on other cancer genes are consistent with the emerging notion that signaling molecules play multiple roles at multiple time, even in the same cell type. However, the biochemical bases for such variations among cancer cells are almost unknown.

In regard to the major pathways and mediators involved in cancer, several investigators have reported a coherent set that are involved in many types of cancers. Vogelstein and Kinzler (2004) noted that major pathways and mediators include p53, RB, WNT, E-cadherin, GL1, APC, ERK, RAS:GTP, P13K,SMAD, RTK BAD, BAX, and H1F1. In regard to coherence and site concordance between animal and human data, the disturbance of a pathway in one species may result in the different expression of tumor pattern in another, but both linked to a common endpoint of cancer. Thus, pathways rather than a single mutation should be the focus of mode of action and cancer as several actions can be manifested by one pathway or change at one time that lead to cancer.

Vogelstein and Kinzler (2004) also noted that pathways that are common to "cancer" are also operative in liver cancer where, as a heterogeneous disease, multiple pathways have been implicated in differing manifestations of this disease. Thus, liver cancer may be an example in its multiple forms that are analogous to differing sites being affected by common pathways

leading to "cancer." Pathway concordance may not always show up as site concordance as expression of cancer between species. Liver cancer may be the example where many pathways can lead a cancer that is characterized by its heterogeneity.

E.3.1.3. Etiology, Incidence, and Risk Factors for HCC

The review article of Farazi and DePinho (2006) provides an excellent summary of the current state of human liver cancer in terms of etiology and incidence. The 5-year survival rate of individuals with liver cancer in the United States is only 8.9% despite aggressive conventional therapy with lethality of liver cancer due in part from its resistance to existing anticancer agents, a lack of biomarkers that can detect surgically respectable incipient disease, and underlying liver disease that limits the use of chemotherapeutic drugs. Chen et al. (2002b) reported that surgical resection is considered the only "curative treatment" but >80 of patients have widespread HCC at the time of diagnosis and are not candidates for surgical treatment. Among patients with localized HCC who undergo surgery, 50% suffer a recurrence. Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality. HCC accounts for between 85 and 90% of primary liver cancers (El-Serag and Rudolph, 2007). Seitz and Stickel (2006) report that epidemiological data from the year 2000 indicate that >560,000 new cases of HCC occurred worldwide, accounting for 5.6% of all human cancers and that HCC is the fifth most common malignancy in men and the eighth in women.

Overall, incidence rates of HCC are higher in males compared to females. In almost all populations, males have higher liver cancer rates than females, with male:female ratios usually averaging between 2:1 and 4:1 and the largest discrepancies in rates (>4:1) found in medium-risk European populations (El-Serag and Rudolph, 2007). Experiments showed a 2–8-fold of control HCC development in male mice as well supporting the hypothesis that androgens influence HCC progression rather than sex-specific exposure to risk factors (El-Serag and Rudolph, 2007). El-Serag and Rudolph (2007) also reported that:

> in almost all areas, female rates peak in the age group 5 years older than the peak age group for males. In low risk population (e.g., U.S.) the highest age-specific rates occur among persons aged 75 and older. A similar pattern is seen among most high-risk Asian populations. In contrast male rats in high-risk African populations (e.g., Gambia) ten to peak between ages 60 and 65 before declining, whereas female rates peak between 65 and 70 before declining.

Age-adjusted incidence rates for HCC are extremely high in East and Southeast Asia and in Africa, but in Europe, there is a gradually decreasing prevalence from South to North. HCC incidence rates also vary greatly among different populations living in the same region and vary by race (e.g., for all ages and sexes in the United States, HCC rates are 2 times higher in Asian than in African Americans, whose rates are 2 times higher than those in whites); ethnic

variability is likely to include differences in the prevalence and acquisition time of major risk factors for liver disease and HCC (El-Serag and Rudolph, 2007).

Worldwide HCC incidence rate doubled during the last two decades and younger age groups are increasingly affected (El-Serag, 2004). The high prevalence of HCC in Asia and Africa may be associated with widespread infection with hepatitis B virus (HBV) and HCV but other risk factors include chronic alcohol misuse, nonalcoholic fatty liver disease (NAFLD), tobacco, oral contraceptives, and food contamination with aflatoxins (Seitz and Stickel, 2006). El-Serag and Rudolph (2007) reported HCC to be the fastest growing cause of cancer-related death in men in the United States with age-adjusted HCC incidence rates increasing more than twofold between 1985 and 2002 and that, overall, 15–50% of HCC patients in the United States have no established risk factors.

Although liver cirrhosis is present in a large portion of patients with HCC, it is not always present. Fattovich et al. (2004) reported that:

> differences of geographic area, method of recruitment of the HCC cases (medical or surgical) and the type of material studied (liver biopsy specimens, autopsy, or partial hepatectomies) may account for the variable prevalence of HCC without underlying cirrhosis (7% to 54%) quoted in a series of studies. Percutaneous liver biopsy specimens are subject to sampling error. However, only a small proportion of patients with HCC without cirrhosis have absolutely normal liver histology, the majority of them showing a range of fibrosis intensity from no fibrosis are all to septal and bridging fibrosis, necroinflammation, steatosis, and liver cell dysplasia.

Farazi and DePinho (2006) noted that for diabetes, a higher indices of HCC have been described in diabetic patients with no previous history of liver disease associated with other factors. El-Serag and Rudolph (2007) reported that in their study of VA patients (173,643 patients with and 650,620 patients without diabetes), that HCC incidence doubled among patients with diabetes and was higher among those with a longer follow-up of evaluation. "Although most studies have been conducted in low HCC rate areas, diabetes also has been found to be a significant risk factor in areas of high HCC incidence such as Japan. Taken together, available data suggest that diabetes is a moderately strong risk factor for HCC."

NAFLD and nonalcoholic steatohepatitis contribute to the development of fibrosis and cirrhosis and therefore, might also contribute to HCC development. The pathogenesis of NAFLD includes the accumulation of fat in the liver, which can lead to reactive oxygen species in the liver with necrosis factor α (TNFα) elevated in NAFDL and alcoholic liver disease (Seitz and Stickel, 2006). Abnormal liver enzymes not due to alcohol, viral hepatitis, or iron overload are present in 2.8–5.5% of the U.S. general population and may be due to NAFLD in 66–90% of cases (Adams and Lindor, 2007). Primary NAFLD occurs most commonly and is associated with insulin-resistant states, such as diabetes and obesity, with other conditions associated with

insulin resistance, such as polycystic ovarian syndrome and hypopituitarism also associated with NAFLD (Adams and Lindor, 2007). The steatotic liver appears to be susceptible to further hepatotoxic insults, which may lead to hepatocyte injury, inflammation, and fibrosis, but the mechanisms promoting progressive liver injury are not well defined (Adams and Lindor, 2007). Substrates derived from adipose tissue such as FFA, TNF-α, leptin, and adiponectin have been implicated, with oxidative stress appearing to be important leading to subsequent lipid peroxidation, cytokine induction, and mitochondrial dysfunction. Liver disease was the 3rd leading cause of death among NAFLD patients compared to the 13th leading cause among the general population, suggesting that liver-related mortality is responsible for a proportion of increased mortality risk among NAFLD patients (Adams and Lindor, 2007).

The RR for HCC in type 2 diabetics has been reported to be approximately 4 and increases to almost 10 for consumption of >80 g of alcohol per day (Hassan et al., 2002). El-Serag and Rudolph (2007) reported that:

> it has been suggested that many cryptogenic cirrhosis and HCC cases represent more severe forms of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steato hepatitis (NASH). Studies in the United States evaluating risk factors for chronic liver disease or HCC have failed to identify HCV, HBV, or heavy alcohol intake in a large proportion of patients (30-40%). Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. Several clinic-based controlled studies have indicated that HCC patients with cryptogenic cirrhosis tend to have clinical and demographic features suggestive of NASH (predominance of women, diabetes, and obesity) as compared with age- and sex-matched HCC patients of well defined vial or alcoholic etiology. The most compelling evidence for an association between NASH and HCC is indirect and come from studies examining HCC risk with 2 conditions strongly associated with NASH: obesity and diabetes. In a large prospective cohort in the US, followed up for 16 years, liver cancer mortality rates were 5 times greater among men with the greatest baseline body mass index (range 35-40) compared with those with a normal body mass index. In the same study, the risk of liver cancer was not as increase in women, with a relative risk of 1.68. Two other population-based cohort studies from Sweden and Denmark found excess HCC risk (increased 2- to 3-fold) in obese men and women compared with those with a normal body mass index…Finally, liver disease occurs more frequently in those with more severe metabolic disturbances, with insulin resistance itself shown to increase as the disease progresses. Several developed countries most notably the United States, are in the midst of a burgeoning obesity epidemic. Although the evidence linking obesity to HCC is relatively scant, even small increase in risk related to obesity could translate into a large number of HCC cases.

Thus, even a small increase in risk related to obesity could result in a large number of HCC cases. and the latency of HCC may make detection of increased HCC risk not detectable for several years.

Other factors are involved, as not every cirrhotic liver progresses to HCC. Seitz and Stickel (2006) suggested that 90–100% of those who drink heavily suffer from alcoholic fatty liver, 10–35% of those evolve to alcoholic steatohepatitis, 8–20% of those evolve to alcoholic cirrhosis, and 1–2% of those develop HCC. HCV infects approximately 170 million individuals worldwide with approximately 20% of chronic HCV cases developing liver cirrhosis and 2.5% developing HCC.

Infection with HBV, a noncytopathic, partially double-stranded hepatotropic DNA virus classified as a member of the hepadnaviridae family, is also associated with liver cancer risk with several lines of evidence supporting the direct involvement of HBV in the transformation process (Farazi and DePinho, 2006). El-Serag and Rudolph (2007) suggested that:

> Epidemiologic research has shown that the great majority of adult-onset HCC cases are sporadic and that many have at lease 1 established non-genetic risk factor such as alcohol abuse or chronic HCV or HBV infection. However, most people with these known environmental risk factors never develop cirrhosis or HCC, whereas a sizable minority of HCC case develop among individuals without any known risk factors…Genetic epidemiology studies in HCC, similar to several other conditions, have fallen short of early expectations that they rapidly and unequivocally would result in identification of genetic variants conveying substantial excess risk of disease and thereby establish the groundwork for effective genetic screening for primary prevention.

E.3.1.4. Issues Associated with Target Cell Identification

Another outstanding and important question in HCC pathogenesis involves the cellular origin of this cancer. The liver is made up of a number of cell types showing different phenotypes and levels of differentiation. Which cell types are targets of hepatocarcinogens and are those responsible for human HCC is a matter of intense debate. Studies over the last decade provide evidence of several types of cells in the liver that can repopulate the hepatocyte compartment after a toxic insult. "Indeed, although the existence of a liver stem cell is often debated, most experts agree that progenitor liver cells are activated, in response to significant exposure to hepatotoxins. Also, progenitor cells derived from nonhepatic sources, such as bone marrow and pancreas, have been demonstrated recently to be capable of differentiating into mature hepatocytes under correct microenvironmental conditions" (Gandillet et al., 2003).

At present, analyses of human HCCs for oval cell markers, comparison of their gene-expression patterns with rat fetal hepatoblasts and the cellular characteristics of HCC from various animal models have provided contrasting results about the cellular origin of HCC and imply dual origins from either oval cells or mature hepatocytes. The failure to identify a clear cell of origin for HCC might stem from the fact that there are multiple cells of origin, perhaps reflecting the developmental plasticity of the hepatocyte lineage. The resolution of the HCC cell of origin issue could affect the development of useful preventative strategies to target nascent

neoplasms, foster an understanding of how HCC-relevant genetic lesions function in that specific cell-development context, and increase our ability to develop more accurate mouse models in which key genetic events are targeted to the appropriate cellular compartment (Farazi and DePinho, 2006). Two reviews by Librecht (2006) and Wu and Chen (2006) provide excellent summaries of the issues involved in identifying the target cell for HCC and the review by Roskams et al. (2004) provided a current view of the "oval cell" its location and human equivalent. Recent reports by Best and Coleman (2007) suggest another type of liver cell is also capable of proliferation and differentiating into small hepatocytes (i.e., small hepatocyte-like progenitor cell).

The review by Librecht (2006) provides an excellent description of the controversy and data supporting different views of the cells of origin for HCC.

> In recent years, the results of several studies suggest that human liver tumors can be derived from hepatic progenitor cells rather than from mature cell types. The available data indeed strongly suggest that most combined hepatocellular-cholangiocarcinomas arise from hepatic progenitor cells (HPCs) that retained their potential to differentiate into the hepatocyte and biliary lineages. Hepatic progenitor cells could also be the basis for some hepatocellular carcinomas and hepatocellular adenomas, although it is very difficult to determine the origin of an individual hepatocellular carcinoma. There is currently not enough data to make statements regarding a hepatic progenitor cell origin of cholangiocarcinoma. The presence of hepatic progenitor cell markers and the presence and extent of the cholangiocellular component are factors that are related the prognosis of hepatocellular carcinomas and combined hepatocellular-cholangiocarcinomas, respectively…The traditional view that adult human liver tumors arise from mature cell types has been challenged in recent decades…HPCs are small epithelial cells with an oval nucleus, scant cytoplasm and location in the bile ductules and canals of Hering. HPCs can differentiate towards the biliary and hepatocytic lineages. Differentiation towards the biliary lineage occurs via formation of reactive bile ductules, which are anastamosing ductules lined by immature biliary cells with a relatively large and oval nucleus surrounded by a small rim of cytoplasm. Hepatocyte differentiation leads to the formation of intermediate hepatocyte-like cells, which are defined as polygonal cells with a size intermediate between than of HPCs and hepatocytes. In most liver diseases, hepatic progenitor cells are "activated" which means that they proliferate and differentiate towards the hepatocytic and/or biliary lineages. The extent of activation is correlated with disease severity…HPCs and their immediate biliary and hepatocytic progeny not only have a distinct morphology, but they also express several markers, with many also present in bile duct epithelial cells. Immunohistochemistry using antibodies against these markers facilitates the detection of HPCs. The most commonly used markers are cytokeratin (CK) 19 and CK7…The proposal that a human hepatocellular carcinoma does not necessarily arise from mature hepatocyte, but could have HPC origin, has classically been based on three different observations. Each of them, however, gives only indirect evidence that can be disputed…Firstly, it has been shown that

> HPCs are the cells of origin of HCC in some animal models of hepatocarcinogenesis, which has led to the suggestion that this might also be the case in humans. However, in other animal models, the HCCs arise from mature hepatocytes and not from HPCs or reactive bile ductular cells (Bralet et al 2002; Lin et al 1995– DEN treated rats). Since it is currently insufficiently clear which of these animal models accurately mimics human hepatocarcinogenesis, one should be careful about extrapolating data regarding HPC origin of HCC in animal models to the human situation…Secondly, liver diseases that are characterized by the presence of carcinogens and development of dysplastic lesions also show HPC activation. Therefore, the suggestion has been made that HPCs form a "target population" for carcinogens, but this is only a theoretical possibility not supported by experimental data…Thirdly, several studies have shown that a considerable proportion of HCCs express one or more HPC markers that are not present in normal mature hepatocytes. Due to the fact that most HPC markers are also expressed in the biliary lineage, the term "biliary marker" has been used in some of these studies. The "maturation arrest" hypothesis states that genetic alterations occurring in a HPC, or its immediate progeny, cause aberrant proliferation and prevent its normal differentiation. Further accumulation of genetic alterations eventually leads to malignant transformation of these incompletely differentiated cells. The resulting HCC expresses HPC markers as evidence of its origin. However, expression of HPC markers can also be interpreted in the setting of the "dedifferentiation" hypothesis, which suggests that the expression of HPC markers is acquired during tumor progression as a consequence of accumulating mutations. For example, experiments in which human HCC cells lines were transplanted into nude mice have nicely shown that the expression of HPC marker, CK19, steadily increased when the tumors became increasingly aggressive and metastasized to the lung, Thus, the expression of CK19 in a HCC does not necessarily mean that the tumor has a HPC origin, but it can also be mutation-induced, acquired expression associated with tumor progression. Both possibilities are not mutually exclusive. For an individual HCC that expresses a HPC marker, it remains impossible to determine whether this marker reflects the cellular origin and/or is caused by tumor progression. This can only be elucidated by determining whether HCC contains cells that are ultrastructurally identical to HPCs in nontumor liver.

Similarly, the review by Wu and Chen (2006) also presents a valuable analysis of these issues and stated:

> The question of whether hepatocellular carcinomas arises from the differentiation block of stem cells or dedifferentiation of mature cells remains controversial. Cellular events during hepatocarcinogenesis illustrate that HCC may arise for cells at various stages of differentiation in the hepatic stem cell lineage…The role of cancer stem cells has been demonstrated for some cancers, such as cancer of the hematopoietic system, breast and brain. The clear similarities between normal stem cell and cancer stem cell genetic programs are the basis of the a proposal that some cancer stem cells could derived from human adult stem cells. Adult mesenchymal stem cells (MSC) may be targets for malignant transformation and undergo spontaneous transformation following long-term *in vitro* culture,

> supporting the hypothesis of cancer stem cell origin. Stem cells are not only units of biological organization, responsible for the development and the regeneration of tissue and organ systems, but are also targets of carcinogenesis. However, the origin of the cancer stem cell remains elusive…Three levels of cells that can respond to liver tissue renewal or damage have been proved (1) mature liver cells, as "unipotential stem cells," which proliferate under normal liver tissue renewal and respond rapidly to liver injury, (2) oval cells, as bipotential stem cells, which are activated to proliferate when the liver damage is extensive and chronic or if proliferation of hepatocytes is inhibited; and (3) bone marrow stem cells, as multipotent liver stem cells, which have a very long proliferation potential. There are two major nonexclusive hypotheses of the cellular origin of cancer; from stem cells due to maturation arrest or from dedifferentiation of mature cells. Research on hepatic stem cells in hepatocarcinogenesis has entered a new era of controversy, excitement and great expectations…The two major hypotheses about the cellular origination of HCC have been discussed for almost 20 years. Debate has centered on whether or not HCC originates from the differentiation block of stem cells or dedifferentiation of mature cells. Recent research suggests that HCC may originate from the transdifferentiation of bone marrow cells. In fact, there might be more than one type of carcinogen target cell. The argument about the origination of HCC becomes much clearer when viewed from this viewpoint: poorly differentiated HCC originate from bone marrow stem cells and oval cells, while well-differentiated HCC originates from mature hepatocytes…The cellular events during hepatocarcinogenesis illustrate that HCC may arise from cells at various stages of differentiation in the hepatocyte lineage. There are four levels of cells in the hepatic stem cell lineage: bone marrow cell, hepato-pancreas stem cell, oval cell and hepatocyte. HSC and the liver are known to have a close relationship in early development. Bone marrow stem cells could differentiate into oval cells, which could differentiate into heptatocytes and duct cells. The development of pancreatic and liver buds in embryogenesis suggests the existence of a common progenitor cells to both the pancreas and liver. All of the four levels of cells in the stem cell lineage may be targets of hepatocarcinogenesis.

Along with the cell types described as possible targets and participants in HCC, Best and Coleman (2007) described yet another type of cell in the liver that can respond to hepatocellular injury, which they term small hepatocyte-like progenitor cells and conclude that they are not the progeny of oval cells, but represent a distinct liver progenitor cell population. Another potential regenerative cell is the small hepatocyte-like progenitor cell (SHPC). SHPCs share some phenotypes with hepatocytes, fetal hepatoblasts, and oval cells, but are phenotypically distinct. They express markers such as albumin, transferring, and alpha-fetoprotein (AFP) and possess bile canaliculi and store glycogen.

A recent review by Roskams et al. (2004) provided a current view of the "oval cell" its location and human equivalent. They concluded that:

> while similarities exist between the progenitor cell compartment of human and rodent livers, the different rodent models are not entirely comparable with the

> human situation, and use of the same term has created confusion as to what characteristics may be expected in the human ductular reaction. For example, a defining feature of oval cells in many rodent models of injury is production of alpha-fetoprotein, whereas ductular reactions in humans rarely display such expression. Therefore we suggest that the "oval cell" and "oval –like cell" no longer be used in description of human liver.

In the chronic hepatitis and cancer model of Vig et al. (2006), it is not the oval cells or SHPCs that are proliferating but the mature hepatocytes, thus supporting theories that it is not only oval cells that are causing proliferations leading to cancer. Vig et al. (2006) also reported that studies in mice and humans indicate that oval cells also may give rise to liver tumors and that oval cells commonly surround and penetrate human liver tumors, including those caused by hepatitis B. Tarsetti et al. (1993) noted that although some studies have suggested that oval cells are directly involved in the formation of HCC, others assert that HCC originates from preneoplastic foci and nodules derived from hepatocytes and report that HCC evolved in their model of liver damage from hepatocytes, presumably hepatocellular nodules, and not from oval cells. They also suggested that proliferation alone may not lead to cancer. Recent studies that follow the progression of hepatocellular nodules to HCC in humans (see Section E.3.1.8) suggest an evolution from nodule to tumor.

E.3.1.5. Status of Mechanism of Action for Human HCC

The underlying molecular mechanisms leading to hepatocarcinogenesis remain largely unclear (Yeh et al., 2007). Although HCC is multistep, and its appearance in children suggest a genetic predisposition exists, the inability to identify most of the predisposing genes and how their altered expression relates to histological lesions that are the direct precursors to HCC, has made it difficult to identify the rate limiting steps in hepatocarcinogenesis (Feitelson et al., 2002). Calvisi et al. (2007) report that although the major etiological agents have been identified, the molecular pathogenesis of HCC remains unclear and that while deregulation of a number of oncogenes (e.g., c-Myc, cyclin D1 and β-catenin and tumor suppressor genes including $P16^{INK4A}$, p53, E-cadherin, DLC-1, and pRb) have been observed at different frequencies in HCC, the specific genes and the molecular pathways that play pivotal roles in liver tumor development have not been identified. Indeed rather than simple patterns of mutations, pathways that are common to cancer have been identified through study of tumors and through transgenic mouse models. Branda and Wands (2006) stated that the molecular factors and interactions involved in hepatocarcinogenesis are still poorly understood but are particularly true with respect to genomic mutations, "as it has been difficult to identify common genetic changes in >20 to 30% of tumors." As well as phenotypically heterogeneous, "it is becoming clear that HCCs are genetically heterogeneous tumors." The descriptions of heterogeneity of tumors and of pathway disruptions common to cancer are also shown for liver

tumors (see Sections E.3.1.6 and E.3.1.8). However, many of these studies focused on the end process and of examination of the genomic phenotype of the tumor for inferences regarding clinical course, aggressiveness of tumor, and consistency with other forms of cancer. As stated above, the events that produce these tumors from patients with conditions that put them at risk, are not known.

El-Serag and Rudolph (2007) suggested that risk of HCC increases at the cirrhosis stage when liver cell proliferation is decreased and that acceleration of carcinogenesis at this stage may result from telomere shortening (resulting in limitations of regenerative reserve and induction of chromosomal instability), impaired hepatocyte proliferation (resulting in cancer induction by loss of replicative competition), and altered milieu conditions that promote tumor cell proliferation.

> When telomeres reach a critically short length, chromosome uncapping induces DNA damage signals, cell-cycle arrest, senescence, or apoptosis. Telomeres are critically short in human HCC and on the single cell level telomere shortening correlated with increasing aneuploidy in human HCC…Chemicals inhibiting hepatocyte proliferation accelerate carcinogen-induced liver tumor formation in rats as well as the expansion and transformation of transplanted hepatocytes. It is conceivable that abnormally proliferating hepatocytes would not expand in healthy regenerating liver but would expand quickly and eventually transform in the growth restrained cirrhotic liver….Liver mass is controlled by growth factors – mass loss through could provide a growth stimulatory macroenvironment. For the microenvironment, cirrhosis activates stellate cells resulting in increased production of extracellular matrix proteins, cytokines, growth factors, and products of oxidative stress.

Like other cancers, genomic instability is a common feature of human HCC with various mechanisms thought to contribute, including telomere erosion, chromosome segregation defects, and alteration in DNA damage-response pathways. In addition to genetic events associated with the development of HCC (p53 inactivation, mutation in β-catenin, overexpression of ErbB receptor family members, and overexpression of the MET receptor whose ligand is HGF), various cancer-relevant genes seem to be targeted on the epigenetic level (methylation) in human HCC (Farazi and DePinho, 2006). Changes in methylation have been detected in the earliest stages of hepatocarcinogenesis and to a greater extent in tumor progression (Lee et al., 2003). Seitz and Stickel (2006) report that aberrant DNA hypermethylation (a silencing effect on genes) may be associated with genetic instability as determined by the loss of heterozygosity and microsatellite instability in human HCC due to chronic viral hepatitis and that modifications of the degree of hepatic DNA methylation have also been observed in experimental models of chronic alcoholism.

Farazi and DePinho (2006) reported that two of the key molecules that are involved in DNA damage response, p53 and BRCA2, seem to have roles in destabilizing the HCC genome (Gollin, 2005). The inactivation of p53 through mutation or viral oncoprotein sequestration is a

common event in HCC and p53 knock in mouse models containing dominant point mutations have been shown to cause genomic instability. However, Farazi and DePinho (2006) noted that despite documentation of deletions or mutations in these and other DNA damage network genes, their direct roles in the genomic instability of HCC have yet to be established in many genetic model systems.

Telomere shortening has been described as a key feature of chronic hyperproliferative liver disease (Rudolf and DePinho, 2001; Miura et al., 1997; Urabe et al., 1996; Kitada et al., 1995), specifically occurring in the hepatocyte compartment. These observations have fueled speculation that telomere shortening associated with chronic liver disease and hepatocyte turnover contribute to the induction of genomic instability that drives human HCC (Farazi and DePinho, 2006). Defects in chromosome segregation during mitosis result in aneuploidy, a common cytogenetic feature of cancer cell including HCC (Farazi and DePinho, 2006).

Several studies have attempted to categorize genomic changes in relation to tumor state. In general, high levels of chromosomal instability seem to correlate with the de-differentiation and progression of HCC (Wilkens et al., 2004). Several studies have suggested certain chromosomal changes to be specific to dysplastic lesions, early-stage and late-stage HCCs, and metastases. It is important to note that the studies that have attempted to compare genomic profiles and tumor state are few in number, often did not classify HCCs on the basis of etiology, and used relatively low-resolution genome-scanning platforms (Farazi and DePinho, 2006). Farazi and DePinho (2006) noted that it should be emphasized that although genome etiology correlates reported in some studies are intriguing, several studies have failed to uncover significant differences in genomic changes between different etiological groups, although the outcome might related to small sample sizes and the low-resolution, genome scanning platform used.

E.3.1.6. Pathway and Genetic Disruption Associated with HCC and Relationship to Other Forms of Neoplasia

In their landmark paper, Hanahan and Weinberg (2000) suggested that the vast catalog of cancer cell genotypes was a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth signals), elevation of programmed cell death (apoptosis), limitless replication potential, sustained angiogenesis, and tissue invasion and metastasis. They proposed that these six capabilities are shared in common by most, and perhaps all, types of human tumors and, while virtually all cancers must acquire the same six hallmark capabilities, their means of doing so would vary significantly, both mechanistically and chronologically. It was predicted that in some tumors, a particular genetic lesions may confer several capabilities simultaneously, decreasing the number of distinct mutational steps required to complete tumorigenesis. Loss of the p53 tumor suppressor was cited as an example that could facilitate both angiogenesis and

resistance to apoptosis and to enable the characteristic of genomic instability. The paths that cells could take on their way to becoming malignant were predicted to be highly variable, and within a given cancer type, mutation of a particular target genes such as ras or p53 could be found only in a subset of otherwise histologically identical tumors. Furthermore, mutations in certain oncogenes and tumor suppressor genes could occur early in some tumor progression pathways and late in others. Genes known to be functionally altered in "cancer" were identified as including Fas,Bcl2, Decoy R, Bax, Smads, TFGβR, p15, p16, Cycl D, Rb, human papilloma virus E7, ARF, PTEN, Myc, Fos, Jun, Ras, Abl, NF1, RTK, transforming growth factor alpha (TGF-α), Integrins, E-cadherin, Src, β-catenin, APC, and WNT.

Branda and Wands (2006) reported that two signal transduction cascades that appear to be very important are insulin/IFG-1/IRS-1/MAPK and Wnt/Frizzled/β-catenin pathways, which are activated in over 90% of HCC tumors (Branda and Wands, 2006). Feitelson et al. (2002) reported that:

> In addition to NF-κB, up-regulated expression of rhoB has been reported in some HCCs. RhoB is in the *ras* gene family, is associated with cell transformation, and may be a common denominator to both viral and non-viral hepatocarcinogenesis. Activation of ras and NF-κB, combined with down regulation of multiple negative growth regulatory pathways, then, may contribute importantly to early steps in hepatocarcinogenesis. Thus viral proteins may alter the patterns of hepatocellular gene expression by transcriptional trans-regulation…Another early event appears to involve the mutation of β-catenin, which is a component of the Wnt signal transduction pathway whose target genes include c-myc, c-jun, cyclin D1, fibronectin, the connective tissue growth factor WISP, and matrix metaolloproteinases.

Boyault et al. (2007) reported that:

> altogether, the principle carcinogenic pathways known to be deregulated in HCC are inactivation of TP53, Wnt/wingless activation mainly through CTNNB1 mutations activating β-catenin- and AXIN1-inactivating mutations, retinoblastoma inactivation through RB1 and CDKN2A promoter methylation and rare gene mutations, insulin growth factor activation through IGF2 overexpression, and IGF2R-inactiving mutations.

El-Serag and Rudolph suggested that "in general, the activation of oncogenic pathways in human HCC appears to be more heterogeneous compared with other cancer types." El-Serag and Rudolph (2007) reported that the p53 pathway is a major tumor-suppressor pathway that: (1) limits cell survival and proliferation (replicative senescence) in response to telomere shortening; (2) induces cell-cycle arrest in response to oncogene activation (oncogene-induced senescence); (3) protects genome integrity; and (4) is affected at multiple levels in human HCC.

"p53 mutations occur in aflatoxin induced HCC (>50%) and with lower frequency (20–40%) in HCC not associated with aflatoxin." In addition,

> the vast majority of human HCC overexpresses gankyrin, which inhibits both Rb checkpoint and p53 checkpoint function…The p16/Rb checkpoint is another major pathway limiting cell proliferation in response to telomere shortening, DNA damage, and oncogene activation. In human HCC the Rb pathway is disrupted in more than 80% of cases, with repression of p16 by promoter methylation being the most frequent alteration. Moreover, expression of gankyrin (an inhibitor of p53 and Rb checkpoint function) is increased in the vast majority of human HCCs, indicating that the Rb checkpoint is dysfunctional in the vast majority of human HCCs…The frequent inactivation of p53 in human HCC indicates that abrogation of p53-dependent apoptosis could promote hepatocarcinogenesis. The role of impairment of p53-independent apoptosis for hepatocarcinogenesis remains to be defined…Activation of the β-catenin pathway frequently occurs in mouse and human HCC involving somatic mutations, as well as transcriptional repression of negative regulators. An activation of the Akt signaling and impaired expression of phosphatase and tensin homolog (PTEN) (a negative regulator of Akt) have been reported in 40-60% of Human HCC.

They suggested that although Myc is a potent oncongene inducing hepatocarcinogenesis in mouse models, the data on human HCC are heterogeneous and further studies are required.

E.3.1.7. Epigenetic Alterations in HCC

The molecular pathogenesis of HCC remains largely unknown, but it is presumed that the development and progression of HCC are the consequence of cumulative genetic and epigenetic events similar to those described in other solid tumors (Calvisi et al., 2006). Calvisi et al. (2007) provided a good summary of DNA methylation status and cancer as well as its status in regard to HCC:

> Aberrant DNA methylation occurs commonly in human cancers in the forms of genome-wide hypomethylation and regional hypermethylation. Global DNA hypomethylation (also known as demethylation) is associated with activation of protooncogenes, such as c-Jun, c-Myc, and c-HA-Ras, and generation of genomic instability. Hypermethylation on CpG islands located in the promoter regions of tumor suppressor genes results in transcriptional silencing and genomic instability. CpG hypermethylation (also known as de novo methylation) acts as an alternative and/or complementary mechanisms to gene mutations causing gene inactivation, and it is now recognized as an important mechanism in carcinogenesis. Although the mechanism(s) responsible for de novo methylation in cancer are poorly understood, it has been hypothesized that epigenetic silencing depends on activation of a number of proteins known as DNA methyltransferases (DNMTs) that posses de novo methylation activity. The importance of DNMTs in CpG methylation was substantiated by the observation that genetic disruption of both DNMT1 and DNMT3b genes in HCT116 cell lines nearly eliminated

methyltransferase activity. However, more recent findings indicate that the HCT116 cells retain a truncated, biologically active form of DNMT1 and maintain 80% of their genomic methylation. Further reduction of DNMT1 levels by a siRNA approach resulted in decreased cell viability, increased apoptosis, enhanced genomic instability, checkpoint defects, and abrogation of replicative capacity. These data show that DNTM1 is required for cell survival and suggest that DNTM1 has additional functions that are independent of its methyltransferase activity. Concomitant overexpression of DNMT1, -3A, and -3b has been found in various tumors including HCC. However, no changes in the expression of DNMTs were found in other neoplasms, such as colorectal cancer, suggesting the existence of alternative mechanisms. In HCC, a novel DNMT3b splice variant, known as DNMT3b4 is overexpressed. DNMT3b4 lacks DNMT activity and competes with DNMT2b3 for targeting of pericentromeric satellite regions in HCC, resulting in DNA hypomethylation of these regions and induction of chromosomal instability, further linking aberrant methylation and generation of genomic alterations.

It is now well accepted that methylation changes occur early and ubiquitously in cancer development. The case has been made that tumor cell heterogeneity is due, in part, to epigenetic variation in progenitor cells and that epigenetic plasticity together with genetic lesions drive tumor progression (Feinberg et al., 2006).

A growing number of genes undergoing aberrant CpG island hypermethylation in HCC have been discovered, suggesting that de novo methylation is an important mechanism underlying malignant transformation in the liver. However, most of the previous studies have focused on a single or a limited number of genes, and few have attempted to analyze the methylation status of multiple genes in HCC and associated chronic liver diseases. In addition, the functional consequence(s) of global DNA hypomethylation and CpG island hypermethylation in human liver cancer has not been investigated to date. Furthermore, to our knowledge no comprehensive analysis of CpG island hypermethylation involving activation of signaling pathways has been performed.

Calvisi et al. (2007) reported that global gene expression profiles show human HCC to harbor common molecular features that differ greatly from those of nontumorous surrounding tissues, and that human HCC can be subdivided into two broad but distinct subclasses that are associated with length of patient survival. They further suggested that aberrant methylation is a major event in both early and late stages of liver malignant transformation and might constitute a critical target for cancer risk assessment, treatment, and chemoprevention of HCC. Calvisi et al. (2007) conducted analysis of methylation status of genes selected based on their capacity to modulate signaling pathways (Ras, Jak/Stat, Wingless/Wnt, and RELN) and/or biologic features of the tumors (proliferation, apoptosis, angiogenesis, invasion, DNA repair, immune response, and detoxification). Normal livers were reported to show the absence of promoter methylation for all genes examined. At least 1 of the genes involved in inhibition of Ras (ARH1, CLU,

DAB2, hDAB21P, HIN-1, HRASL, LOX, NORE1A, PAR4, RASSF1A, RASSF2, RASSF3, RASSF4, RIG, RRP22, and SPRY2 and -4), Jak/Stat (ARH1,CIS, SHP1, PIAS-1, PIAS-γ, SOCS1, -2, and -3, SYK, and GRIM-19), and Wnt/β-catenin (APC, E-cadherin, γ-catenin, SFRP1, -2, -4, and -5, DKK-1 and -3, WIF-1 and HDPR1) pathways were affected by de novo methylation in all HCC. A number of these genes were also reported to be highly methylated in the surrounding nontumorous liver. In contrast, inactivation of at least one of these genes implicated in the RELN pathway (DAB1, reelin) was detected differentially in HCC of subclasses of tumors that had differences in tumor aggressiveness and progression. Epigenetic silencing of multiple tumor suppressor genes maintains activation of the Ras pathway with a major finding in the Calvisi et al. (2007) study to be the concurrent hypermethylation of multiple inhibitors of the Ras pathway with Ras was significantly more active in HCC than in surrounding or normal livers. Also important was the finding that no significant associations between methylation patterns and specific etiologic agents (i.e., HVB, HVC, ethanol, etc.) were detected, further substantiating the conclusion that aberrant methylation is a ubiquitous phenomenon in hepatocarcinogenesis.

> Current evidence suggests that hypomethylation might promote malignant transformation via multiple mechanisms, including chromosome instability, activation of protooncogenes, reactivation of transposable elements, and loss of imprinting…The degree of DNA hypomethylation progressively increased from nonneoplastic livers to fully malignant HCC, indicating that genomic hypomethylation is an important prognostic factor in HCC, as reported for brain, breast, and ovarian cancer.

Calvisi et al. (2007) also reported that regional CpG hypermethylation was also enhanced during the course of HCC disease and that the study of tumor suppressor gene promoters showed that CpG methylation was frequently detected both in surrounding nontumorous livers and HCC.

E.3.1.8. Heterogeneity of Preneoplastic and HCC Phenotypes

A very important issue for the treatment of HCC in humans is early detection. Research has focused on identification of lesions that will progress to HCC and to also determine from the phenotype of the nodule and genetic expression its cell source, likely survival, and associations with etiologies and modes of action. As with rodent models where preneoplastic foci have been observed to be associated with progression to adenoma and carcinoma, nodules observed in humans with high risk for HCC have been observed to progress to HCC. In humans, histomorphology of HCC is notoriously heterogeneous (Yeh et al., 2007). Although much progress has been made, there is currently not universally accepted staging system for HCC partly because of the natural course of early HCC is unknown and the natural progression of intermediated and advanced HCC are quite heterogeneous (Thorgeirsson, 2006). Nodules are

heterogeneous as well, with differences in potential to progress to HCC. Chen et al. (2002b) reported that standard clinical pathological classification of HCC has limited valued in predicting the outcome of treatment as the phenotypic diversity of cancer is accompanied by a corresponding diversity in gene expression patterns. There is also histopathological variability in the presentation of HCC in geographically diverse regions of the world with some slow growing, differentiated HCC nodules surrounded by a fibrous capsule are common among Japanese but, in contrast, a "febrile" form of HCC, characterized by leukocytosis, fever, and necrosis within a poorly differentiated tumor to be common in South African blacks (Feitelson et al., 2002).

A multistep process is suggested histologically, where HCC appears within the context of chronic hepatitis and/or cirrhosis within regions of the liver cell dysplasia or adenomatous hyperplasia (Feitelson et al., 2002). Kobayashi et al. (2006) reported that the higher the grade of the nodule, the higher the percentage that will progress to HCC with 18.8% of all nodules and regenerative lesions going on to become HCC, 53.3% remaining unchanged, and 27.9% disappearing in the observation period of 0.1–8.9 years. Borzio et al. (2003) reported that the rate of liver malignant transformation was 40% in larger regenerative nodules, low-grade dysplastic, and high-grade dysplastic nodules with higher grade of dysplasia extranodular detection of large cell change and hyperchronic pattern associated with progression to HCC. Yeh et al. (2007) reported that nuclear staining for Ki-67 and Topo II-α (a nuclear protein targeted by several chemotherapeutic agents) significantly increased in the progression from cirrhosis, through high-grade dysplastic nodules to HCC, whereas the scores for TGF-α in these lesions showed an inverse relationship. "In comparison with 18 HCC arising in noncirrhotic livers, the expression of TGF-α is significantly stronger in cirrhotic liver than in noncirrhotic parenchyma and its expression is also stronger in HCC arising in cirrhosis than in HCC arising in noncirrhotic patients." They concluded that initiation in cirrhotic and noncirrhotic liver may have different pathways with transforming growth factor-α (a mitogen activated the EFGR) playing a relative more important role in HCC from cirrhotic liver. Overexpression of TGF-α in the liver of transgenic mice induced increased proliferation, dysplasia, adenoma, and carcinoma. Yeh et al. (2007) concluded that such high-grade dysplastic nodules are precursor lesions in hepatocarcinogenesis and that TGF-α may play an important role in the early events of liver carcinogenesis.

Moinzadeh et al. (2005) reported in a meta-analysis of all available (n = 785) HCCs that gains and losses of chromosomal material were most prevalent in a number of chromosomes and that amplifications and deletions occurred on chromosomal arms in which oncogenes (e.g., MYC and 8q24) and tumor suppressor genes (e.g., RB1 on 13q14) are located as well as modulators of the WNT-signaling pathway. However, in multifocal HCC, nodules arising de novo within a single liver have a different spectrum of genetic lesions. "Hence, there are likely to be many paths to HCC, and this is why it has been difficult to assign specific molecular alterations to

changes in hepatocellular phenotype, clinical, or histopathological changes that accompany tumor development" (Feitelson et al., 2002).

Serum AFP is commonly used as a tumor marker for HCC. Several reports have linked HCC to cytokines in an attempt to find more specific markers of HCC. Jia et al. (2007) reported that AFP marker allows for identification of a small set of HCC patients with smaller tumors, and these patients have a relatively long-term survival rate following curative treatment.

> Presently the only approach to screen for the presence of HCC in high-risk populations is the combination of serum AFP and ultrasonagraphy. However, elevated AFP is only observed in about 60 to 70% of HCC patients and to a lesser extent (33-65%) in patients with smaller HCCs. Moreover, nonspecific elevation of serum AFP has been found in 15% to 58% of patients with chronic hepatitis and 11% to 47% of patients with liver cirrhosis.

Soresi et al. (2006) reported that serum IL-6 levels are low in physiological conditions, but increase considerably in pathological conditions such as trauma, inflammation, and neoplasia. In tumors, IL-6 may be involved in promoting the differentiation and growth of target cells. "Many works have reported high serum IL-6 levels in various lifer diseases such as acute hepatitis, primary biliary cirrhosis, chronic hepatitis (hepatitis C) and HCV-correlated liver cirrhosis and in hepatocellular carcinoma." Soresi et al. (2006) reported that patients with HCC group had higher IL-6 values than those with cirrhosis and that "higher-staged" patients had the highest IL-6 levels. Hsia et al. (2007) also examined IL-6, IL-10 and hepatocyte growth factor (HGF) as potential markers for HCC.

> The expression of IL-6 or IL-10 or higher level of HGF or AFP was observed only 0-3% of normal subjects. Patients with HCC more frequently had higher IL-6 and IL-10 levels, where as HGF levels in HCC patients were not significantly elevated compared to patients with chronic hepatitis or non-HCC tumors (but greater than controls). Among patients with low AFP level, IL-6 or IL-10 expression was significantly associated with the existence of HCC. Patients with large HCC (>5 cm) more often had increased IL-6, IL-10 or AFP levels. Serum levels of IL-6 and IL-10 are frequently elevated in patients with HCC but not in benign liver disease or non-HCC tumors.

Nuclear DNA content and ploidy have also been the subjects of several studies through the years for identification of pathways for prediction of survival or origin of tumors. Nakajima et al. (2004) report that p53 loss can contribute to the propagation of damaged DNA in daughter cells through the inability to prevent the transmission of inaccurate genetic material, considered to be one of the major mechanisms for the emergence of aneuploidy in tumors with inactivated p53 protein and the increasing ploidy in HCC was associated with disturbance in p53. McEntee et al. (1992) reported that specimens from 74 patients who underwent curative resection for

primary HCC and analyzed for DNA content, (i.e., tumors were classified as DNA aneuploid if a separate peak was present from its standard large diploid peak [2C] and tetraploid peak [4C]) 33% were DNA diploid, 30% were DNA tetraploid/polyploidy, and 37% were aneuploid of the primary tumors examined. Nontumor controls were diploid and survival was not different between patients with diploid vs. nondiploid tumors. Zeppa et al. (1998) reported ploidy in 84 HCCs diagnosed by fine-needle aspiration biopsy to have 68 cases that were aneuploid and 16 euploid (9 diploid and 7 polyploid), with median survival of 38 months for patients with diploid HCC and 13 months for aneuploid HCC. Lin et al. (2003) reported in their study of fine-needle aspiration of HCC that:

> the ratio of S and G2/M periods of DNA, which reflect cell hyperproliferation, in the group with HCC tumors>3 cm in diameter were markedly higher than those of the group with nodules<3 cm in diameter and the group with hyperplastic nodules…DNA analysis of aspiration biopsy tissues acquired from intrahepatic benign hyperplastic nodules showed steady diploid (2c) peak that stayed in G1 period. DNA analysis of aspiration biopsy tissues acquired from HCC nodules showed S period of hyperproliferation and G2/M period. The DNA analysis of HCC nodules showed aneuploid peak.

They concluded that in regard to the biological behavior of the cell itself, that the normal tissue, reactive tissue, and benign tumor all have normal diploid DNA but, like most other malignant tumors, "HCC appears to have polyploid DNA, especially aneuploid DNA."

Attallah et al. (1999) reported small needle liver biopsy data to show HCC to be 21.4% diploid, 50% aneuploid, and 28.6% tetraploid and that higher ploidies (aneuploid and tetraploid) were observed in human liver cancer than residual tissues, although in some cases, there was increased aneuploidy (cirrhosis, 37%, hepatitis ~50%). Of note for the study is the lack of appropriate control tissue and uncertainty as to how some of their diploid cells could have been binucleate tetraploid cells. Anti et al. (1994) reported reduction in binuclearity in the chronic hepatitis and cirrhosis groups that was significantly correlated with a rise in the diploid/ polyploidy ratio and that precancerous and cancerous nodules within cirrhotic liver show an increased tendency toward diploidy or the emergence of aneuploid populations. They noted that a number of investigators have reported significantly increased hepatocyte diploidization during the early stages of chemically induced carcinogenesis in rat liver, but other experimental findings indicate that malignant transformation can occur after any type of alteration in ploidy distribution.

On the other hand, Melchiorri et al. (1994) noted that several studies using flow cytometric or image cytometric methods reported high DNA ploidy values in 50–77% of the examined HCCs and that the presence of aneuploidy was significantly related to a poor patient prognosis. They reported that the DNA content of mononucleated and binucleated hepatocytes, obtained by ultrasound-guided biopsies of 10 macroregenerative nodules without histologic signs

of atypia from the lesions with the greater fraction of mononucleated hepatocytes were diagnosed as HCCs during the clinical follow-up, with results also suggesting that diploid and tetraploid stem cell lines are the main lines of the HCCs as well as a reduction in the percentage of binucleated hepatocytes in HCC. Gramantieri et al. (1996) reported that the percentage of binucleated cells was reduced in most of the HCC that they studied (i.e., the mean percentage of binucleated cells 9% in comparison to 24% found in normal liver) and that most HCC, as many other solid neoplasms, showed altered nuclear parameters.

Along with reporting pathways that are perturbed in HCC, emerging evidence also shows that signatures of pathway are predictive of clinical characteristics of HCC. A number of studies have examined gene expression in tumors to try to determine which pathways may have been disturbed in an attempt to predict survival and treatment options for the patients and to investigate possible modes of action for the tumor induction and progression. Chen et al. (2002b) described a systematic characterization of gene expression patterns in human liver cancers using cDNA microarrays to study tumor and nontumor liver tissues in HCC patients, and of note did quality assurance on their microarray chips (many studies do not report that they have done so), and examined the effects of hepatitis virus on its subject and identified people with it. Most importantly, Chen et al. (2002b) provided phenotypic anchoring of each tumor with its genetic profile rather than pooling data.

The hierarchical analysis demonstrated that clinical samples could be divided into two major clusters, one representing HCC samples and the other with a few exceptions, representing nontumor liver tissues. Most importantly, expression patterns varied significantly among the HCC and nontumor liver samples and that samples from HBV-infected, hepatitis C virus infected, and noninfected individuals were interspersed in the HCC branch. Thus, tumors from people infected with HVB, HVC, and noninfected people with HCC were interspersed in the HCC pattern and could be discerned based on etiology. One cluster of genes was highly expressed in HCC samples compared with nontumor liver tissues included a "proliferation cluster" comprised of genes whose functions are required for cell-cycle progression and whose expression levels correlate with cellular proliferation rates with most of the genes in this cluster are specifically expressed in the G2/M phase. Gene profiles for HCC were consistent with fewer molecular features of differentiated normal hepatocytes.

Chen et al. (2002b) noted that both normal and liver tumors are complex tissue compose of diverse cells and that distinct patterns of gene expression seemed to provide molecular signatures of several specific cell types including expression of two clusters of genes associated with T and B lymphocytes, presumably reflecting lymphocytic infiltration into liver tissues, and genes associated with stellate cell activation. This important finding acknowledges that HCC is not only heterogeneous in hepatocyte phenotype but is also made up of many other nonparenchymal cell types and that gene expression patterns reflect that heterogeneity. A gene cluster was also identified at a higher level in HCC that included several genes typically

expressed in endothelial cells, including CD34, which is expressed in endothelial cells in veins and arteries but not in the endothelial cells of the sinusoids in nontumor liver and which may reflect disruption of the molecular program that normally regulate blood vessel morphogenesis in the liver.

Of great importance was the investigation by Chen et al. (2002b) of whether samples from multiple sites in a single HCC tumor, or multiple separate tumor nodules in one patient, would share a recognizable gene expression signature. With a few instructive exceptions, all of the tumor samples from each patient clustered were reported to cluster together. To further examine the relationship among multiple tumor samples from individual patients, they calculated the pairwise comparison for all pairs of samples and samples some primary tumors multiple times. Tumor patterns of gene expression were more highly correlated with those seen in samples from the same patient than other patients but every tumor had a distinctive and characteristic gene expression pattern, recognizable in all samples taken from different areas of the same tumor.

For multiple discrete tumor masses obtained from six patients, three of these patients had multiple tumors with a shared distinctive gene expression pattern but in three other patients, expression patterns varied between tumor nodules and the difference providing new insights into the sources of variation in molecular and biological characteristics of cancers. Thus, in some patients, multiple tumors were from the same clone, as demonstrated by a similar gene expression profile, but for some patients, multiple tumors were arising from differing clones within the same liver. In regard to whether the distinctive expression patterns characteristic of each tumor reflect the individuality of the tumor or are determined by the patient in whom the tumor arose, analysis of the expression patterns observed in the two tumor nodules from one patient showed that the two tumors were not more similar than those of an arbitrary pair of tumors from different patients. These results show the heterogeneity of HCC and that "one gene pattern" will not be characteristic of the disease.

However, HCC did have a pattern that differed from other cancers. Chen et al. (2002b) analyzed the expression patterns of 10 randomly selected HCC samples and 10 liver metastases of other cancers and reported that the HCC samples and the metastatic cancers clustered into two distinct groups, based on difference in their patterns of gene expression. Although some of the HCC samples were poorly differentiated and expressed the genes of the liver-specific cluster at very low levels compared to with either normal liver or well-differentiated HCC, the genes of the liver-specific cluster were reported to be consistently expressed at higher levels in HCC than in tumors of nonliver origin. Metastatic cancers originating from the same tissue typically clustered together, expressing gene characteristic of the cell types of origin. Thus, liver cancer was distinguishable from other cancer even though very variable in expression and differentiation state.

In an attempt to create molecular prognostic indices that can be used for identification of distinct subclasses of HCC that could predict outcome, Lee et al. (2004a) reported two subclasses of HCC patients characterized by significant differences in the length of survival. They also identified expression profiles of a limited number of genes that accurately predicted the length of survival. Total RNAs from the 19 normal livers, including "normal liver in HCC patients," were pooled and used as a reference for all microarray experiments and thus variations between patients, and especially differences due to conditions predisposing HCC, were not determined. DNA microarray data using hierarchical clustering was reported to yield two major clusters, one representing HCC tumors, and the other representing nontumor tissues with a few exceptions that were not characterized by the authors. Lee et al. (2004a) reported that, along with two distinctive subtypes of gene expression patterns in HCC, there was heterogeneity among HCC gene expression profiles and that one group had an overall survival time of 30.8 months and the other 83.7 months. Only about half the patients in each group were reported to have cirrhosis. Expression of typical cell proliferation markers such as PCNA and cell cycle regulators such as *CDK4*, *CCNB1*, *CCNA2*, and *CKS2* was greater in one class than the other of HCC.

The report by Boyault et al. (2007) attempted to compare etiology and genetic characterization of the tumors they produce and confirmed the heterogeneity of HCC, some without attendant genomic instability. Boyault et al. (2007) reported that genetic alterations are indeed closely associated with clinical characteristics of HCC that define two mechanisms of hepatocarcinogenesis.

> The first type of HCC was associated with not only a high level of chromosome instability and frequent TP53 and AXIN1 mutations but also was closely linked to HBV infections and a poor prognosis. Conversely, the second subgroup of HCC tumors was chromosome-stable, having a high incidence of activating β-catenin alteration and was not associated with viral infection.

Boyault et al. (2007) reported that in a series of 123 tumors, mutations in the CTNNB1 (encoding β-catenin), TP53, ACIN1, TCF1, PIK3CA and KRAS genes in 34, 31, 13, 5, 2, and 1 tumors were identified, respectively. No mutations were found in NRAS, HRAS, or EGFR. Hypermethylation of the CDKN2A and CDH1 promoter was identified in 35 and 16% of the tumors, respectively. Boyault et al. (2007) grouped tumors by genomic expression as well as other factors. HCC groups associated with high rate of chromosomal instability were reported to be enriched with overexpression of cell-cycle/proliferation/DNA metabolism genes. They concluded that "the primary clinical determinant of class membership is HBV infection and the other main determinants are genetic and epigenetic alterations, including chromosome instability, CTNNB1 and TP53 mutations, and parental imprinting. Tumors related to HCV and alcohol abuse were interspersed across subgroups G3-G6." Boyault et al. (2007) suggested that their results indicated that HBV infection early in life leads to a specific type of HCC that has

immature features with abnormal parental gene imprinting selections, possibly through the persistence of fetal hepatocytes or alternatively through partial dedifferentiation of adult hepatocytes. "These G1 tumors are related to high-risk populations found in epidemiological studies."

E.3.2. Animal Models of Liver Cancer

There are obvious differences between rodents and primate and human liver, and there is a difference in background rates of susceptibility to hepatocarcinogenesis. With strains of mice, there are large differences in responses to hepatotoxins (e.g., acetaminophen) and to hepatocarcinogens as well as background rates of hepatocarcinogenecity. Boyault et al. (2007) reported that modulators of murine hepatocarcinogenesis, such as diet, hormones, oncogenes, methylation, imprinting, and cell proliferation/apoptosis are among multiple mechanistically associated factors that impact this target organ response in control as well as in treated mice, and suggested that there is no one simple paradigm to explain the differential strain sensitivity to hepatocarcinogenesis. Because of the variety of studies with differing protocols used to generate susceptibility data, direct comparisons among strains and stocks is problematic but in regard to susceptibility to carcinogenicity, the C3H/HeJ and C57BL/6J mouse have been reported to have up to a 40-fold difference in liver tumor multiplicity (Boyault et al., (2007).

However, as noted above, TCE causes liver tumors in C6C3F1 and Swiss mice with studies of TCE metabolites DCA, TCA, and CH suggesting that both DCA and TCA are involved in TCE-induced liver tumorigenesis. Many effects reported in mice after DCA exposure are consistent with conditions that increase the risk of liver cancer in humans and can involve GST Xi, histone methylation, and overexpression of insulin-like growth factor-II (IGF-II) (Caldwell and Keshava, 2006). The heterogeneity of liver phenotype observed in mouse models is also consistent with human HCC. These data lend support to the qualitative relevance of the mouse model for TCE-induced cancer risk.

Bannasch et al. (2003) made important observations that have implications regarding the differences in susceptibility between rodent and human liver cancer. They stated that:

> Although the classification of such nodular liver lesions in rodents as hyperplastic or neoplastic has remained controversial, persistent nodules of this type are considered neoplasms, designated as adenomas. In human pathology, the situation appears to be paradoxical because adenomas are only diagnosed in the noncirrhotic liver, yet a confusing variety terms avoiding the clearcut classification as an adenoma has been created for nodular lesions in liver cirrhoses, not withstanding that the vast majority hepatocellular carcinomas develop in cirrhotic livers. Even if a portion of these nodular lesions would be regarded as adenomas, being integrated into an adenoma-carcinoma sequence as observed in many animal experiments, clinical and epidemiological records of liver neoplasms, including both benign and malignant forms, would increase

> considerably. This would not only bring hepatic neoplasia further into focus of human neoplasia in general, but also shed new light on the classification of some chemicals producing high incidence of liver neoplasms in rodents, but appearing harmless to humans according to epidemiological evaluations solely based on the incidence of hepatocellular carcinoma in exposed populations.

Thus, in humans, only HCCs are recorded, but in animals, adenomas are counted as neoplasms, indicating that the scope of the problem of liver cancer in humans may be underestimated.

Tumor phenotype differences have been reported for several decades through the work of Bannasch et al. The predominant cell line of foci of altered hepatocytes (FAH) have excess glycogen storage early in development that appears to be similar to that shown by DCA treatment. Bannasch et al. (2003) reported that "the predominant glycogenotic-basophilic cell line FAH reveals that there is an overexpression of the insulin receptor, the IGF-1 receptor, the insulin receptor substrates-1/2 and other components of the insulin-stimulated signal transduction pathway." Bannasch et al. (2003) stated that foci of this type have increased expression of GST-π and insulin has also been shown to induce the expression of GST-pi, but that hyperinsulin-induced foci do not show increased GST-π. Cellular dedifferentiation during progression from glycogenotic to basophilic cell populations is associated with downregulation in insulin signaling. The amphophilic-basophilic cell lineage of peroxisome proliferators and hepadnaviridae were reported to have foci that mimic effects of thyroid hormone with mitochondrial proliferation and activation of mitochondrial enzymes. Bannasch et al. (2003) stated that:

> the unequivocal separation of 2 types of compounds, usually classified as initiators and promoters, remains a problem at the level of the foci because at least the majority of chemical hepatocarcinogens seem to have both initiating and promoting activity, which may differ in quantitative rather than qualitative terms from one compound to another…Whereas genetic mutations have been predominantly postulated to initiate hepatocarcinogenesis for many years, more recently epigenetic changes have been increasingly discussed as a plausible cause of the evolution of preneoplastic foci characterized by metabolic changes including the expression of GSTpi.

Su and Bannasch (2003) reported that glycogen-storing foci represent early lesions with the potential to progress to more advance glycogen-poor basophilic lesions through mixed-cell foci and resulting hyperproliferative lesions and are associated with HCC in man. Small-cell change (SCC) of liver parenchyma (originally called liver cell dysplasia of small cell size) is reported to share cytological and histological similarities to early well defined HCC. Close association between SCC and more advanced (basophilic) foci indicates that foci often progress to HCC through SCC in humans. SCC was reported to be present in all basophilic foci.

Previous studies were cited that showed that the biochemical phenotype of human FAH, mainly including glycogen storing clear cell foci and clear cell-predominated mixed cell foci, were observed in >50% of cirrhotic livers with or without HCC. FAH of clear and mixed cell types were observed in almost all livers bearing HCC, and in chronic liver diseases without HCC but at a lower frequency. Su and Bannasch (2003) reported that:

> the finding of mixed cell foci (MCF) mainly in livers with high-risk or cryptogenenic cirrhosis indicates that these are more advanced precursor lesions in man, in line with earlier observations in experimental animals. Considering their preferential emergence in cirrhotic livers of the high-risk group, their unequivocally elevated proliferative activity, and the resulting large size with frequent nodular transformation, we suggest that mixed cell populations are endowed with a high potential to progress to HCC in humans, as previously shown in rats.

In human HCC, irregular areas of liver parenchyma with marked cytoplasmic amphophilia, phenotypically similar to the amphophilic preneoplastic foci in rodent liver exposed to different hepatocarcinogenic chemicals (e.g., DHEA a peroxisome proliferator) or the hepadnaviruses, were reported to present in 45% of the specimens from cirrhotic livers examined. "However, more data are needed to elucidate the nature of the oncocytic and amphophilic lesions regarding their role in HCC development."

With respect to the ability respond to a mitogenic stimulus, differences between primate and rodent liver response to a powerful stimulus, such as partial hepatectomy, have been noted that indicate that primate and human liver respond differently (and much more slowly) to such a stimulus. Gaglio et al. (2002) reported after 60% partial hepatectomy in Rhesus macaques (*Macaca mulatto*), the surface area of the liver remnant was restored to its original preoperative value over a 30-day period. The maximal liver regeneration occurred between days 14 and 21, with thickening of liver cell plates, binucleation of hepatocytes, Ki-67 and PCNA expression (occurring in hepatocytes throughout the lobule at a maximum labeling index of 30%), and mitoses parallel increased most prominently between posthepatectomy days 14 and 30.

However, cytokines associated with inducing proliferation were elevated much earlier. TGF-α, IL-6, HGF, IL-6, and TNF-α mRNA persisted until Day 14, with peak elevations of IL-6 and TNF-α, occurring 24 hours later surgery, and IL-6 reduced to control levels by day 14. Gaglio et al. (2002) suggested that their results clearly indicate that the pattern and timing of liver regeneration observed in this nonhuman primate model are significantly different when comparing different species (e.g., peak expression of Ki-67 in a 60% partial hepatectomy model in rats occurs within hours following partial hepatectomy) and that the difference in timing and pattern of maximal hepatocellular regeneration cannot be explained simply by differences in size of animals (e.g., 60% partial hepatectomy in dogs produced liver regeneration peaks at 72 hours with weights approximating the weights of the Rhesus macaques). They noted that previous

studies in humans, who underwent 40–80% partial hepatectomy, reveal a similar delay in peak liver regeneration based on changes in serum levels of ornithine decarboxylase and thymidine kinase, further highlighting significant interspecies differences in liver regeneration.

For C57BL/6 X 129 mice, Fujita et al. (2001) reported that after partial hepatectomy, the liver had recovered >90% of its weight within 1 week. This difference in response to a mitogenic stimulus has impacts on the interpretations of comparisons between rodent and primate liver responses to chemical exposures which give a transient increases in DNA synthesis or cell proliferation such as PPARα agonists. Also, as stated above, the primate and human liver, while having a significant polyploidy compartment, do not have the extent of polyploidization and the early onset of that has been observed in the rodent. However, as noted by Lapis et al. (1995), exposure to DEN has proven to be a highly potent hepatocarcinogen in nonhuman primates, inducing malignant tumors in 100% of animals with an average latent period of 16 months when administered at 40 mg/kg i.p. every 2 weeks.

In regard to species extrapolation of epigenomic changes between humans and rodents, Weidman et al. (2007) cautioned that:

> Although we do predict some overlap between mouse and human candidate imprinted genes identified through our machine-learning approach, it is likely that the most significant criterion in species-specific identification will differ. This difference underscored the importance for increased caution when assessing human risk from environmental agents that alter the epigenome using rodent models; the molecular pathways targeted may be independent.

Despite species differences, the genome of the mouse has been sequenced and many transgenic mouse models are being used to study the consequences of gene expression modulation and pathway perturbation to study human diseases and treatments. However, the use of transgenic models must be used with caution in trying to determine to determine modes of action and the background effects of the transgene (including background levels of toxicity) and specificity of effects must be taken into account for interpretation of mode-of-action data, especially in cases where the knockout in the mouse causes significant liver necrosis or steatosis (Caldwell et al., 2008b; Caldwell and Keshava, 2006; Keshava and Caldwell, 2006). For the determination of effects of pathway perturbation and similarity to human HCC phenotype, mouse transgenic models have been particularly useful with tumors produced in such models shown to correlate with tumor aggressiveness and survival to human counterparts.

E.3.2.1. Similarities with Human and Animal Transgenic Models

Mice transgenic for transforming growth factor α (a member of the EGF family and a ligand for the ErfB receptors) develop HCCs (Farazi and DePinho, 2006). Compound TGFα and MYC transgenic mice show increase hepatocarcinogenesis that is associated with the disruption

of TGF-β1 signaling and chromosomal losses, some of which are syntenic to those in human HCCs that include the retinoblastoma (RB) tumor suppressor locus (Sargent et al., 1999).

Lee et al. (2004b) investigated whether comparison of global expression patterns of orthologous genes in human and mouse HCCs would identify similar and dissimilar tumor phenotypes, and thus allow the identification of the best-fit mouse models for human HCC. The molecular classification of HCC on the basis of prognosis in Lee et al. (2004a) was further compared with gene-expression profiles of HCCs from seven different mouse models (Lee et al., 2004b). Lee et al. (2004b) characterized the gene expression patters of 68 HCC from seven different mouse models; two chemically induced (Ciprofibrate and diethylnitrosamine), and four transgenic (targeted overexpression of Myc, E2F1, Myc and E2F1, and Myc and Tgfa in the liver). HCCs from some of these mice (MYC, E2F1, and MYC-E2F1 transgenics) showed similar gene-expression patterns to the ones of HCCs from patients with better survival. Murine HCCs derived for MYC-TGF-α transgenic model or diethylnitrosamine-treated mice showed similar gene-expression patterns to HCCs from patients with poor survival. The authors reported that Myc Tgfa transgenic mice typically have a poor prognosis, including earlier and higher incident rates of HCC development, higher mortality, higher genomic instability and higher expression of poor prognostic markers (e.g., AFP) and that Myc and Myc/E2f1 transgenic mice have relatively higher frequency of mutation in β-catenin (*Catnb*) and nuclear accumulation of β-catenin that are indicative of lower genomic instability and better prognosis in human HCC.

Lee et al. (2004b) indentified three distinctive HCC clusters, indicating that gene expression pattern of mouse HCC are clearly heterogeneous and reported that Ciprofibrate-induced HCCs and HCCs from Acox -/- mice were closely clustered and well separated from other mouse models. However, there are several issues regarding this study that give limitations to some of its conclusions regarding the Acox -/- mouse and Ciprofibrate treatment. The Acox -/- mouse is characterized by profound hepatonecrosis, which confounds conclusions regarding gene expression related to PPARα agonism made by the authors. There was very limited reporting of the animal models (DEN and Clofibrate) protocols used. Only three tumors were examined for Clofibrate treatment and it is unknown if the tumors were from the same animals. Similarly, only three tumors were examined from DEN treatment, which has been shown to produce heterogeneous tumors and to produce necrosis in some paradigms of exposure. Myc/E2F1 and E2F1 mice were split in both clusters that were compared with human HCCs. The authors used previously published data from Meyer et al. (2003) for tumors from $Acox1^{-1-}$ null mice, DENA-treated mice, and Ciprofibrate-treated mice.

Meyer et al. (2003) examined three tumors from two C57BL/6j mice fed Ciprofibrate for 19 months and three tumors from two C57BL/6j mice injected with DEN at 2–3 months, but the age at which tumors appeared was not given by the authors. Pooled mRNA from animals of varying age (5–15 months old) was used for controls. mRNAs that differed by twofold in tumors were reported to have: 60 genes upregulated and 105 genes downregulated in $Acox1^{-1-}$ null mice

tumors; 136 genes upregulated and 156 genes downregulated in Ciprofibrate-induced tumors; and 61 genes upregulated and 105 genes downregulated in DEN-induced tumors. The authors stated that "Each tumor class revealed a somewhat different unique expression pattern." There were "genes that were general liver tumor markers in all three types of tumors" with 38 genes commonly deregulated in all three tumor types. Of note, the cell cycle genes (CDK4, CDC25Am CDC7, and MAPK3) cited by Lee et al. (2004b) as being more highly expressed in DEN-induced tumors were not reported to be changed in DEN tumors in Meyer et al. (2003) or to be altered in the $Acox1^{-1-}$ null mice or mice treated with Ciprofibrate. Finally, the distinction between groups may be dominated by gene expression changes in a large number of genes that are related to PPAR activation, but not related to hepatocarcinogenesis.

Calvisi et al. (2004a) used transgenic mice to study pathway alterations and tumor phenotype and to further examine the premise that genomic alterations (genetic and epigenetic) characteristic of HCC can describe tumors into two broad categories, the first category characterized by activation of the Wnt/Wingless pathway via disruption of β-catenin function and chromosomal stability and the second by chromosomal instability. Increased coexpression of c-Myc with TGF-α or E2F-1 transgenic mice was reported to result in a dramatic synergistic effect on liver tumor development when compared with respective monotransgenic lines, including shorter latency period, and more aggressive phenotype. β-catenin activation is relatively common in HCCs developed in c-Myc and c-Myc/TGF-β1 transgenic mice and rare in the c-Myc/TGF-α transgenic line which also has genomic instability.

Calvisi et al. (2004a) also reported that β-catenin staining correlated with histopathologic type of liver tumors. Eosinophilic tumors with abnormal nuclear staining of β-catenin were predominant in neoplastic lesions characteristic of c-Myc and c-Myc/E2F1 lesions. Poorly differentiated HCCs with basophilic or clear-cell phenotypes developed more frequently in c-Myc/TGF-α and TGF-α mice and often showed a reduction or loss of β-catenin immunoreactivity. β-catenin mutation was associated with a more benign phenotype. These observations regarding tincture and aggressiveness are consistent with those of Bannasch (1996) and Carter et al. (2003). Calvisi et al. (2004a) noted that the relationship between β-catenin activation, tumor grade, and clinical outcome in human HCC remains controversial.

> There are studies that show a significant correlation between β-catenin nuclear accumulation, a high grade of HCC tumor differentiation, and a better prognosis, whereas others find that nuclear accumulation of β-catenin may be associated with poor survival or that it does not affect clinical outcome.

Calvisi et al. (2004b) reported that for E-cadherin, a variety of morphologenetic events, including cell migration, separation, and formation of boundaries between cell layers and differentiation of each cell layer into functionally distinct structures. Loss of expression of E-cadherin was reported to result in dedifferentiation, invasiveness, lymph node, or distant

metastasis in a variety of human neoplasms including HCC and that the role of E-cadherin might be more complex than previously believed.

> In order to elucidate the role of E-cadherin in the sequential steps of liver carcinogenesis, we have analyzed the expression patterns of E-cadherin in a collection of preneoplastic and neoplastic liver lesions from c-Myc, E2F1, c-Myc/TGF-α and c-Myc/E2F1 transgenic mice. In particular, we have investigated the relevance of genetic, epigenetic, and transcriptional mechanisms on E-cadherin protein expression levels. Our data indicate that loss of E-cadherin contributes to HCC progression in c-Myc transgenic mice by promoting cell proliferation and angiogenesis, presumably through the upregulation of HIF-1α and VEGF proteins.

The c-Myc line was most like wild-type and lost E-cadherin in the tumors. c-Myc/TGF-α dysplastic lesions were reported to show overexpression of E-cadherin mainly in pericentral areas with E2F1 clear cell carcinoma showed intense staining of E-cadherin. Reduction or loss of E-cadherin expression is primarily determined by loss of heterozygosity at the E-cadherin locus or by its promoter hypermethylation in human HCC. Calvisi et al. (2004b) determined the status of the E-cadherin locus and promoter methylation in wild-type livers and tumors from transgenic mice by microsatellite analysis and methylation specific PCR, respectively.

> Wild-type livers and HCCs, regardless of their origins, showed the absence of LOH at the E-cadherin locus. E-cadherin promoter was not hypermethylated in wild-type, c-Myc/TGF-α and E2F1 livers. No E-cadherin promoter hypermethylation was detected in c-Myc and c-Myc/E2F1 HCCs with normal levels of E-cadherin protein. In striking contrast, seven of 20 (35%) of c-Myc and two of four (50%) c-Myc/E2F1 HCCs with downregulation of E-cadherin displayed E-cadherin promoter hypermethylation. These results suggest that promoter hypermethylation might be responsible for E-cadherin downregulation in a subset of c-Myc and c-Myc/E2F1 HCCs…The molecular mechanisms underlying down-regulation of E-cadherin in c-Myc tumors remain poorly understood at present. No LOH at the E-cadherin locus was detected in the c-Myc HCCs whereas only a subset of c-Myc tumors displayed hypermethylation of the E-cadherin promoter. Furthermore, no association was detected between E-cadherin downregulation and protein levels of transcriptional repressors, Snail, Slug or the tumor suppressor WT1, in disagreement with the finding that overexpression of Snail suppresses E-cadherin in human HCC…E-cadherin might play different and apparently opposite roles, which depend on specific tumor requirements in both human and murine liver carcinogenesis.

Importantly, the results of Calvisi et al. (2004b) showed that hypermethylation of promoters can be associated with downregulation of a gene in mouse liver tumors similar to human HCC and that tumors can have the same behavior with methylation change as with loss of hetererozygosity.

This report also gave evidence of the usefulness of the mouse model to study human liver cancer as it shows the similarity of dysfunctional regulation in mouse and human cancer and the heterogeneity within and between mouse lines tumors with differing dysfunctions in gene expression. These findings parallel human cancer where there is heterogeneity in tumors from one person and every tumor has its own signature. Finally, this report correlates differing pathway perturbations with mouse liver phenotypes similar to those reported in experimental carcinogenesis models and for TCE and its metabolites.

Farazi and DePinho (2006) suggested that:

> as comparative array CGH analysis of various murine cancers has shown that such aberrations often target syntenic loci in the analogous human cancer type, we further suggest that comparative genomic analysis of available mouse model of mouse HCC might be particularly helpful in filtering through the complex human cancer genome. Ultimately, mouse models that share features with human HCCs could serve as valuable tools for gene identification and drug development. However, one needs to keep in mind key differences between mice and humans. For example, as noted in certain human HCC cases, telomere shortening might drive the genomic instability that enables the accumulation of cancer-relevant changes for hepatocarcinogenesis. As mice have long telomeres, this aspect of hepatocarcinogenesis might be fundamentally different between the species and provide additional opportunities for model refinement and testing of this mechanism through use of a telomere deficient mouse model. These and other cross-species difference, and limitations in the use of human cell-culture systems, must be considered in any interpretation of data from various model systems (Farazi and DePinho, 2006).

Thus, these mouse models of liver cancer inductions are qualitatively able to mimic human liver cancer and support the usefulness of mouse models of cancer.

E.3.3. Hypothesized Key Events in HCC Using Animal Models

E.3.3.1. Changes in Ploidy

As stated in Section E.1.1, increased polyploidization has been associated with numerous types of liver injury and appears to result from exposure to TCE and its metabolites as well as changes in the number of binucleate cells. Hortelano et al. (1995) reported that cytokines and NO can affect ploidy and further suggest a role of these changes for carcinogenesis in general. Vickers and Lucier (1996) noted that while both DEN and 17 α-ethinylestradiol have been reported to enhance the proportion of diploid hepatocytes, initiators like *N*-nitrosomorpholine are reported to increase the proportion of hypertrophied and polyploidy hepatocytes. The relationship of such changes to cancer induction has been studied in transgenic mouse models and in models involved with mitogens of differing natures.

Melchiorri et al. (1993) reported the response pattern of the liver to acute treatment with primary mitogens in regard to ploidy changes occurring in rat liver following two different types

of cell proliferation: compensatory regeneration induced by surgical partial hepatectomy (PH) and direct hyperplasia induced by the mitogens lead nitrate and Nafenopin (a PPARα agonist) in 8-week-old male Wistar rats. Feulgen stain was used and DNA content was quantified by image cytometry in mononucleated and binucleated cells. Mitotic index was determined in the same samples. The term "diploid" was used to identify cells with a single, diploid nucleus and tetraploid for cells containing two diploid nuclei or one tetraploid nucleus referred (bi- and mononucleated, respectively). Octoploid cells were identified as either binucleate or mononucleate.

> During liver regeneration following surgical PH an increase in the mitotic index with a peak at 24 hours was observed. The most striking effect associated with the regenerative response was the almost complete disappearance of binucleate cells, tetraploid (2 X 2c) as well as octoploid (4 X 2c) with only < 10% of the control values being present 3 days after PH…Concomitantly, an increase in mononucleate tetraploid (4c) as well as mononucleate octoploid (8c) cells was observed, resulting at 3 days after PH in a population made up of almost entirely (98%) by mononucleated cells.

Lead nitrate treatment was reported to induce rapid increases in the formation of binucleated cells occurring 3 days after treatment, their number accounting for 40% of the total cell population vs. 22% binucleate cells in control rats and 2% in PH animals killed at the same time point. The increased binuclearity was reported to be observed only in the 4 × 2c cells (25 vs. 6% of the controls) and in 8 × 2c cells (3.7 vs. 0.1% of controls). The increase in 4 × 2c and 8 × 2c cells was reported to be accompanied by a concomitant reduction in 2 × 2c cells with the change induced in cellular ploidy by lead nitrate resulting in 37% of cells being either 8c or 16c. However, at the same time point, cells having a ploidy higher than 4c were reported to account for only 11% in PH rats and 9% in control animals. Changes in the ploidy pattern were reported to be preceded by an increased mitotic activity, which was maximal 48 hours after treatment with lead nitrate. The increase in mitotic index in lead nitrate-treated rats was associated with a striking increase in the labeling index of hepatocytes (60.1 vs. 3% of control rats) and to an almost doubling of hepatic DNA content in 3 days after lead nitrate.

Melchiorri et al. (1993) concluded that the entire cell cycle appeared to be induced by lead nitrate but that the finding of a high increase of binucleated cells suggested that lead nitrate-induced liver growth, unlike liver regeneration induced by partial hepatectomy, was characterized by an uncoupling between cell cycle and cytokinesis. This raised questions on whether lead nitrate-induced liver growth resulted in a true increase in cell number or is only the expression of an increased hepatocyte ploidy. They reported that part of the increase in DNA content observed 3 days after lead nitrate was indeed expression of polyploidizing process due to acytokinetic mitoses, but that a consistent increase in cells number (+26%) was also induced by lead nitrate treatment.

After Nafenopin treatment, Melchiorri et al. (1993) reported that the increase in DNA content was increased 22% over controls and was much lower than induced by lead nitrate and that Nafenopin did not induce significant changes in binucleated cell number. However, a shift towards a higher ploidy class (8c) was reported to be observed following Nafenopin and the 21% increase in DNA content seen after Nafenopin treatment was almost entirely due to increase in the ploidy state with only 7% increase in cell number.

Melchiorri et al. (1993) examined whether hepatocytes characterized by high ploidy content (highly differentiated cells) would be preferentially eliminated by apoptosis. An increase in apoptotic bodies was reported to be associated with the regression phase after lead nitrate treatment (when liver mass is reduced) but despite the elimination of excess DNA, the changes in ploidy distribution induced by lead nitrate were found to persist suggested that polyploidy cells were not preferentially eliminated by apoptosis during the regression phase of the liver. Melchiorri et al. (1993) noted that other studies in rats exposed to the mitogen, cyproterone acetate (CPA), and the peroxisome proliferator, MCP, also reported a very strong decline in binucleated cells with a concomitant increase in mononucleated tetraploid cells in the liver similar to the pattern described after partial hepatectomy.

Lalwani et al. (1997) reported the results of 1,000 ppm WY-14,643 exposure in male Wistar rats after 1, 2, and 4 weeks and suggested that an early wave of nuclear division occurred at the early stages of exposure without cumulative effects on cell proliferation. Consistent with hepatomegaly, WY-14,643-treated rats were reported to exhibit multifocal hepatocellular hypertrophy and karyomegaly by routine microscopic analysis. For binucleated hepatocytes, there were no reported differences between WY-14,643-treated and control groups for days 4 and 11 but an increase in the number at day 25 in WY-14,643-treated animals compared to controls. Increases in the diameter of nuclei were shown by WY-14,643-treatment from days 11 and 25 with increasing numbers of cells displaying larger nuclear diameters. The mitotic index was reported not to be significantly changed in WY-14,643-treated rats compared to controls. Mitotic figures did not appear to survive the treatment necessary for flow cytometric analyses. PCNA was increased on day 4 in WY-14,643-treated animals compared to controls whereas no differences were found on days 11 and 25.

However, immunohistochemistry was reported to show remarkable increases in BrdU-labeled nuclei in liver sections after 4 days of labeling, with the populations of BrdU-labeled cell declining over the course of treatment. The labeling index was high and approximately 80% of the BrdU-labeled cells were in periportal areas. PCNA-expressing cells were increased in the periportal area of the liver. Intense nuclear staining of PCNA was evident as an indicator of DNA replication in S phase. Microscopic examination showed BrdU labeling only in periportal hepatocytes, whereas no significant labeling was observed in nonparenchymal cells, indicating that the replicative activity was confined to the liver cells.

Lalwani et al. (1997) suggested that their results showed that events related to cell proliferation occur in the initial phase of WY-14,643 treatment in rats but not followed by changes in the rate of DNA synthesis as the treatment progressed. They note that Marsman et al. (1988) observed constant increases in DNA synthesis by [^{3}H]-thymidine authoradiography with up to 1 year of continuous administration of WY-14,643, whereas the rate of DNA synthesis or the BrdU labeling index in their study declined after the first 4 weeks of treatment. They suggest that the increased percentage of cells appearing in G2-M phase and the analysis of liver nuclear profiles suggest that the progression of these additional cells (i.e., cells that are stimulated to enter the cell cycle by the test agent) through the cell cycle is arrested in the late stages of the cell cycle. They state:

> Unlike BrdU labeling, which demonstrated DNA synthesis activity over the 4-day labeling period, the PCNA labeling index represents levels of the protein product at an interval post treatment. PCNA expression in cells exposed to chemicals or to WY may not provide true representation of S phase or proliferative activity because PCNA-expressing nuclei were also found in G0=G1 and G2-M phases.

Lalwani et al. (1997) concluded that cell proliferation alone does not appear to constitute a determining process leading to tumors in most tissues and sustained cell replication may not be a primary feature of peroxisome proliferator-induced hepatocarcinogenesis.

Miller et al. (1996) noted that studies with MCP in Alpk:AP rats indicate that DNA synthesis occurs primarily in one hepatocyte subpopulation as defined by ploidy status, the binucleated tetraploid (2 × 2N) hepatocytes, and that this preferential hepatocyte DNA synthesis is manifested by dramatic alterations in hepatocyte ploidy subclasses (i.e., significant increases in mononucleate tetraploid [4N] hepatocytes concomitant with decreases in 2 × 2N hepatocytes).

They reported results in male F344 rats that were 13 weeks old (an age in which polyploidization had reached a plateau) exposed to 1,000 ppm WY-14,643 and MCP (gavage via corn oil at 8 mg/mL or 25 mg/kg MCP once daily) for 2, 5, and 10 days (n = 4). WY-14,643 and MCP were reported to induce significant increases in the octoploid hepatocyte class that coincided with decreases in the tetraploid hepatocyte class. However, MCP did not induce this shift until day 5 of exposure. These results showed an approximate doubling of mononuclear octoploid (8N) hepatoctyes but still a very low number of the total hepatocyte population that did not reach >7% and was still only approximately twice that of control values. Thus, this finding does not indicate a very large target population. There was no real effect on 4N hepatocytes due to these treatments and the percent of hepatocytes that were 4N stayed ~70% and were thus the major cell type in the liver. Miller et al. (1996) noted the importance of maturation and/or strain for these analyses; there are maturation-dependent differences in the distribution and mitogenic sensitivity of hepatoctyes in the various subclasses.

Hasmall and Roberts (2000) noted that despite their differing abilities to induce liver cancer, both DCB (a nonhepatocarcinogen in F344 rats) and DEHP, at the doses and routes used in the NTP bioassays, induced similar profiles of S-phase LI. A large and rapid peak during the first 7 days (1,115 and 1,151% of control for DEHP and DCB, respectively) was followed by a return to control levels. They suggested that the size of the S-phase response does not necessarily determine hepatocarcinogenic risk and that the subpopulation in which S-phase is induced may be a better correlate with subsequent hepatocarcinogenecity.

They compared the effects on polyploidy/nuclearity and on the distribution of S-phase labeled cells with ETU, the peroxisome proliferator: MCP and phenobarbitone. Male F334 rats 7–9 weeks old were exposed to MCP (0.1% in diet), ETU (83 ppm in diet), or phenobarbitone (500 mg/mL in drinking water) for 7 days. The number of rats for the 7-day study was not given by the authors. Hasmall and Roberts (2000) reported that treatment of rats with MCP, ETU, or phenobarbitone for 7 days had no significant effect on the ploidy profile as compared with corn oil controls (data not shown) but that MCP and phenobarbitone did induce significant changes in nuclearity. MCP reduced the 2 × 2N population and increased the 8N population. Phenobarbitone similarly increased the proportion of cells in the 4N population. ETU had no effect on the nuclearity profile as compared with control. However, what the authors describe for their results in polidy and nuclearity are different than those presented in their figures. There were significant differences between controls that the authors did not characterize and there appeared to be a greater difference between controls than some of the treatments.

Gupta (2000) reported that in transgenic mice with overexpression of TGF-α, liver-cell turnover increases, along with the onset of hepatic polyploidy, whereas HCC originating in these animals contain more diploid cells. Coexpression of c-Myc and TGF-α transgenes in mouse hepatocytes was associated with greater degrees of polyploidy as well as increased development of HCC. Gupta (2000) noted that in the presence of ongoing liver injury and continuous depletion of parenchymal cells, hepatic progenitor cells (including oval cells) are eventually activated but what roles polyploid cells play in this process requires further study. In the working model by Gupta (2000), sustained disease by chronic hepatitis, metabolic disease, toxins, etc., may lead to hepatocyte polyploidy and loss, and the emergence of rapidly cycling progenitor or escape cell clones with the onset of liver cancer.

Conner et al. (2003) described the development of transgenic mouse models in which E2F1 and/or c-Myc was overexpressed in mouse liver. The E2F1 and c-Myc transcription factors are both involved in regulating key cellular activities including growth and death and, when overexpressed, are capable of driving quiescent cells into S-phase in the absence of other mitogenic stimuli and are potent inducers of apoptosis operating at least through one common pathway involving p53. Deregulation of their expression is also frequently found in cancer cells (Conner et al., 2003). Conner et al. (2003) reported that although both c-Myc and E2F1 mono-transgenic mice were prone to liver cancer, E2F1 mice developed HCC more rapidly and with a

higher frequency and that the combined expression of these two transcription factors dramatically accelerated HCC growth compared to either E2F1 or c-Myc mono-transgenic mice. All three transgenic lines were reported to show a low but persistent elevation of hepatocyte proliferation before an onset of tumor growth. Ploidy was shown to be affected differently by c-Myc and E2F1, and suggested distinct differences by which these two transcription factors control liver proliferation/maturation. Both transgenic alterations induced liver cancer but had differing effects on polyploidization suggestive that liver cancer can arise from either type of mature hepatocyte.

c-Myc single-transgenic mice showed a continuous high cell proliferation that preceded the appearance of preneoplastic lesions, which was also true, although to a lesser extent, in the E2F1 mice. At 15 weeks of age, all of the transgenic mouse lines were reported to have a high incidence (>60%) of hepatic dysplasia with mitotic indices equivalent in c-Myc/E2F1, and c-Myc livers, but twofold higher than the mitotic index in E2F1 and very low in wild-type mice. Thus, the combination of the two transgenes did not have an additive effect on proliferation. An analysis of the DNA content in hepatocyte nuclei isolated from 4- to 15-week-old mice was reported to show that in young wild-type livers, the majority of nuclei had a diploid DNA content with a smaller proportion of tetraploid nuclei. As the mice aged, the number of tetraploid and octoploid nuclei increased consistent with the previous findings of others.

However, c-Myc mice were reported to demonstrate a premature polyploidization with the number of 2N nuclei in c-Myc livers almost 2-fold less, while the proportion of 4N nuclei increased >2.5-fold at 4 weeks of age. The most prominent ploidy alteration was an increase in the fraction of hepatocytes with octaploid nuclei (~200-fold higher). The percentage of polyploidy cells was reported to continue to rise in 15-week-old c-Myc livers. The majority of hepatocytes had nuclei with 4N and 8N DNA content, with an attendant increase in binucleated hepatocytes and increase in average cell size.

In striking contrast, E2F1 hepatocytes were reported not to undergo normal polyploidization with aging. The majority of E2F1 nuclei were reported to remain in the diploid state and to be almost identical in E2F1 mice at 4 and 15 weeks of age. The percentage of binucleated hepatocytes was also reduced. In c-Myc/E2F1 mice, the age-related changes in ploidy distribution were reported to resemble those found in both c-Myc and in E2F1 single transgenic mice.

At a young age, c-Myc/E2F1 mice, similar to E2F1 mice, were reported to retain significantly more diploid nuclei than c-Myc mice. However, as mice aged, the majority of c-Myc/E2F1 hepatocytes, similar to c-Myc cells but in contrast to findings in E2F1 cells, became polyploid. Consistent with a more progressive polyploidization, the DNA content was significantly higher in both c-Myc/E2F1 and c-Myc livers. Conner et al. (2003) reported that other known modulators of ploidy in the liver are the tumor suppressor p53, pRb, and the cell

cycle inhibitor p21 as well as genes involved in the control of the cell cycle progression such as cyclin A, cyclin B, cyclin D3, and cyclin E.

Along with increased liver cancer, Conner et al. (2003) noted that the C-Myc mice also experienced a persistent liver injury as evidenced by significant elevation of circulating levels of AST, ALT, and ALP along with the appearance of a frequent oval/ductular proliferation. However, oval cell proliferation may be a marker of hepatocyte damage but not be the cells responsible for tumor induction (Tarsetti et al., 1993). Conner et al. (2000) reported that if E2F1 is overexpressed in the liver, there is both oncogenic and tumor-suppressive properties. In regard to liver morphological changes, E2F1 transgenic mice were reported to uniformly develop pericentral dysplasia and foci adjacent to portal tracts followed by the abrupt appearance of adenomas and subsequent malignant conversion with all of the animals having foci by 2–4 months, and by 8–10 months, most having adenomas with dysplastic changes remaining confined to the pericentral regions of the liver lobule.

In regard to phenotype, the majority of the foci were composed of small round cells, with clear-cell phenotype but eosinophilic, mixed, and basophilic foci were also seen. In adenomas with malignant transformation to HCC, there appeared to be high mitotic indices, blood vessel invasion, and central collection of deeply basophilic cells with large nuclei giving a "nodule-in-nodule" appearance. Macrovesicular hepatic steatosis was first noted in some E2F1 transgenic livers at 6–8 months, and by 10–12 months, 60% of animals had developed prominent fatty change. Hepatic steatosis has been noted in several transgenic mouse models of liver carcinogenesis (Conner et al., 2000). These results raise interesting points of regional difference in tumor formation which can be lost in analyses using whole liver and that the phenotype of foci and tumors are similar to those seen from chemical carcinogenesis. The occurrence of hepatotoxicity in these transgenic mice is also of note.

E.3.3.2. Hepatocellular Proliferation and Increased DNA Synthesis

Caldwell et al. (2008b) presented a discussion of the role of proliferation in cancer induction. They stated that:

> in the case of CCl4 exposure, hepatocyte proliferation may be related to its ability to induce liver cancer at necrogenic exposure levels, but the nature of this proliferation is fundamentally different from peroxisome proliferators or other primary mitogens that cause hepatocyte proliferation without causing cell death (Columbano and Ledda-Columbano, 2003; Ledda-Columbano et al., 2003; Ledda-Columbano et al., 1998; Menegazzi et al., 1997; Coni et al., 1993; Ledda-Columbano et al., 1993). After initiation with a mutagenic agent, the transient proliferation induced by primary mitogens has not been shown to lead to cancer-induction, while partial hepatectomy or necrogenic treatments of CCl4 result in the development of tumors (Gelderblom et al., 2001; Ledda-Columbano et al., 1993).

Roskams et al. (2003) noted that partial hepatectomy does not cause HCC in normal mice without initiation. Melchiorri et al. (1993) reported that a series of studies has shown that acute proliferative stimuli provided by primary mitogens, unlike those of the regenerative type such as those elicited by surgical or chemical partial hepatectomy, do not support the initiation phase and do not effectively promote the growth of initiated cells (Columbano et al., 1990; Ledda-Columbano et al., 1989; Columbano et al., 1987). They noted that the finding that most of these chemicals, with the exception of WY, induce only a very transient increase in cell proliferation raises the question whether such a transient induction of liver cell proliferation might be related to liver cancer appearing 1–2 years later. They noted that mitogen-induced liver growth differs from compensatory regeneration in several aspects: (1) it does not require an increased expression of hepatocyte growth factor mRNA in the liver; (2) it is not necessarily associated with an immediate early genes such as c-Fos and c-Jun; and (3) it results in an excess of tissue and hepatic DNA content that is rapidly eliminated by apoptotic cell death following withdrawals of the stimulus.

Other studies have questioned the importance of a brief wave of DNA synthesis in induction of liver cancer. Chen et al. (1995) noted that Jirtle et al. (1991) and Schulte-Hermann et al. (1986) reported that during a 2-week period of treatment with lead, DNA synthesis was increased most in centrolobular hepatocytes and that the predominantly centrilobular distribution of the labeled nuclei may have been due largely to the brief wave of mitogenic response, because from the fifth day onward, DNA synthesis activity returned to control level even though lead nitrate treatment continued. They concluded that sustained cell proliferation may be more important than a brief wave of increased DNA synthesis. Chen et al. (1995) also noted that a number of different agents acting via differing modes of action will induce periportal proliferation.

Vickers and Lucier (1996) reported that mitogenic response induced by acute 17 α-ethinylestradiol administration is randomly distributed throughout the hepatic lobule, while continuous administration increases the proportion of diploid cells. Richardson et al. (1986) reported that the lobular distribution of the correlation of hepatocyte initiation and akylation reported in their model of carcinogenicity did "not support that early proliferation is associated with cancer as at 7 days there is a transient increase in the lobes least likely to get a tumor and no difference between the lobes at 14 and 28 days DEN although there is a difference in tumor formation between the lobes." Thus, cells undergoing DNA synthesis may not be in the same zone of the liver where other hypothesized "key events" take place.

Tanaka et al. (1992) noted that the distribution of hepatocyte proliferation in the periportal area was in contrast to the distribution of peroxisome proliferation in the centrilobular area of Clofibrate-treated rats. Melnick et al. (1996) noted that replicative DNA synthesis commonly has been evaluated by measurement of the fraction of cells incorporating BrdU or

tritiated thymidine into DNA during S-phase of the cell cycle (S-phase labeling index), but that the S-phase labeling index would not be identical to the cell division rate when replication of DNA does not progress to formation of two viable daughter cells. "The general view at an international symposium on cell proliferations and chemical carcinogenesis was that although cell replication is involved inextricably in the development of cancers, chemically enhanced cell division does not reliably predict carcinogenicity" (Melnick et al., 1993). They noted that the finding that enzyme-altered hepatic foci were not induced in rats fed WY-14,643 for 3 weeks followed by partial hepatectomy indicates that early high levels of replicative DNA synthesis and peroxisome proliferation are not sufficient activities for initiation of hepatocarcinogenesis.

Baker et al. (2004) reported that, similar to the pattern of transient increases in DNA synthesis reported for TCE metabolites, Clofibrate exposure induced the upregulation of a variety of cell proliferation-associated genes (e.g., G2/M specific cyclin B1, cyclin-dependent kinase 1, DNA topoisomerase II alpha, c-Myc protooncogene, pololike serien-threonine protein kinase, and cell divisions control protein 20) began on or before day 1 and peaked at some point between days 3 and 7. By day 7, cell proliferation genes were downregulated. The chronology of this gene expression agrees with the histologic diagnosis of mitotic figures in the tissue, where an increase in mitotic figures was detected in the day 1 and most notably day 3 high and low-dose groups. However, by day 7, the incidence of mitotic figures had decreased. The clustering of genes associated with the G2/M transition point suggests that in the rats, the polyploid cells arrested at G2/M are those that are proceeding through the cell cycle.

A dose-response for increased DNA-synthesis also seems to be lacking for the model PPARα agonist, WY-14,643 suggesting that the transient increases in DNA synthesis reported by Eacho et al. (1991) for this compound at lower levels that then increase later at necrogenic exposure levels, are not related to its carcinogenic potential. Wada et al. (1992) reported that in male F344 rats exposed to a range of WY-14,643 concentrations (5–1,000 ppm), liver weight gain occurred at the lowest dose that gave a sustained response for many weeks but gave increased cell labeling only in the first week. Peroxisomes proliferation, as measure by electron microscopy, increases started at 50 ppm exposures. By enzymatic means, peroxisomal activities were elevated at the 5 ppm dose. Of note is the reported difference in distribution in hepatocellular proliferation, which was not where the hypertrophy or where the lipofuscin increases were observed. The authors noted that these data suggest that 50 and 1,000 ppm WY-14,643 should give the same carcinogenicity if peroxisome proliferation or sustained proliferation are the "key events."

The study of (Marsman et al., 1992) is very important in that it not only shows that clofibric acid (another PPAR α agonist) does not have sustained proliferation, but it also shows that it and WY-14,643 at 50 ppm did not induce apoptosis in rats. It is probable that use of WY-14,643 at high concentrations may induce apoptosis in a manner not applicable to other peroxisome proliferators or to treatment with WY-14,643 at 50 ppm. This study also confirmed

that exposure to WY-14,643 at 50 ppm and WY-14,643 at 1,000 ppm induces similar effects in regards to hepatocyte proliferation and peroxisomal proliferation.

The study by Eacho et al. (1991) also gave a reference point for the degree of hepatocytes undergoing transient DNA synthesis from WY-14,643 and Clofibrate and how much smaller it is for TCE and its metabolites, which generally involve <1% of hepatocytes.

> The labeling index of BrdU was 7.2% on day 3 and 15.5% on day 6 after clofibric acid but by day 10 and 30 labeling index was the same as controls at ~1-2%.…For WY the labeling index was 34.1% at day 3 and 18.6% at day 6. At day 10 the labeling index was 3.3% and at day 30 was 6%, representing 6.6- and 15-fold of respective controls. Control levels were ~0.5 to 1%.…The labeling index was increased to 32% by 0.3% LY171883 and to 52% by 0.05% Nafenopin. The 0.005% and 0.1% dietary doses of WY increased the 7 day labeling index to a comparable level (55% - 58%).

Yeldandi et al. (1989) reported that until foci appear, cell proliferation has ceased to increase over controls after the first week for Ciprofibrate-induced hepatocarcinogenesis. The results also showed the importance of using age-matched controls and not pooled controls for comparative purposes of proliferation as well as how low proliferative rates are in control animals.

The results of Barrass et al. (1993) are important in suggesting that age of animals is important when doing quantitation of labeling indexes. Studies such as that conducted by Pogribny et al. (2007) that only give the replication rate as a ratio to control will make the proliferation levels look progressive when, in fact, they are more stable with time as it is just the controls that change with age as a comparison point.

E.3.3.3. Nonparenchymal Cell Involvement in Disease States Including Cancer

The recognition that not only parenchymal cells but also nonparenchymal cells play a role in HCC has resulted in studies of their role in initiation as well as progression of neoplasia. The role of the endothelial cell in controlling angiogenesis, a prerequisite for neoplastic progression, and the role of the Kupffer cell and its regulation of the cytokine milieu that controls many hepatocyte functions and responses have been reported. However, as pointed out by Pikarsky et al. (2004) and by the review by Nickoloff et al. (2005), the roles of inflammatory cytokines in cancer are context- and timing-specific and not simple. For TCE, nonparenchymal cell proliferation has been observed after inhalation (Kjellstrand et al., 1983a) and gavage (Goel et al., 1992) exposures of ~4 weeks duration.

E.3.3.3.1. Epithelial Cell Control of Liver Size and Cancer—Angiogenesis

The epithelium is key in controlling restoration after partial hepatectomy and not surprisingly HCC growth. Greene et al. (2003) hypothesized that the control of physiologic

organ mass was similar to the control of tumor mass in the liver and that specifically, the proliferation of hepatocytes after partial hepatectomy, like the proliferations of neoplastic cells in tumors, requires the synthesis of new blood vessels to support the rapidly increasing mass. They reported that a peak in hepatocyte production of vascular endothelial growth factor (VEGF), an endothelial mitogen, corresponds to an increase of VEGF receptor expression on endothelial cells after partial hepatectomy and the rate of endothelial proliferation. Fibroblast growth factor and transforming growth factor-alpha (TGfox), which stimulate endothelial cells, are secreted by hepatoctyes 24 hours after partial hepatectomy. However, endothelial cells were reported to secrete hepatocyte growth factor, a potent hepatocyte mitogen, that is also proangiogenic. The secretion of transforming growth factor-beta by (TGfox) endothelial cells 72 hours after partial hepatectomy was reported to inhibit hepatocyte proliferation. Thus, Greene et al. (2003) suggested that endothelial cells and hepatocytes of the regenerating liver influence each other, and both populations are required for the regulation of the regenerative process.

E.3.3.3.2. Kupffer Cell Control of Proliferation and Cell Signals, Role in Early and Late Effects

Vickers and Lucier (1996) have reported that Kupffer cells are increased in number in preneoplastic foci but are decreased in HCC, and that other studies have demonstrated that both sinusoidal endothelial cells and Kupffer cells within HCC cells in humans stain positive for mitotic activity although the number of nonparenchymal cells compared to parenchymal cells may be reduced. Lapis et al. (1995) reported that Kupffer cells contain lysozyme in their cytoplasmic granules, vacuoles, and phagosomes, some cells show a positive reaction in the rough endoplasmic reticulum, perinuclear cisternae, and the Golgi zone, and that in human monocytes, the lysozyme is colocalized with the CD68 antigen and myeloperoxidase. They also reported that, in rodent hepatocarcinogenesis, increased numbers of Kupffer cells were observed in preneoplastic foci, whereas abnormally low numbers were present following progression to HCC. They also noted that "the Kupffer cell count in human HCC has also been shown to be very low and varies with different histological form." They reported that for monkey HCCs, the proportion of endothelial elements remained constant (the parenchymal/endothelial cell ratio); however, there was a striking reduction in the areas occupied by Kupffer cells. While healthy control livers contained the highest number of Kupffer cells, in the tumor-bearing cases, the nonneoplastic, noncirrhotic liver adjacent to the HCC nodules had a significantly lower number of Kupffer cells and the number decreased further in the nonneoplastic portions of cirrhotic livers. Within HCC nodules, the Kupffer cell count was greatly reduced with no significant changes observed between the cirrhotic areas and the carcinomas; however, the tumors contained fewer lysozyme and CD68 positive cells. Lapis et al. (1995) noted that:

since other cell types within the liver sinusoids (monocytes and polypmorphs) and portal macrophage were also positive, it was important to identify the star-like morphology of the Kupffer cells. The results of the two independent observers assessment of the morphology and enumeration of Kupffer cells were quite consistent and differed by only 3%." "The loss of Kupffer cells in the HCC may possibly result from capillarization of the sinusoids, which has been observed during the process of liver cirrhosis and carcinogenesis. Capillarization entails the sinusoidal lining endothelial cells losing their fenestrations.

E.3.3.3.3. Nf-kB and TNF-α—Context, Timing and Source of Cell Signaling Molecules

A large body of literature has been devoted to the study of nuclear factor κ B for its role not only in inflammation and a large number of other processes, but also in carcinogenesis. However, the effects of these cytokines are very much dependent on their cellular context and the timing of their modulation. As described by Adli and Baldwin (2006):

> The classic form of NF-kB is composed of a heterodimer of the p50 and p65 subunits, which is preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the IkB family. Following exposure to a variety of stimuli, including inflammatory cytokines and LPS, IkBs are phosphorylated by the IKKα/β complexes then accumulate in the nucleus, where they transcriptionally regulate the expression of genes involved in immune and inflammatory responses.

The five members of the mammalian NF-kB family, p65 (RelA), RelB, c-Rel, P50/p105 (NF-KB1), and p52/p100 (NF-kB2), exist in unstimulated cells as homo- or heterodimers bound to IkB family proteins. Transcriptional specificity is partially regulated by the ability of specific NF-kB dimmers to preferentially associate with certain members of the IkB family. Individual NF-kB responses can be characterized as consisting of waves of activation and inactivation of the various NF-kB members (Hayden and Ghosh, 2004). While the function of NF-kB in many contexts have been established, it is also clear that there is great diversity in the effects and consequences of NF-kB activation with NF-kB subunits not necessarily regulating the same genes in an identical manner and in all of the different circumstances in which they are induced. The context within which NF-kB is activated, be it the cell type or the other stimuli to which the cell is exposed, is therefore, a critical determinant of the NF-kB behavior (Perkins and Gilmore, 2006).

Balkwill et al. (2005) reported that:

> the NF-κB pathway has dual actions in tumor promotion: first by preventing cell death of cells with malignant potential, and second by stimulating production of proinflammatory cytokines in cells of infiltrating myeloid and lymphoid cells. The proinflammatory cytokines signal to initiated and/or otherwise damaged epithelial cells to promote neoplastic cell proliferation and enhance cell survival.

> However, the tumor promoting role of NF-κB may not always predominate. In some cases, especially early cancers, activation of this pathway may be tumor suppressive (2004). Inhibiting NF-κB in keratinocytes promotes squamous cell carcinogenesis by reducing growth arrest and terminal differentiation of initiated keratinocytes (Seitz et al., 1998).

Other inflammatory mediators have also been associated with oncogenesis. Balkwill et al. (2005) reported that TNFα is frequently detected in human cancers (produced by epithelial tumor cells, as in for instance, ovarian and renal cancer) or stromal cells (as in breast cancer). They also report that the loss of hormonal regulation of IL-6 is implicated in the pathogenesis of several chronic diseases, including B cell malignancies, RCC, and prostate, breast, lung, colon, and ovarian cancers. Over 100 agents, such as antioxidants, proteosome inhibitors, NSAIDs, and immunosuppressive agents are NF-κB inhibitors with none being entirely specific (Balkwill et al., 2005). Thus, alterations in these cytokines, and the cells that produce them, are implicated as features of "cancer" rather than specific to HCC.

Balkwill et al. (2005) reported that:

> Two mouse models of inflammation-associated cancer now implicate the gene transcription factor NF-κB and the inflammatory mediator known as tumor-necrosis factor α (TNF- α) in cancer progression. Using a mouse model of inflammatory hepatitis that predisposes mice to liver cancers, Pikarsky et al. present evidence that the survival of hepatocytes - liver cells - and their progression to malignancy are regulated by NF-κB. NF-κB is an important transcription factor that controls cell survival by regulating programmed cell death, proliferation, and growth arrest. Pikarsky et al. find that the activation state of NF-κB, and its localization in the cell, can be controlled by TNF-α produced by neighboring inflammatory cells (collectively known as stromal cells).

Pikarsky et al. (2004) reported that that the inflammatory process triggers hepatocyte NF-κB through upregulation of TNF-α in adjacent endothelial and inflammatory cells. Switching off NF-κB in mice from birth to 7 months of age, using hepatocyte-specific inducible IκB-super repressor transgene, had no effect on the course of hepatitis, nor did it affect early phases of hepatocyte transformation. By contrast, suppressing NF-κB inhibition through anti-TNF-α treatment or induction of the IκB-super repressor in later stages of tumor development resulted in apoptosis of transformed hepatocytes and failure to progress to HCC. The Mdr2 knockout hepatocytes in Pikarsky's model of hepatocarcinogenicity were distinguishable from wild-type cells by several abnormal features: high proliferation rate, accelerated hyperploidy and dysplasia. Pikarsky et al. (2004) reported that NF-κB knockout and double mutant mice displayed comparable degrees of proliferation, hyperploidy, and dysplasia, implying that NF-κB is not required for early neoplastic events. Thus, activation of NF-κB was not important in the early stages of tumor development, but was crucial for malignant conversion.

It was noted that:

> Greten et al. reporting in Cell, come to a similar conclusion by studying a mouse colitis-associated cancer model. Their work does not directly implicate TNF-α, but instead found enhanced production of several pro-inflammatory mediators (cytokines) including TNF-α, in the tumor microenvironment during the development of cancer. An important feature of both studies is that NF-κB activation was selectively ablated in different cell compartments in developing tumor masses, and at different stages of cancer development.

Balkwill et al. (2005) also noted that TNF-α and NF-κB have many different effects, depending on the context in which they are called into play and the cell type and environment.

In contrast, El-Serag and Rudolph (2007) noted that "the influence of inflammatory signaling on hepatocarcinogenesis can be context dependent; deletion of Nf-κB-dependent inflammatory responses enhanced HCC formation in carcinogen treated mice (Sakurai et al., 2006)." Similarly, deletion of Nf-κB essential modulator/I kappa β kinase (NEMO/IKK), an activator of Nf-κB, induced steatohepatitis and HCC in mice (Luedde et al., 2007).

Maeda et al. (2005) reported that hepatocyte-specific deletion of IKKβ (which prevents NF-kB activation) increased DEN-induced hepatocarcinogenesis and that a deletion of IKKβ in both hepatocytes and hematopoietic-derived cells, however, had the opposite effect, decreasing compensatory proliferation and carcinogenesis. They suggested that these results differ from previous suggestion that the tumor-promoting function of NF-kB is excreted in hepatocytes (Pikarsky et al., 2004), and suggest that chemicals or viruses that interfere with NF-kB activation in hepatocytes may promote HCC development.

Alterations in NF-kB levels have been suggested as a key event for the hepatocarcinogenicity by PPARα agonists. The event associated with PPAR effects has been the extent of NF-kB activation as determined through DNA binding. As reported by Tharappel (2001), NF-kB activity is assayed with electrophoretic mobility shift assay with nuclear extracts prepared from frozen liver tissue as a measure of DNA binding of NF-kB. Increased transcription of downstream targets of NF-kB activity has also been measured. It has been suggested that PPARα may act as a protective mechanism against liver toxicity. Ito et al. (2007) cite repression of NF-kB by PPARα to be the rationale for their hypothesis that PPARα-null mice may be more vulnerable to tumorigenesis induced by exposure to environmental carcinogens. However, as shown in Section E.3.4.1.2, although DEHP was reported to also induce glomerulonephritis more often in PPARα-null mice, as suggested (Kamijima et al., 2007) to be due of the absence of PPARα-dependent anti-inflammatory effect of antagonizing the oxidative stress and NF-κB pathway, there was no greater or lesser susceptibility to DEHP-induced liver carcinogenicity in the PPARα null mice.

Because PPARα is known to exert anti-inflammatory effects by inducing expression of IκBα, which antagonizes NFκB signaling, the expression of IκBα has been measured in some studies (Kamijima et al., 2007), as well as expression of TNR1 mRNA to evaluate the sensitivity to the inflammatory response. Ito et al. (2007) reported that in wild-type mice, there did not appear to be a difference between controls and DEHP treatment for p65 immunoblot results. DEHP treatment was also reported to not induce p65 or p52 mRNA either or influence the expression levels of TNFα, IkBα, IkBβ, and IL-6 mRNA in wild-type mice.

Tharappel et al. (2001) treated rats with WY-14,643, Gemfibrozil, or dibutyl phthalate and reported elevated NF-kB DNA binding in rats with WY-14,642 to have sustained response but not others. WY-14,643 increased DNA binding activity of NF-kB at 6, 34, or 90 days. Gemfibrozil and DEHP increased NF-kB activity to a lesser extent and not at all times in rats. For Gemfibrozil, there was only a twofold increase in binding at 6 days with no increase at 34 days and an increase only in low dose at 90 days. In rats treated with dibutyl phthalate, there was no change at 6 days; at 34 days, there was an increase at high and low dose and at 90 days, only low-dose animals showed a change. In pooled tissue from WY-14,643-treated animals, the complex that bound the radiolabeled NF-kB fragment did contain both p50 and p65. Both WY-14,643 and Gemfibrozil were reported to produce tumors in rats with dibutyl phthalate untested in rats for carcinogenicity. Thus, early changes in NF-kB were not supported as a key event and WY-14,643 to have a pattern that differed from the other PPARα agonists examined.

In regard to the links between inflammation and cancer, Nickoloff et al. (2005), in their review of the issue, cautioned that such a link is not simple. They noted that:

> dissecting the mediators of inflammation in cutaneous carcinogenic pathways has revealed key roles for prostaglandins, cyclooxygenase-2, tumor necrosis factor-α, AP-1, NF-κB, signal transducer and activator of transcription (STAT)3, and others. Several clinical conditions associated with inflammation appear to predispose patients to increased susceptibility for skin cancer including discoid lupus erythematosus, dystrophic epidermolysis bullosa, and chronic wound sites. Despite this vast collection of data and clinical observations, however, there are several dermatological setting associated with inflammation that do not predispose to conversion to lesions into malaignancies such as psoriasis, atopic dermatitis, and Darier's disease.

Nickoloff et al. (2005) suggested that such a

> link may not be as simple as currently portrayed because certain types of inflammatory processes in skin (and possibly other tissues as well) may also serve a tumor suppressor function. Over the past few months, several publications in leading biomedical journals grappled with an important issue in oncology, namely defining potential links between chronic tissue damage, inflammation, and the development of cancer. Balkwill and Coussens (2004) reviewed the role of the NF-κB signal transduction pathway that can regulate inflammation and also

promote malignancy. Their review summarized the latest findings revealed in a letter to Nature by Pikarsky et al. (2004). Using Mdr2 knockout mice in which hepatitis is followed by hepatocellular carcinoma, Pikarsky et al. implicated TNFα upregulation in tumor promotion of HCC, and suggest that TNFα and NF-κB are potential targets for cancer prevention in the context of chronic inflammation. A similar conclusion was reached with respect to NF-κB by an independent group of investigators using a model of experimental dextran sulfate-induced colitis, in which inactivation of the IκB kinase resulted in reduced colorectal tumors (Greten et al., 2004). Although there are many other clinical condition supporting the concept of inflammation is a critical component of tumor progression (e.g., reflux esophagitis/esophageal cancer; inflammatory bowel disease/colorectal cancer), there is at least one notable example that does not fit this paradigm. As described below, psoriasis is a chronic cutaneous inflammatory disease, which is seldom if ever accompanied by cancer suggesting the relationship between tissue repair, inflammation, and development may not be as simple as portrayed by the aforementioned reviews and experimental results. Besides psoriasis, other noteworthy observations pointing to more complexity include the observation that in the Mdr2 knockout mice, we rarely detect bile duct tumors despite extensive inflammation, NF-κB activation, and abundant proliferation of bile ducts in portal spaces (Pikarsky et al., 2004). Moreover, in a skin-cancer mouse model, NF-κB was shown to inhibit tumor formation (Dajee et al., 2003). Thus, the composition of inflammatory mediators, or the properties of the responding epithelial cells (e.g., signaling machinery, metabolic status), may dictate either tumor promotion or tumor suppression. Chronic inflammation and tissue repair can trigger pro-oncogenic events, but also that tumor suppressor pathways may be upregulated at various sites of injury and chronic cytokine networking.

One cannot easily dismiss the many dilemmas raised by the psoriatic plaque that confound a simple link between the tissue repair, inflammation, and carcinogenesis. Since it is easily visible to the naked eye, and patients may suffer from such lesions for decades, it is difficult to argue that various skin cancers such as squamous cell carcinoma, basal cell carcinoma, or melanoma actually do develop within plaques by are being overlooked by patients and dermatologists. Remarkably, psoriatic plaques are intentionally exposed to mutagenic agents including excessive sunlight, topical administration of crude coal tar, or parenteral DNA cross-linking agent –psoralen followed by ultraviolet light. Moreover these treatments are known to induce skin cancer in nonlesional skin. Thus since psoriatic skin is characterized by altered differentiation, angiogenesis, increased telomerase activity, proliferative changes, and apoptosis resistance, one would expect that each and every psoriatic plaque would be converted to cancer, or at least serve as fertile soil for the presence of non-epithelial skin cancers over time….In conclusion, it would seem prudent to remember the paradigm proposed by Weiss (1971) in which he suggested that premalignant cells do not comprise an isolated island, but are a focus of intense tissue interactions. The myriad inflammatory effects of the tumor microenvironment are important for understanding tumor development, as well as tumor suppression and senescence, and for the design for efficacious prevention strategies against inflammation-associate cancer (Nickoloff et al., 2005).

E.3.3.4. Gender Influences on Susceptibility

As discussed previously, male humans and rodents are generally more likely to get HCC. The increased risk of liver tumors from estrogen supplements in women has been documented. In mice, TCE exposure has been shown not only to have greater variability in response and greater effects on body weight in males (Kjellstrand et al., 1983a; Kjellstrand et al., 1983b) but also to induce dose-related increases in liver weight and carcinogenic response in female mice as well as males (see Section E.2.2). Recent studies have attempted to link differences in inflammatory cytokines and gender differences in susceptibility.

Lawrence et al. (2007) suggested that:

> studies of Naugler et al. (2007) and Rakoff-Nahoum and Medzhitov (2007), advance our understanding of the mechanisms of cancer-related inflammation. They describe an important role for an intracellular signaling protein called MyD88 in the development of experimental liver and colon cancers in mice. MyD88 function has been well characterized in the innate immune response (Akira and Takeda, 2004), relaying signals elicited by pathogen-associated molecules and by the inflammatory cytokine interleukin-1 (IL-1)….The conclusion from Naugler et al. (2007) and Rakoff-Nahoun and Medzhitov is that MyD88 may function upstream of NF-κB in cells involved in inflammation-associated cancer. Immune cells infiltrate the microenvironment of a tumor. Naugler et al. (2007) and Rakoff-Nahoun and Medzhitov (2007) suggest that the development of liver and intestinal cancers in mice may depend on a signaling pathway in infiltrating immune cells that involved the protein MyD88, the transcription factor NF-κB, and the pro-inflammatory cytokine Il-6. TLR binds a ligand which acts on MyD88 which acts on NF-κB which leads to secretion of inflammatory cytokine IL-6 which leads to promotion of tumor cell survival and proliferation.

Naugler et al. (2007) suggested gender disparity in MyD88-dependent IL-6 production was linked to differences in cancer susceptibility using the DEN model (a mutagen with concurrent regenerative proliferation at a single high dose) with a single injection of DEN. Partial hepatectomy was reported to induce no gender-related difference in IL-6 increase. After DEN treatment, the male mouse had 275 ng/mL as the peak IL-6 levels 12 hours after DEN, and for female mice, the peak was reported to be 100 ng/mL 12 hours after DEN administration. This is only about a 2.5-fold difference between genders. IL-6 mRNA induction was reported for mice 4 hours after DEN, at a time when there was no difference in serum IL-6 between male and female mice. It was not established that the 4-hour results in mRNA translated to the differences in serum at 12 hours between the sexes. The magnitude of mRNA differences does not necessarily hold the same relationship as the magnitude in serum protein. In fact, there was not a linear correlation between mRNA induction and IL-6 serum levels.

A number of issues complicate the interpretation of the results of the study. The study examined an acute response for the chronic endpoint of cancer and may not explain the differences in gender susceptibility for agents that do not cause necrosis. The DEN was administered in 15-day-old mice (which had not reached sexual maturity) for tumor information at a much lower dose than used in short-term studies of inflammation and liver injury in which mature mice were used. If large elevations of IL-6 are the reason for liver cancer, why does not a partial hepatectomy induce liver cancer in itself?

The percentage of proliferation at 36 and 48 hours after partial hepatectomy was the same between the sexes. If a 2.5-fold difference in IL-6 confers gender susceptibility, it should do so after partial hepatectomy and lead to cancer. For female mice, partial hepatectomy showed alterations in a number of parameters. However, partial hepatectomy does not cause cancer alone. The 5-fold increase 4 hours after DEN induction of IL-6 mRNA in male mice is in sharp contrast to the 27-fold induction of IL-6 1 hour after partial hepatectomy (in which at 4 hours, the IL-6 had diminished to 6-fold). There appeared to be variability between experiments. For example, the difference in males between experiments appears to be the same magnitude as the difference between male and female in one experiment and the baseline of IL-6 mRNA induction appeared to be highly variable between experiments as well as absolute units of ALT in serum 24 and 48 hours after DEN treatment that tended to be greater that the effects of treatments. The experiments used very few animals (n = 3) for most treatment groups. Of note is that the MyD88 -/- male mice still had a background level of necrosis similar to that of WT mice at 48 hours after DEN treatment, a time, long after the peak of IL-6 mRNA induction and IL-6 serum levels were reported to have peaked.

One of the key issues regarding this study is whether difference in IL-6 reported here lead to an increase in proliferation and does that difference within 48 hours of a necrotizing dose of a carcinogen change the susceptibility to cancer? This report shows that male and female mice have a difference in necrosis after carbon tetrachloride and a difference in proliferation. Are early differences in IL-6 at 4 hours related to the same kind of stimulus that leads to necrosis and concurrent proliferation? The amount of proliferation (as measured by DNA synthesis) between male and female mice 48 hours after DEN was very small and the study was conducted in a very few mice (n = 3). At 36 hours, the degree of proliferation was almost the same between the genders and about 0.6% of cells. The baseline of proliferation also differed between genders, but the variation and small number of animals made it insignificant statistically. At 48 hours the differences in proliferation between the male and female mouse were more pronounced, but were still quite low (2% for males and ~1% for females). Is the change in proliferation just a change in damage by the agent? Given the large variation in serum ALT and by inference necrosis, is there an equal amount of variability in proliferation? This study gives only limited information for DEN treatment.

The difference in incidence of HCC was reported to be greater than that of "proliferation" between genders and of other parameters, although differences in tumor multiplicity or size between the genders are never given in the paper. Most importantly, comparisons between the short-term changes in cytokines and indices of acute damage are for adult animals that are sexually mature and at doses that are 4 times (100 vs. 25 mg/kg) that of the sexually immature animals that are going through a period of rapid hepatocyte proliferation (15-day-old animals).

It is therefore difficult to extrapolate between the two paradigms to distinguish the effects of hormones and gender on the response. Finally, the work of Rakoff-Nahoum and Medzhitov (2007) showed that it is the effect of tumor progression and not initiation that is affected by MyD88 (a signaling adaptor to Toll-like receptors). Thus, examination of parameters at the initiation phase at necrotic doses for liver tumors may not be relevant.

E.3.3.5. Epigenomic Modification

There are several examples of chemical exposure to differing carcinogens that have led to progressive loss of DNA methylation (i.e., DNA hypomethylation) including TCE and its metabolites. The evidence for TCE and its metabolites is specifically discussed in Section E.3.4.2.2. Other examples of carcinogen exposures or conditions that have been noted to change DNA methylation are early stages of tumor development include ethionine feeding, phenobarbitol, arsenic, dibromoacetic acid, and stress. However, it has not yet been established whether epigenetic changes induced by carcinogens and found in tumors play a causative role in carcinogenesis or are merely a consequence of the transformed state (Tryndyak et al., 2006).

Pogribny et al. (2007) reported the effects of WY-14,643 on global mouse DNA hypomethylation exposed at 1,000 ppm for 1 week, 5 weeks, or 5 months. What is of particular note in this study is that at this exposure level, one commonly used for mode-of-action studies using WY-14,643 to characterize the effects of PPARα agonists as a class, there was significant hepatonecrosis and mortality reported by Woods et al. (2007a).

Both wild-type and PPARα -/- null mice were examined. In wild-type mice DNA syntheses was elevated 3-, 13-, and 22-fold of time-matched controls after 1 week, 5 weeks, and 5 months of WY 14,543 treatment. Changes in ploidy were not examined. After 5 weeks of exposure, the ratio of unmethylated CpG cites in whole-liver DNA was the same for WY-14,643 treatment and control but by 5 months, there was an increase in hypomethylation in WY-14,643 treated wild-type mice. The authors did not report whether foci were present or not, which could have affected this result. The similarity in hypomethylation at 5 days and 5 weeks, a time point that also had a small probability of foci development, is suggestive of foci affecting the result at 5 months.

For PPAR -/- mice, there was increased hypomethylation reported at 1 and 5 weeks after WY-14,643 treatment that was not statistically significant with so few animals studied. At 5 months, the null mice had decreased hypomethylation compared to 1 and 5 weeks. The authors

noted that methylation of c-Myc genes was reported to not be affected by long-term dietary treatment with WY-14,643 even though WY-14,643-related hypomethylation of c-Myc gene early after a single dose of WY-14,643 has been observed (Ge et al., 2001a). The authors concluded "thus, alterations in the genome methylation patterns with continuous exposure to nongenotoxic liver carcinogens, such as WY, may not be confined to specific cell proliferation-related genes."

Pogribny et al. (2007) reported Histone H3 and H4 trimethylation status in wild-type and PPAR null mice to show a rapid and sustained loss of histone H3K9 and histone H4K20 trimethylation in wild-type mice fed WY-14,643 from 1 week to 5 months. There was no progressive loss in histone hypomethylation, with the same amount of demethylation occurring at 5 days, 5 weeks, and 5 months in wild-type mice fed WY-14,643. The change from control was ~60% reduction. The control values with time were not reported and all controls were pooled to give one value (n = 15). For PPAR -/- l mice, there was a slight decrease with WY-14,643 treatment (~15%) reported. In wild-type mice, WY-14,643 treatment was reported to have no effect on the major histone methyltransferase, Suv39h1, while expression of another (PRDM/Riz1) increased significantly as early as one week of treatment and remained elevated for up to 5 months. The effect on expression of Suv420h2 (responsible for histone H4K20 trimethylation) was more gradual and the amounts of this protein in livers of mice fed WY-14,643 were reported to be lower than in control.

The authors did not examine these parameters in the null mice, so the relationship of these effects to receptor activation cannot be determined. Pogribny et al. (2007) reported hypomethylation of retroelements (LTR IAP, LINE1, and LINE2 retrotransposons) following long-term exposure to WY-14,643, which the authors concluded can have effects on the stability of the genome. Again, these results are for whole liver that may contain foci.

Nevertheless, these findings raise questions about other target organs and a more general mechanism for WY-14,643 effects than a receptor mediated one. The lack of effects on c-Myc and the irrelevance of the transient proliferation through it reported here gives more evidence of the irrelevance of a mode of action dependent on transient proliferation. The authors noted that studies show that a sustained loss of DNA methylation in liver is an early and indispensable event in hepatocarcinogenesis induced by long-term exposure of both genotoxic and nongenotoxic carcinogens in rodents. Thus, this statement argues against making such a distinction in mode of action for "genotoxic" and "nongenotoxic" carcinogens. Finally, the use of a dose that Woods et al. (2007a) demonstrate to have significant hepatonecrosis and mortality, limits the interpretation of these results and their relevance to models of carcinogenesis without concurrent necrosis.

Strain sensitivity to hepatocarcinogenicity has been investigated in terms of short-term changes in methylation. Bombail et al. (2004) reported that a tumor-inducing dose of phenobarbital reduced the overall level of liver DNA methylation in a tumor-sensitive ($B6C3F_1$)

mouse strain but that the same dose of phenobarbital did not alter the global methylation level in a more tumor-resistant strain (C57BL/6), although the compound increased hepatocyte proliferation as measured by increased DNA synthesis in both strains (Counts et al., 1996). Bombail et al. reported that "In a similar study, Watson and Goodman (2002) used a PCR-based technique to measure DNA methylation changes specifically in GC-rich regions of the mouse genome." Watson and Goodman (2002) found that, that in these areas of the genome, exposure to phenobarbital caused an increase in methylation in dosed animals compared with control animals. Again, the change was more pronounced in tumor-prone C3H/He and $B6C3F_1$ strains than in the less sensitive C57BL/6 strain. They also reported increased DNA synthesis in C57BL/6 mice but decreased global methylation in the $B6C3F_1$ strain after phenobarbitol administration for 1–2 weeks. The lifetime spontaneous tumor rates were reported to be <5% in C57BL/6 mice but up to 80% in C3H/He mice.

Counts et al. (1996) reported cell proliferation and global hepatic methylation status in relatively liver tumor susceptible $B6C3F_1$ with relatively resistant C57BL6 mice following exposure to phenobarbitol and/or chlorine/methionine deficient (CMD) diet. Cell proliferation (i.e., DNA synthesis) was reported to be higher in C57BL/6 mice, while transient hypomethylation occurred to a greater extent in $B6C3F_1$ mice after phenobarbital treatment. Dual administration of CMD and phenobarbitol led to enhanced cell proliferation and greater global hypomethylation with similar trends in terms of strain sensitivities in comparison to with either treatment alone (i.e., greater increase in cell proliferation in C57BL/6 and greater levels of hypomethylation in $B6C3F_1$). Thus, the authors concluded that $B6C3F_1$ mice have relatively low capacity to maintain the nascent methylation status of their hepatic DNA.

However, on the whole, the control values for methylation for the C57BL/6 mice appear to be slightly higher than the $B6C3F_1$ mice. Claims that the liver tumor sensitive $B6C3F_1$ had more global hypomethylation after a promoting stimulus, which could be related to tumor sensitivity, are tempered by the fact that the resistant strain had a higher control baseline of methylation. The baseline level of LI or hepatocyte proliferation also appears to be slightly higher in the C57BL/6 mouse. In addition, the largest strain difference in hypomethylation after a CMD diet was at week 12 (135% of control for the $B6C3F_1$ strain and 151% of control for the C57BL/6 strain) and this pattern was opposite that for the 1-week time point. Thus, the suggestion by Counts et al. (1996), that the inability to maintain methylation status by the $B6C3F_1$ strain, is also not supported by the longer duration data for CMD diet.

E.3.4. Specific Hypothesis for Mode of Action of TCE Hepatocarcinogenicity in Rodents

E.3.4.1. PPARα Agonism as the Mode of Action for Liver Tumor Induction—The State of the Hypothesis

PPARα receptor activation has been suggested to be the mode of action for TCA liver tumor induction and for TCE liver tumor induction to occur primarily as a result of the presence

of its metabolite TCA (NRC, 2006). However, as discussed previously (see Section E.2.1.10), TCE-induced increases in liver weight have been reported in male and female mice that do not have a functional PPARα receptor (Ramdhan et al., 2010; Nakajima et al., 2000). The dose-response for TCE-induced liver weight increases differs from that of TCA (see Section E.2.4.2). The phenotype of the tumors induced by TCE have been described to differ from those by TCA and to be more like those occurring spontaneously in mice, those induced by DCA, or those resulting from a combination of exposures to both DCA and TCA (see Section E.2.4.4). As to whether TCA-induced tumors are induced through activation of the PPARα receptor, the tumor phenotype of TCA-induced mouse liver tumors has been reported to have a pattern of H-ras mutation frequency that is opposite to that reported for other peroxisome proliferators (see Section E.2.4.4.; Bull et al., 2002; Stanley et al., 1994; Hegi et al., 1993; Fox et al., 1990). While TCE, DCA, and TCA are weak peroxisome proliferators, liver weight induction from exposure to these agents has not correlated with increases in peroxisomal enzyme activity (e.g., PCO activity) or changes in peroxisomal number or volume. However, liver weight induction from subchronic exposures appears to be a more accurate predictor of carcinogenic response for DCA, TCA, and TCE in mice (see Section E.2.4.4). The database for cancer induction in rats is much more limited than that of mice for determination of a carcinogenic response to these chemicals in the liver and the nature of such a response.

The mode of action for peroxisome proliferators has been the subject of research and debate for several decades. It has evolved from an "oxidative damage" due to increased peroxisomal activity to a mode-of-action framework example developed by Klaunig et al. (2003) that described causal inferences for hepatocarcinogenesis after a chemical exposure was shown to activate of the PPAR-α receptor with concurrent perturbation of cell proliferation and apoptosis, and selective clonal expansion. Of note, although inhibition of apoptosis was proposed as part of the sequellae of PPARα activation, as noted in Section E.2.4.1, no changes in apoptosis in mice exposed to TCE have been reported, with the exception of mild enhanced apoptosis at a 1,000 mg/kg-day dose. More importantly, for mice, the rate of apoptosis decreases as mice age and appears to be lower than that of rats. While DCA exposure has been noted to reduce apoptosis, the significance of DCA-induced reduction in apoptosis from a level that is already inherently low in the mouse, is difficult to apply as the mode of action for DCA-induced liver cancer.

Klaunig et al. (2003)based causal inferences on the attenuation of these events in PPAR-α-null mice in response to the prototypical agonist WY-14,643 with a number of intermediary events considered to be associative (e.g., expression of peroxisomal and nonperoxisome genes, peroxisome proliferation, inhibition of gap junction intracellular communication, hepatocyte oxidative stress as well as Kupffer cell-mediated events). The data set for DEHP was prominently featured as an example of "PPAR-α induced hepatocarcinogenesis." For DEHP, PPAR-α activation was described as the initial key event with evidence lacking for a direct effect

but primarily supported by evidence from PPAR-α-knockout mice treated with WY-14,643. Klaunig et al. (2003) concluded that "…all of the effects observed are due only to the activation of this receptor and the downstream events resulting from this activation and that no other modes of action are operant"

Although that PPARα receptor activation is the sole mode of action for DEHP has been cited by several reports (including IARC, 2000), several articles have questioned the adequacy of this proposed mode of action (Guyton et al., 2009; Caldwell et al., 2008b; Melnick et al., 2008; Keshava et al., 2007; Caldwell and Keshava, 2006; Keshava and Caldwell, 2006); FIFRA SAP, 2004). New information is now available that also questions several of the assumptions inherent in the proposed mode of action by Klaunig et al. (2003) and the dismissal of PPARα agonists as posing a health risk to humans. These issues were recently examined in Guyton et al. (2009) and are discussed below. Furthermore, IARC has recently concluded that additional mechanistic information has become available, including studies with DEHP in PPAR-α-null mice, studies with several transgenic mouse strains, carrying human PPARα or with hepatocyte-specific constitutively activated PPARα and a study in humans exposed to DEHP from the environment that has changed its conclusions regarding the relevance of rodent tumor data to human risk (Grosse et al., 2011). Data from these new studies suggest that many molecular signals and pathways in several cell types in the liver, rather than a single molecular event, contribute to cancer development in rodents, with IARC concluding that the human relevance of the molecular events leading to DEHP-induced cancer in several target tissues (e.g., liver and testis) in rats or mice could not be ruled out, resulting in the evaluation of DEHP as a Group-2B agent, rather than Group 3.

Specific questions have been raised about the use of WY-14,643 as a prototype for PPARα (especially at necrogenic doses) and use of the PPARα -/- null mouse in abbreviated bioassays to determine carcinogenic hazard.

E.3.4.1.1. Heterogeneity of PPARα Agonist Effects and Inadequacy of WY-14,643 Paradigm as Prototype for Class

Inferences regarding the carcinogenic risk posed to humans by PPARα agonists have been based on limited epidemiology studies in humans that were not designed to detect such effects. However, as noted by Nissen et al. (2007), the PPARα receptor is pleiotropic, highly conserved, has "cross talk" with a number of other nuclear receptors, and plays a role in several disease states. "The fibrate class of drugs, which are PPARα agonists intended to treat dyslipidemia and hypercholesterolemia, have recently been associated with a number of serious side effects." While these reports of clinical side effects are for acute or subchronic conditions and are not (and would not be expected to be) able to detect liver cancer from fibrate treatment, they clearly demonstrate that compounds activating the PPAR receptors may produce a spectrum of effects in humans and the difficulty in studying and predicting the effects from PPAR

agonism. Graham et al. (2004) recently reported significantly increased incidence of hospitalized rhabdomyolysis in patients treated with fibrates both alone and in combination with statins. Even though pharmaceutical companies have spent a great deal of effort to develop agonists that are selective for desired effects, the pleiotropic nature of the receptor continues to be an obstacle.

Also, fibrates, WY-14,643, and other PPARα agonists are pan agonists for other PPARs. Shearer and Hoekstra (2003) noted that fibrates, including Fenofibrate, Clofibrate, Bezafibrate, Ciprofibrate, Gemfibrozil, and Beclofibrate are all drugs that were discovered prior to the cloning of PPARα and without knowledge of their mechanism of action but with optimization of lipid lowering activity carried out by administration of candidates to rodents. They report that many PPARα ligands, including most of the common fibrate ligands, show only modest selectivity over the other subtypes with, for example, fenofibric acid and WY-14,643 showing <10-fold selectivity for activation of human PPARα compared to PPARγ and/or PPARδ. In human receptor transactivation assays, they report:

> Human receptor transactivation assays of median effective concentration (EC_{50}):
> WY-14,643 = 5.0 μm for PPARα, 60 μm for PPAR γ, 35 μm for PPARδ.
> Clofibrate = 55 μm for PPARα, ~500 μm for PPAR γ, inactive at 100 μm for PPARδ.
> Fenofibrate = 30 μm for PPARα, 300 μm for PPAR γ, inactive at 100 μm for PPARδ.
> Bezafibrate = 50 μm for PPARα, 60 μm for PPAR γ, 20 μm for PPARδ.
>
> Murine receptor transactivation assay of EC_{50}:
> WY = 0.63 μm for PPARα, 32 μm for PPAR γ, inactive at 100 μm for PPARδ.
> Clofibrate = 50 μm for PPARα, ~500 μm for PPAR γ, inactive at 100 μm for PPARδ.
> Fenofibrate = 18 μm for PPARα, 250 μm for PPAR γ, inactive at 100 μm for PPARδ.
> Bezafibrate = 90 μm for PPARα, 55 μm for PPAR γ, 110 μm for PPARδ.

Thus, these data show the relative effective concentrations and "potency for PPAR activity" of various agonists in humans and rodents, rodent and human responses may vary depending on agonist, agonists vary in what they activate between the differing receptors, and there is a great deal of transactivation of these drugs.

For fibrates specifically, a study by Nissen et al. (2007) reported that in current practice, two fibrates, Gemfibrozil and Fenobibrate, are still widely used to treat a constellation of lipid abnormalities known as atherogenic dyslipidemia and note that currently available fibrates are weak ligands for the PPARα receptor and may interact with other PPAR systems. They noted that the pharmaceutical industry has sought to develop new, more potent and selective agents within this class but, most importantly, that none of the novel PPARα agonists has achieved regulatory approval and that according to a former safety officer in the U.S. Food and Drug Administration (El-Hage, 2007) that >50 PPAR modulating agents have been discontinued due

to various types of toxicity (e.g., elevations in serum creatinine, rhabdomylosis, "multi-species, multi-site increases in tumor with no safety margin for clinical exposures," and adverse cardiovascular outcomes) but without scientific publications describing the reasons for termination of the development programs. Nissen et al. (2007) reported differences in effect between a more highly selective and potent PPARα agonist and the less potent and specific one in humans. They noted:

> a recent large study of Fenofibrate in patients with diabetes showed no significant reduction in morbidity but a trend toward increased all-cause mortality (Keech et al., 2006; Keech et al., 2005). Whether this potential increase in mortality is derived from compound specific toxicity of Fenofibrate or is an adverse effect of PPARα activation remains uncertain."
>
> In addition to the lack of publication of effects from PPAR agonists in human trials in which toxicity can be examined as noted by Nissen et al., the Keech study is illustrative of the problem in trying to ascertain liver effects from fibrate treatment in humans as the focus of the outcomes was coronary events in a study of 5 years duration in a older diabetic population. As stated above, the challenges the pharmaceutical industry and the risk assessor face in determining the effects of PPAR agonists is "that these compounds and drugs modulate the activity of a large number of genes, some of which produce unknown effects."

Nissen et al. further noted that:

> Accordingly, the beneficial effects of PPAR activation appear to be associated with a variety of untoward effects which may include, oncogenesis, renal dysfunction, rhabdomylosis, and cardiovascular toxicity. Recently, the FDA began requiring 2-year preclinical oncogenicity studies for all PPAR-modulating agents prior to exposure of patients for durations of longer than 6 months (El-Hage, 2007).

Guyton et al. (2009) further explored the status of the PPARα epidemiological database and describe its inability to discern a cancer hazard from the available data. Thus, while existing evidence for liver cancer in humans is null rather than negative, there remains a concern for oncogenicity and many obstacles for determining such effects through human study. The heterogeneity in response to PPARα agonists and the heterogeneity of effects they cause (Keshava and Caldwell, 2006) are evident from these reports.

Many studies have used the effects of WY-14,643 at a very high dose and extrapolated those findings to PPARα agonists as a class. However, this diverse group of chemicals has varying potencies and effects for the "key events" described by Klaunig et al. (Keshava and Caldwell, 2006; 2003). The standard paradigm used with WY-14,643 to induced liver tumors in all mice exposed to 1 year (an abbreviated bioassay), uses a large dose that has also has been

reported to produced liver necrosis, which can have an effect of cell proliferation and gene expression patterns, and to also induce premature mortality (Woods et al., 2007a).

As stated above, WY-14,643 also has a short peak of DNA synthesis that peaks after a few days of exposure, recedes, and then unlike most PPARα agonists studied (e.g., Clofibrate, clofibric acid, Nafenopin, Ciprofibrate, DEHP, DCA, TCA and LY-171883), has a sustained proliferation at the doses studied (David et al., 1999; Carter et al., 1995; Barrass et al., 1993; Lake et al., 1993; Marsman et al., 1992; Tanaka et al., 1992; Eacho et al., 1991; Sanchez and Bull, 1990; Yeldandi et al., 1989; Marsman et al., 1988). Clofibrate has been shown to have a decrease in proliferation gene expression shortly after its peak (see Section E.3.3.2).

As shown above for WY-14,643, hepatocellular increases in DNA synthesis did not appear to have a dose-response (see Section E.3.3.2), only WY-14,643 had a sustained elevation of Nf-κB (gem and dibutyl phthalate did not) (see Section E.3.3.3.3). The effects on DNA methylation occurred at 5 months and not earlier time points (when foci were probably present) and effects of histone trimethylation were observed to be the same from 1 weeks to 5 months (see Section E.3.3.5). Such effects on the epigenome suggest that other effects of WY-14,643, other than receptor activation, are not specific to just WY-14,643 and are found in a number of conditions leading to cancer and in tumor progression (see Sections E.3.1.1 and E.3.1.7).

In their study of PPARα-independent short-term production of reactive oxygen species from induced by large concentrations of WY-14,643 and DEHP in the diet, Woods et al. (2007a) examined short-term exposures to 0.6% w/w DEHP or 0.05% or 500 pm WY-14,643 for 3 days, 1 weeks or 3 weeks and reported that WY-14,643 induced a dramatic increase in bile flow that was not observed from DEHP exposure. By 1 week of exposure, there was a 5% increase in bile flow for DEHP treatment but a 240% increase in bile flow for WY-14,643 treatment. By 3 weeks, the difference in bile volume between treated and control was 12% for DEHP and 1,100% for WY-14,643 treated animals.

In this study, oxygen radical formation, as measured by spin trapping in the bile, was reported to be decreased after 3 days of DEHP and WY-14,643 treatment. However, the large changes in bile flow by WY-14,643 treatment limit the interpretation of these data along with a small number of animals examined in this study (e.g., six control and DEHP animals and three animals exposed to WY-14,643 at 3 days), a 30% variation in percent liver/body weight ratios between control groups, and the insensitivity of the technique. In an earlier study, oxidative stress appears to be correlated with neither cell proliferation nor carcinogenic potency (Woods et al., 2006). Woods et al. (2006) reported WY-14,643Y or DEHP to induce an increase in free radicals at 2 hours, a decrease at 3 days then an increase at 3 weeks for both. However, radical formation did not correlate with the proliferative response, as DEHP fails to produce a sustained induction of proliferative response in rodent liver but WY-14,643 does, and both WY-14,643 and DEHP gave a similar pattern of radical formation that did not vary much from controls, which is in contrast to their carcinogenic potency.

Although assumed to be a reflection of cell proliferation in many studies of WY-14,643 and by Klaunig et al. (2003), DNA synthesis recorded using the standard exposure paradigm for WY-14,643 can also be a reflection of hepatocyte, nonparenchymal cell, or inflammatory cell mitogenesis (in the case of necrosis induced inflammation), from changes in hepatocyte ploidy, or a combination of all. Other peroxisome proliferators have been shown to have a decrease in proliferation gene expression shortly after their peaks (e.g., Clofibrate, see Section E.3.3.2) and both Methylclofenapate and Nafenopin have been shown to increase cell ploidy, with Nafenopin having the majority of its DNA synthesis as a reflection of increased ploidy with only a small percentage as increases in cell number (see Section E.3.4.1). Several authors have also noted increases in ploidy for WY-14,643 (see Section E.3.4.1).

The Tg.AC genetically modified mouse was used to study 14 chemicals administered by the topical and oral (gavage and/or diet) routes by Eastin et al. (2001). Clofibrate was considered clearly positive in the topical studies but not WY-14,643, regardless of route of administration. Based on the observed responses, it was concluded by the workgroup (Assay Working Groups) that the Tg.AC model was not overly sensitive and possesses utility as an adjunct to the battery of toxicity studies used to establish human carcinogenic risk. The difference in result between Clofibrate and WY-14,643 is indicative of a different mode of action for the two compounds.

Similarly, at large exposure concentrations, Boerrigter (2004) investigated the response of male and female lacZ-plasmid transgenic mice treated at 4 months of age with 6 doses of 2,333 mg/kg DEHP, 200 mg/kg WY-14,643, or 90 mg/kg Clofibrate over a 2-week period. Mutation frequencies were assayed at 21 days following the last exposure. DEHP and WY-14,643 were shown to significantly elevate the mutant frequency in both male and female liver DNA, while Clofibrate, at the dose level studied, was apparently nonmutagenic in male and female liver (i.e., six-dose exposure to DEHP or WY-14,643 over a 2-week period significantly increased the mutant frequency in liver of both female and male mice by approximately 40%). The author noted that:

> the laxZ plasmid-based transgenic mouse mutation assay is somewhat unique among other commercially available models (e.g. mutamouse and big blue), by virtue of its ability to accurately quantify both point mutations and large deletions including those which originate in the lacZ plasmid catamer and extend into the 3' flanking genomic region. It should be noted that to date there is no single, agreed upon protocol for conducting mutagenicity assays with transgenic rodents although several aspects have been upon by the Transgenic Mutation Assays workgroup of the International Workshop on Genotoxicity Procedures.

For several chemicals, both rats and mice demonstrate evidence of receptor activation through peroxisome proliferation and peroxisome-related gene expression, but only one develops cancer. The herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is a striking example of the problems that would be associated with only using evidence of PPARα receptor activation to

make conclusions about the mode of action of liver tumors. 2,4-D is structurally similar to the PPARα agonist Clofibrate and has been shown at similar concentrations to increase peroxisome number and size, increase hepatic carnitine acetyltransferase activity and catalase, and decrease serum triglycerides and cholesterol in rats (Vainio et al., 1983). Peroxisome number was also increased in Chinese hamsters to a similar level as with Clofibrate at the same exposure concentration after 9 days of exposure to 2,4-D (Vainio et al., 1982). In mice, Lundgren et al. (1987) reported that 2,4-D exposure statistically increased the liver-somatic index over controls after a few days of exposure and increased mitochondrial protein, microsomal protein, carnitine acetyltransferase, PCO activity, cytochrome oxidase, cytosolic epoxide hydrolase, microsomal epoxide hydrolase, microsomal P450 content, and hepatic cytosolic epoxide hydrolase in mouse liver. Thus, 2,4-D activates the PPARα receptor, with associated changes in peroxisome-related gene expression, in multiple species and at similar doses to Clofibrate. However, Charles et al. (1996) and Charles and Leeming (1998) reported that in several 2-year studies, there were no 2,4-D-induced increases in liver tumors in F344 rats, CD-1 rats, B6C3F_1 mice, or CD-1 mice.

Another example, is provided by Gemfibrozil, known as (5-2[2,5-dimethylphenoxy] 2,2-dimethylpentanoic acid) and [2,2-dimethyl-5-(2,5-xylyoxy) valeric acid], a therapeutic agent that activates the PPARα receptor and is a peroxisome proliferator, but is carcinogenic only in male rats but not female rats, nor in either gender of mouse (Contrera et al., 1997). Gemfibrozil causes tumors in pancreas, liver, adrenal, and testes of male rats and causes increases in absolute and relative liver weights in both rats and mice (Fitzgerald et al., 1981). Gemfibrozil is a highly effective lipid and cholesterol lowering drugs in humans and in mice (Olivier et al., 1988). However, although Gemfibrozil activates the PPARα receptor and induces peroxisome proliferation in mice, it does not induce liver tumors in that species.

In the long-term study of Bezafibrate, Hays et al. (2005) noted that the role of this receptor in hepatocarcinogenesis has only been examined using one relatively specific PPARα agonist (WY-14,643) and report that Bezafibrate can induce the expression of a number of PPARα target genes (acyl CoA oxidase and CYP4a) and increased liver weight in PPARα knockout mice that is not dependent on activation of PPARβ or PPARγ. As noted by Boerrigter (2004):

> In contrast to DEHP and WY-14,643, Clofibrate produced hepatocellular carcinomas in rats only while no increase in the incidence of tumors was reported in mice (Gold and Zeiger, 1997). However, Clofibrate induces peroxisome proliferation in both rats and mice (Lundgren and DePierre, 1989) but only produced hepatocellular carcinomas in rats (Gold and Zeiger, 1997).

Melnick et al. (1996) noted that similar levels of peroxisomal induction were observed in rats exposed to DEHP and di(2-ethylhexyl) adipate (DEHA) at doses comparable to those used in the bioassays of these chemicals. However, DEHP but not DEHA gave a positive liver tumor

response in 2-year studies in rats. In an evaluation of the carcinogenicity of tetrachloroethylene, an expert panel of the IARC concluded that the weak induction of peroxisome proliferation by this chemical in mice was not sufficient to explain the high incidence of liver tumors observed in an inhalation bioassay.

In adult animals, apoptosis acts as a safeguard to prevent cells with damaged DNA from progressing to tumors, but like cell proliferation, alterations in apoptosis are common to many modes of action. In addition, only short-term data are available on changes in apoptosis due to PPARα agonists, and long-term changes have not been investigated (Rusyn et al., 2006). For example, although a decrease in apoptosis has also been suggested to be an important additional molecular event that may affect the number of cells in rodent liver following exposure to the peroxisome proliferator DEHP, apoptosis rates have not investigated past 4 days of exposure and thus, the time-course of this event is uncertain. The antiapoptotic effects of PPAR agonists appear to be also dependent on nonparenchymal cells (i.e., Kupffer cells), which do not express PPARα and could be a transient event (Rusyn et al., 2006). Morimura et al. (2006) reported evidence for exposure to WY-14,643 that does not support a role for PPARα–mediated apoptosis in tumor formation (see Section E.3.4.1.3) as well as appearing to be specific to WY-14,643 (see Section E.3.3.3.3).

The lack of a causal relationship of transient DNA synthesis increases and hepatocarcinogenesis has been raised by many (Caldwell et al., 2008b) and is discussed in Section E.3.4.2 as well as the changes in ploidy (see Section E.3.4.1). In regard to gene expression profiles, many studies have focused on gene profiles during the early transient proliferative phase or have identified genes primarily associated with peroxisome proliferation as "characteristic" or relevant to those associated with tumor induction. Several have focused on the number of genes whose expression "goes up" or "goes down" from a small number of animals. Caldwell and Keshava (2006) presented information on WY-14,643, dibutyl phthalate, Gemfibrozil, and DEHP, and noted inconsistent results between PPARα agonists, paradoxes between mRNA and protein expression, strain, gender, and species differences in response to the same chemical, and time-dependent differences in response for several enzymes and GSH.

E.3.4.1.2. New Information on Causality and Sufficiency for PPARα Receptor Activation

In its review of the U.S. EPA's draft risk assessment of perfluorooctanoic acid (PFOA), the Science Advisory Panel (FIFRA SAP, 2004) expressed concerns about whether PPARα agonism constitutes the sole mode of action for PFOA effects in the liver and the relevance to exposed fetuses, infants, and children. In part based on uncertainties regarding the Klaunig et al. (2003) proposed mode of action, they concluded that the tumors induced by PFOA were relevant to human risk assessment. The hypothesis that activation of the PPARα receptor is the sole mode-of-action of hepatocarcinogenesis induced by DEHP and many other chemicals is further

called into question by recent studies. In the case of DEHP, Klaunig et al. (2003) assumed that WY-14,643 and DEHP would operate through the same key events and that long-term bioassays of DEHP in PPARα -/- knockout mice would be negative and hence demonstrate the need for receptor activation for hepatocarcinogenesis from DEHP.

The fallacy of these assumptions is illustrated by the recent report of the first 2-year bioassay of DEHP in PPARα -/- knockout mice (Sv/129 background strain) that reported DEHP-induced hepatocarcinogenesis (Ito et al., 2007). Further discussion was provided by Guyton et al. (Guyton et al., 2009). Similar to other studies, the PPAR -/- mice had slightly increased liver weights in comparison to controls and treated wild-type mice (~12% increase over controls). In fact, statistical analysis of the incidence data show that adenomas were significantly increased in PPARα -/- mice compared with wild-type mice exposed to 500 ppm DEHP and that a significant dose-response trend for adenomas and adenomas plus carcinomas was observed in PPARα -/- mice (Figure E-5). Overall, the cancer incidences were consistent with a previous study of DEHP (David et al., 1999) in $B6C3F_1$ mice at the same doses for nearly the same exposure duration. A strength of this study is that it was conducted at much lower, more environmentally relevant doses that did not significantly increase liver enzymes as indications of toxicity.

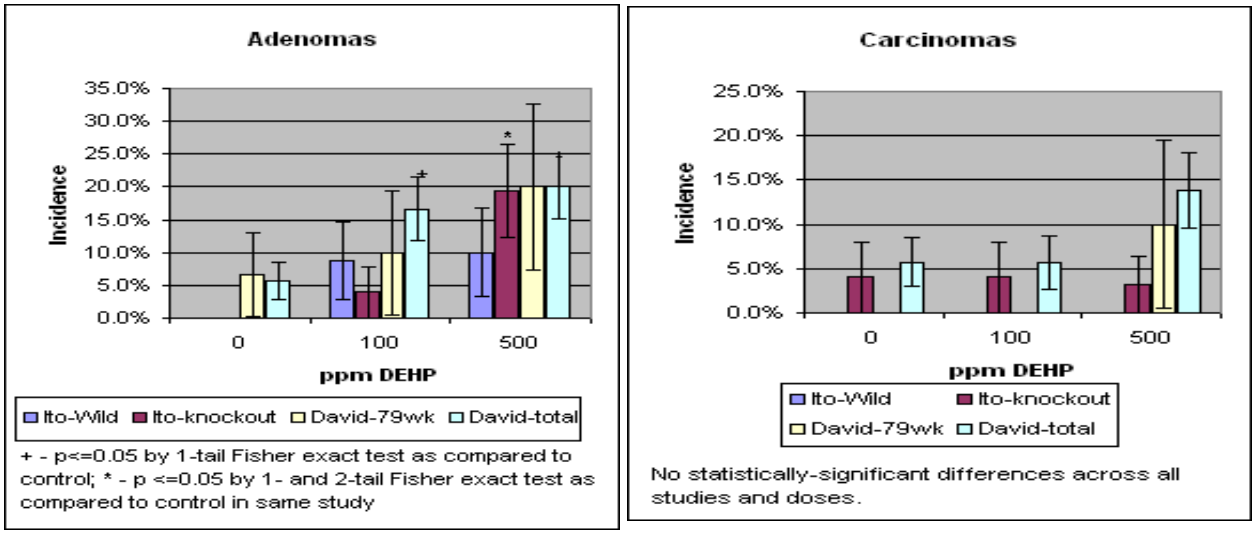


Figure E-5. Comparison of Ito et al. and David et al. data for DEHP tumor induction from (Guyton et al., 2009).

As noted by Kamijo et al. (2007), DEHP was reported also to induce glomerulonephritis more often in PPARα-null mice because of the absence of PPARα-dependent anti-inflammatory effect of antagonizing the oxidative stress and NF-κB pathway (Kamijo et al., 2007). Thus, these data support that hypothesis that there is no difference in liver tumor incidences between PPARα -/- mice and wild-type mice in a standard nonabbreviated exposure bioassay that does

not exceed the maximal tolerated doses and that DEHP can induce hepatotoxicity as well as other effects independent of action of the PPARα receptor.

The study of Yang et al. (2007) informs as to the sufficiency of PPARα receptor activation and subsequent molecular event for hepatocarcinogenesis in mice. The study used a VP16PPARα transgene under control of the liver-enriched activator protein (LAP) promoter to activate constitutively the PPARα receptor in mouse hepatocytes. LAP-VP16PPARα transgenic mice showed a number of effects associated with PPARα receptor activation including decreased serum triglycerides and free fatty acids, peroxisome proliferation, enhanced hepatocyte DNA synthesis, and induction of cell-cycle genes and those described as "PPARα targets" to comparable levels reported for WY-14,643 exposure. Hepatocyte proliferation, as determined by the labeling index of hepatocyte nuclei, was increased after 2 weeks of WY-14,643 treatment over controls (20.5 vs. 1.6% in control livers) with the LAP-VP16PPARα mice giving a similar results (20.8 vs. 1.0% in control livers).

The authors noted that transgenic mice did not appear to have positive labeling of nonparenchymal cell nuclei that were present in the WY-14,643 treated animals. The transferase-mediated dUTP nick end-labeling assay results were reported to show that there was no difference in apoptosis in wild-type mice treated with WY-14,643, the transgenic mice, or controls. In a small number of animals, microsomal genes (CYP4A) and peroxisomal (Acox, BIEN—the bifunctional enzyme) and mitochondrial fatty oxidation genes (LCAD—long chain acyl CoA dehydrogenase and VLCAD) were expressed in the transgenic mice, with WY-14,643 also increasing expression of these genes in wild-type mice but with less lipoprotein lipase (LPL) than the transgenic mice. Hepatic CoA oxidation was increased to a similar level in wild-type mice treated with WY-14,643 and the transgenic mice (n = 3–4) and was statistically different than controls. LAP-VP16PPARα transgenic mice (8 weeks of age) exhibited hepatomegaly (~50 increase percent body/liver weight over controls) and an accumulation of lipid due to triglycerides but not cholesterol.

However, compared to wild-type mice exposed to WY-14,643 for 2 weeks, the extent of hepatomegaly was reduced (i.e., percent liver/body weight increase of ~2.5-fold with WY-14,643 treatment), no hepatocellular hypertrophy or eosinophilic cytoplasms were noted, and no evidence of nonparenchymal cell proliferation was observed in the LAP-VP16PPARα transgenic mice.

At ~1 year of age, Yang et al. (2007) reported there to be no evidence of preneoplastic lesions or hepatocellular neoplasia in LAP-VP16PPARα transgenic mice, in contrast to results after 11 months of exposure to WY-14,643 in wild-type mice. Microscopic examination of liver sections were consistent with the gross findings, as HCCs and hepatic lesions were observed in the long-term WY-14,643 treated wild-type mice, but not in >20 LAP-VP16PPARα mice at the age of over 1 year in the absence of DOX. There was no quantitative information on tumors given nor of foci development in the WY-14,643 mice. As noted by Yang et al. (2007), PPARα

activation only in mouse hepatocytes is sufficient to induce peroxisome proliferation and increased DNA synthesis, but not to induce liver tumors.

Thus, "hepatocyte proliferation" indentified by Klaunig et al. (2003) as a "causal event" in their PPARα mode of action is not sufficient to induce hepatocarcinogenesis. These data not only call into question the adequacy of the mode-of-action hypothesis proposed by Klaunig et al. (2003), but also suggest that multiple mechanisms and multiple cell types may be involved in hepatocarcinogenicity caused by chemicals that are also PPARα agonists.

E.3.4.1.3. Use of the PPAR -/- Knockout and Humanized Mouse

Great importance has been attached to the results reported for PPARα -/- mice and their humanized counterparts with respect to inferences regarding the mode of action or peroxisome proliferators and whether short-term chemical exposures or abbreviated bioassays conducted with these mice can show that a PPARα mode of action is involved. Consequently, the use of these models warrants scrutiny.

Compared to untreated wild-type mice, liver weights in knockout mice or humanized mice have been reported to be elevated (Morimura et al., 2006; Voss et al., 2006; Laughter et al., 2004) and within 10% of each other (Peters et al., 1997). In order to be able to assign effects to a chemical tested in knockout mice, a better characterization is needed of the baseline differences between PPARα -/- knockout and wild-type mice. This is particularly important for examining weak agonists because the changes they induce may be small and need to be confidently distinguished from differences due to the loss of the receptor alone. As shown by the Ito et al. (2007) study and as noted by Maronpot et al. (2004), there is a need for lifetime studies to characterize background or spontaneous tumor patterns and lifespans (including those of the background strain). While the original work by Lee et al. (1995) describes "the mice homozygous for the mutation were viable, healthy, and fertile and appeared normal," the authors did not describe the survival curves for this model nor their background tumor rate. In fact, further work has shown that they carry a background of chronic conditions, including: (1) chronic diseases such as obesity and steatosis (Akiyama et al., 2001; Costet et al., 1998); (2) altered hepatic of hepatocellular structure and function, such as vacuolated hepatocytes (Voss et al., 2006; Anderson et al., 2004), also seen in "humanized" mice (Cheung et al., 2004); and (3) altered lipid metabolism, including reduced glycogen stores, blunted hepatic and cardiac fatty acid oxidation enzyme system response to fasting, elevated plasma free fatty acids, fatty liver (steatosis), impaired gluconeogenesis, and significant hepatic insulin resistance (Lewitt et al., 2001). Howroyd et al. (2004) reported decreased longevity and enhancement of age-dependent lesions in PPARα -/- mice.

These baseline differences from wild-type mice may render them more susceptible to toxic responses or shorten their lifespans with chemical exposure. For example, after administration of 250 µL carbon tetrachloride/kg, all male and 40% of female PPARα knockout

mice were dead or moribund after 2 days of treatment, whereas 25% of male wild-type mice and none of the female wild-type mice exhibited outward signs of toxicity (Anderson et al., 2004). Hays et al. (2005) reported that 100% of PPARα knockout have cholestasis after 1 year of Bezafibrate treatment with higher bile acid concentration than wild-type mice. As described in Section E.2.1.15, Ramdhan et al. (2010) have provided data not only that indicated greater susceptibility of TCE liver toxicity in PPARα-null mice and humanized null mice, but also that there is a background dysregulation of the number of gene and protein expressions and triglyceride accumulation in the liver in these strains.

Lewitt et al. (2001) noted that male knockout mice have more marked accumulation of hepatic fat and hypercholesterolemia and are particularly sensitive to fasting, with some dying if fasted for >24 hours. Sexual dimorphism, especially increased susceptibility of the male mouse, has been reported for knockout mice with pure Sv/129 backgrounds (Anderson et al., 2004; Lewitt et al., 2001) as well as those with a suggested C57BL/6N background (Costet et al., 1998; Djouadi et al., 1998). Akiyama et al. (2001) showed an apparent greater sexual dimorphism in mice with a pure Sv/129 background than C57BL/6N in regard to weight gain from 2 to 9 months, but not in changes in body weight or liver weight between wild-type and knockout animals. Adipose tissue, serum triglycerides, and cholesterol were altered in the knockout animals. Given that the experiment was only carried out for 9 months, changes in body fat, liver weight, and lipid levels may be greater as the animals get older and steatosis is more prevalent.

The dramatic effect on survival as well as gender difference by the increased expression of lipoprotein lipase in the PPARα knockout mouse with further genetic modification is demonstrated by Nöhammer et al. (2003), who reported 50% mortality in 6 months and 100% mortality within 11 months of age while females survived. These differences could affect the results of tumor induction for PPARα agonists with less potency than WY-14,643 that do not produce tumors so rapidly.

In addition, these studies suggest the need for careful consideration of the effects of use of different background strains for the knockout and the need for careful characterization of the background responses of the mouse model and the effects of the use of different background strains for the knockout. Morimura et al. (2006) reported that, using the B6 background strain, there were only foci at time periods but knockouts with the SV129 background had multiple tumors after WY-14,643 treatment.

PPARα knockout mice have also been used to examine the dependence of PPARα on changes in cell signaling, protein production, or liver weight. However, to be useful, the changes incurred just by loss of the PPARα should also be well described. Reported differenced between PPARα-knockout and wild-type mice can impact the sensitivity and specificity of these markers of for the hypothesized mode of action.

In regards to altered cell signaling, Wheeler et al. (2003) note that in normal cells, $p21^{waf}$ and $p27^{kip1}$ inhibit the Cdk/cyclin complexes responsible for cell cycle progression through G1/S

transition. While these cellular signaling molecules are downregulated in response to partial hepatectomy in normal mice, they remain elevated in PPARα knockout mice along with decreased DNA synthesis.

Fumonisins are hepatocarcinogens that have been associated changes in apoptosis and tissue generation, and increased acyl-CoA oxidase and CYP4A (markers of PPARα activation) (Martinez-Larrañaga et al., 1996). Voss et al. (2006) report that the average number of hepatic apoptotic foci per mouse induced by Fumonisins were threefold higher and liver mitotic figures counts were twofold lower in PPARα knockout in comparison to wild-type mice, thus illustrating a difference in proliferative response in the mice. PPARα-null mice have been reported to have increased apoptosis and decreased mitosis with fumonisin treatment.

Voss et al. (2006) also report several differences in gene expression in wild-type and PPARα knockout mice that ranged from 0.3 to 483% of the activity of wild-type mice. The complex expression patterns of gene expression and determination of their mechanistic implications in regard to hepatotoxicity and carcinogenicity are difficult. Certainly the large number of genes whose expression is affected by WY-14,643 (1,012 genes as cited by Voss et al., 2006) illustrates such complexity. Voss et al. (2006) concluded that studies should consider dose- and time course-related effects as well as species and strain-related differences in the expression of gene products.

The "humanized" PPARα mouse has a human copy of PPARα inserted into a PPARα knockout mouse. It is inserted in a tetracycline response system so that in the absence of DOX, only human PPARα is transcribed in humanized mouse liver and not in other tissues. A rigorous examination of newly emerging studies regarding the "humanized" mouse is warranted. The humanized PPARα mouse has been studied in the reports of Cheung et al. (2004), Morimura et al. (2006), and Ramdhan et al. (2010) (see Section E.2.1.15). Many of the issues described above for PPARα -/- mice are of concern for the humanized knockout mouse. In addition, the placement of the humanized PPAR gene is a potential confounding factor, as discussed by Morimura et al. (2006):

> It also cannot be ruled out that the hPPARα mice are resistant to the hepatotoxic effects of peroxisome proliferators due to the site of expression of the human receptor. The cDNA was placed under control of the tetracycline regulatory system and the liver-specific Cebp/B promoter that is preferentially expressed in hepatocytes.

In the Cheung (2004) report, **t**he humanized mouse was fed WY-14,643 for 2 or 8 weeks (age not given for the mice). WY-14,643 and Fenobrate were reported to decrease serum total triglyceride levels in wild and humanized mice to about the level seen in PPARα -/- mice (which were already suppressed without treatment). Hepatomegaly and increase in hepatocyte size were observed in the PPARα-humanized mice fed WY-14,643 for 2 weeks but less than that of wild

mice. By contrast, Morimura et al. (2006) stated that the humanized mice did not exhibit hepatomegaly after treatment with WY-14,643.

Cheung et al. (2004) present figures that showed increased vacuolization of hepatocytes in a control humanized mouse in comparison to wild-type mice. Vacuolization increased with WY-14,643 treatment in the humanized mouse. Therefore, there was a background level of liver dysfunction in these mice even with humanized PPARα. Vacuolization is consistent with fatty liver observed in the nonhumanized PPARα -/- mouse. As reported by Ramdhan et al. (2010), untreated humanized mice had increased triglyceride levels in their livers in comparison to untreated wild type mice.

The authors reported that the humanized mouse did not have increased numbers of peroxisomes after WY treatment. However, they present a figure for genes encoding peroxisomal, mitochondrial, and microsomal fatty acid oxidation enzymes that shows they were still markedly increased in PPARα -humanized mice following 8 weeks of exposure to WY-14,643. Therefore, there is a paradox in these reported results.

Morimura et al. (2006) provided a useful example to illustrate the many issues associated with interpreting studies with genetically-altered animals. While this study is suggestive of a difference in susceptibility to tumor induction between wild-type and PPARα humanized mice, a conclusion that human PPARα is refractory to liver tumor induction is not sufficiently supported.

This study had uneven durations of exposure and follow-up and reported substantial toxicity or mortality that limit the interpretation of the observed tumor rates. For example, the 6-week-old male "humanized" mice had a 44-week experimental period, but for wild-type mice, that period was 38 weeks. In addition, for humanized mice, 10 mice were treated with 0.1% WY-14,643 with 20 controls, but for wild-type mice, 9 mice were given 0.1% WY with 10 controls. Furthermore, wild-type, WY-14,643-treated animals had suppressed growth and only a 50% survival to 38 weeks, so an effective LD_{50} has been used for this length of exposure. Specifically, of the 10 wild-type WY-14,643 treated mice, 3 died of toxicity and 2 were killed due to morbidity and their tissues were examined. Humanized mice had similar growth for animals treated with WY-14,643 or controls with only one mouse killed because of morbidity. Therefore, the reported results, including tumor numbers, are for a mixture of different exposure durations and ages of animals. In addition, the results of the study were reported for only on exposure level.

Furthermore, it is interesting that while control humanized mice had no adenomas, WY-14,643 treated humanized mice had one. Morimura et al. (2006) noted that this adenoma had a morphology "similar to spontaneous mouse liver tumor with basophilic and clear hepatocytes," whereas the tumors in wild-type mice treated with WY-14,643 were more diffusely basophilic. If the humanized animals were allowed to live their natural lifespan, this raises the possibility that WY-14,643 may induce tumors that are similar to other carcinogens rather than those that have been described as "characteristic" of peroxisome proliferators (see

Section E.3.4.1.5) when human PPARα is present. Therefore, the humanized PPARα rather than mouse PPARα may have an association with a tumor phenotype characteristic of other modes of action but this study needed to be carried out for a longer period of exposure and with more animals to make that determination.

The baseline tumor response of PPARα humanized mice needs to be characterized as well as the tumor response following exposure to WY-14,643 or other carcinogens acting through differing modes of action. The numbers of foci were not reported, but "altered foci" were detected in one humanized mouse with WY-14,643 treatment and one without treatment. The phenotypes of the foci were not given by the authors.

As discussed above, changes in liver weights have been associated with susceptibility to liver tumor induction and the issues regarding baseline differences in PPARα -/- mice are equally relevant for PPARα humanized mice. Morimura et al. (2006) reported that absolute liver weight for control humanized mice at 44 weeks was 1.57 g (n = 10). The absolute liver weight for wild control mice was 1.1 g (n = 9) at 38 weeks. The final body weights differed by 14% but liver weights differed by 30%. Therefore, even though comparing different aged mice, the control humanized mice had greater liver size than the wild-type control mice on an absolute and relative basis. This is consistent with humanized knockout mice having greater sized livers and a baseline of hepatomegaly. Ramdhan et al. (2010) reported significantly elevated liver/body weight ratios in untreated humanized mice.

With treatment, Morimura et al. (2006) reported that PPARα humanized mice treated with WY-14,643 had greater absolute and relative liver weights than controls but less elevations than wild-type treated animals. However, because half of the wild-type animals died, it is difficult to discern if liver weights were reported for moribund animals sacrificed as well as animals that survived to 38 weeks for wild-type mice treated with WY-14,643. However, it appears that moribund animals were included that were sacrificed early for treated groups and that values from the animal killed at 27 weeks were added in with those surviving until 45 weeks in the PPARα humanized mice treated with WY-14,643.

With respect to the gene expression results reported by Morimura et al. (2006), it is important to note that they are for liver homogenates with a significant portion of the nuclei from nonparenchymal cell of the liver (e.g., Kupffer and stellate cells). Thus, the results represent changes resulting from a mixture of cell types and from differing zones of the liver lobule, with potentially different gene changes merged together. Livers without macroscopic nodules were used for western blot, but could have contained small foci in the homogenate as well. The gene expression results were also reported for an exposure level of WY-14,643 that is an LD_{50} in wild-type mice and could reflect toxicity responses rather than carcinogenic ones. The samples were also obtained at the end of the experiment (with a mix of durations of exposure) and may not reflect key events in the causation of the cancer but events that are downstream.

These limitations notwithstanding, it is interesting that expression of p53 gene was reported by Morimura et al. (2006) to be increased in PPARα humanized mice treated with WY-14,643 compared to all other groups. Furthermore, of the cell cycle genes that were tested (i.e., CD-1, Cyclin-dependent Kinases 1 and 4, and c-Myc), there was a slightly greater level of c-Myc and CD-1 in control PPARα humanized mice than control wild-type mice as a baseline. This could indicate that there was already increased cell cycling going on in the control PPARα humanized mouse and could be related to the increased liver size. Treatment with WY-14,643 induced an increase in cycling genes in wild-type mice in relation to its control, but whether that induction was greater than control levels for PPARα humanized mice for c-Myc and CDk4 was not reported by the authors.

Apoptosis genes were reported to have little difference between control PPARα humanized and wild-type mice but to have a greater response induced by WY-14,643 in humanized mice for p53 and p21. There was no consistent or large change in apoptosis genes in response to exposure to WY-14,643 in wild-type mice. The increased response of apoptosis genes in PPARα humanized mice without corresponding tumor formation does not support that response as a key event in the mode of action (neither does the lack of response from WY-14,643 in wild-type mice). For genes associated with PPARα peroxisomal (Acox), microsomal (CYP4a), mitochondrial fatty oxidation (Mcad) and especially malic enzyme, there was a greater response in wild-type than PPARα humanized mouse after treatment with WY-14,643. However, this is somewhat in contrast to Cheung et al. (2004), who reported increases in some genes encoding peroxisomal, mitochondrial, and microsomal fatty oxidation enzymes in the PPARα humanized mouse after treatment with WY-14,643.

The results reported by Yang et al. (2007) use another type of "humanized" mouse to study PPARα effects. Yang et al. (2007) used a PPARα humanized transgenic mouse on a PPAR -/- background that has the complete human PPARα (hPPARα) gene on a PAC genomic clone, introduced onto the mouse PPARα-null background and expressed hPPARα not only in the liver but also in other tissues. Mice were administered WY-14,643 or Fenofibrate (0.1 or 0.2% [w/w]). The authors showed a figure representing expression of the hPPARα for two mice with the tissue used for the genotyping exhibiting great variation in expression between the two cloned mice as indicated by intensity of staining. The authors stated that in agreement with mRNA expression, hPPARα protein was highly expressed in the liver of hPPARα^{PAC} mice to an extent similar to the mPPARα in wild-type mice. They reported that following 2 weeks of Fenofibrate treatment, a robust induction of mRNA expression of genes encoding enzymes responsible for peroxisomal (Acox), mitochondrial (MCAD and LCAD), microsomal (CYP4A) and cytosolic (ACOT) fatty acid metabolism were found in liver, kidney, and heart of both wild-type and hPPARα^{PAC} mice, indicating that hPPARα functions in the same manner as mPPARα to regulate fatty acid metabolism and associated genes.

However, the authors did no measures in Fenofibrate-treated animals, only WY-14,643, raising the issue of whether there was a difference in the relative mRNA expression of genes for ACOX etc. and lipids between the two peroxisomal proliferator treatments. The expression of enzymes associated with PPARα induction was presented only for mice treated with Fenofibrate. However, the lipid results were presented only for mice treated with WY-14,643. Therefore, it cannot be established that these two agonists give the same response for both parameters. Also for the enzymes, the relative expressions compared to wild-type controls, the absolute expression, and variation between animals is not reported.

It appears that the peroxisomal enzyme induction by Fenofibrate is the same in the wild-type and transgenic mice. However, in Figure 4 of the paper, the mice treated with WY-14,643 instead of Fenofibrate were presented for the peroxisomal membrane protein 70 (PMP70) in total liver protein gel. There appears to be more PMP70 in the transgenic mice than wild-type mice as a baseline. The PMP70 appeared to be similar after WY-14,643 treatment. However, only one gel was given and no other quantitation was given by the authors.

The authors stated that "in addition WY-14,643 and Fenofibrate treatment produced similar effect to the liver specific humanized PPARα mouse line (Cheung et al., 2004)." However, the results were not the same between Fenofibrate and WY-14,643 and the mouse line used by Cheung et al. (Cheung et al., 2004) had background differences in response and pathology. In one figure in the paper, there appears to be a difference in background level of serum total triglyceride between the wild-type and hPPARα^{PAC} mice that the authors did not note. The power of using such few mice does not help discern any significant differences in background level of triglycerides.

The authors note that WY-14,643 treatment also resulted in decreased serum triglycerides levels in hPPARα^{PAC} mice, consistent with the induction of expression of genes encoding fatty acid metabolism, and that the hypolipidemic effects of fibrates are generally explained by increased expression of LPL and decreased expression of apolipoprotein C- III (Apo C-III) (Auwerx et al., 1996). However, the alteration of these genes by WY-14,643 treatment was only observed in wild-type mice and not in hPPARα^{PAC} mice, suggesting that the hypolipidemic effect observed in hPPARα^{PAC} mice are not through LPL and APO C-III. The authors do not note that there could be a difference in the regulation of these pathways by the transgene rather than how the normal gene is regulated and the pathways it affects. The rationale for examining this question with WY-14,643 treatment rather than with Fenofibrate treatment is not addressed by the authors, especially since the other "markers" of peroxisomal gene induction appear to be affected by Fenofibrate in the wild-type and hPPARα^{PAC} mice.

Hepatomegaly was reported to be observed in the hPPARα^{PAC} mice following 2 weeks of WY-14,643 treatment as revealed by the increase liver to body weight ratio compared to untreated hPPARα^{PAC} mice, but to be markedly lower when compared to wild-type mice under the same treatment.

Histologically, the livers of the wild-type mice treated with WY-14,643 were hypertrophic with clear eosinophilic regions. These phenotypic effects were observed in both wild-type and hPPARα^{PAC} mice. The percent liver/body weight was reported to increase from ~4% in wild-type mice to ~9% after WY-14,643 treatment and from ~4% in hPPARα^{PAC} to little less that 6% after treatment with WY-14,643.

In wild-type mice treated with WY-14,643, the labeling index was 21.8% compared with 1.1% in untreated wild-type controls. In hPPARα^{PAC} mice, WY-14,643 treatment was reported to give an average labeling index of 1.0% compared with 0.8% in the untreated control hPPARα^{PAC} mice. Treatment with WY-14,643 treatment was reported to result in a marked induction in the expression of CDK4 and cyclin D1 in the livers of wild-type mice but to be unaffected hPPARα^{PAC} mice treated with WY-14,643. These data were reported to be in agreement with the liver-specific PPARα-humanized mice that showed no increase in incorporation of BrdU into hepatocytes upon treatment with WY-14,643 (Cheung et al., 2004) and further confirmed that activation of hPPARα does not induce hepatocyte proliferation.

However, the authors present a figure as an example with one liver each with no quantitation given by the authors for BrdU incorporation. It is not clear whether the pictures were taken from the same area of the liver or how representative they are. The numbers of mice were never reported for the labeling index. The data presented do suggest that there was hypertrophy and hepatomegaly in the humanized mice, but not proliferation in this particular WY-14,643 model. Of interest would be investigation of proliferation by other peroxisome proliferators besides WY-14,643 at this necrogenic dose, as it is WY-14,643 that is the anomaly to continue to induce proliferation or DNA synthesis at 2 weeks. The photomicrographs presented by the authors are so small and at such low magnification that little detail can be discerned from them. There are no portal triads or central veins to orient the reader as to what region of the liver has been affected and where, if any, there would be hepatocellular vacuolization.

To determine whether peroxisome proliferation occurred in the hPPARα^{PAC} mice upon administration of peroxisome proliferators, Yang et al. (2007) examined by Western Blot analysis the protein levels of the major PMP70 a marker of peroxisome proliferation). After 2 weeks of treatment with 1,000 ppm WY-14,643, induction of PMP70 was reported to be observed in the wild-type mice as well as in hPPARα^{PAC} mice. The authors suggested that this result indicates that peroxisomal proliferator treatment induced peroxisomal proliferation in hPPARα^{PAC} mice. The results of this study indicate that hepatomegaly and peroxisome proliferation occur in this humanized mouse model when treated with large concentrations of WY-14,643. Thus, these results are inconsistent with claims that peroxisome proliferators cannot cause hepatomegaly or peroxisome proliferation in humans or that humans are refractory to these effects. Like the lipid effects, they suggest that a broader spectrum of effects may occur in humans and decreases the specificity of these effects as species specific. However, due to the

model compound being WY-14,643 at a necrogenic dose of 1,000 ppm, the effect may not be seen in humans using the lower potency peroxisome proliferators. It would have been useful for this study to include an examination of these effects with Fenofibrate rather than WY-14,643 and then attempting to extrapolate such effects to other peroxisome proliferators. The authors often attributed the effects of peroxisome proliferators to those reactions induced by WY-14,643 and did not acknowledge that the changes induced by WY-14,643 may be different. This is especially true in regards to hepatocellular DNA synthesis in which other peroxisome proliferators can cause liver tumors without the sustained proliferation that WY-14,643 induces, especially at a necrogenic dose.

Yang et al. (2007) reported the results of induction of various genes by WY-14,643 in wild-type and hPPARα^{PAC} mice by microarray analysis followed by confirmation and quantitation by qPCR and report that more genes were induced by WY-14,643 in wild-type mice than in hPPARα^{PAC} mice. They reported that:

> importantly, the oncogene c-myc was not induced in hPPARα^{PAC} mice. Moreover, genes encoding cell surface proteins such as Anxa2, CD39, CD63, Ly6D, and CD24a, and several other genes such as *Cidea, Cidec, Dhrs8* and *Hsd11b* were also not induced in hPPARα^{PAC} mice. Interestingly, Sult2a1 was only induced in hPPARα^{PAC} mice and not in WT mice; this gene is also induced in human hepatocytes by PP (Fang et al., 2005). The regulation of several of these genes has previously been demonstrated through a PPARα-dependent mechanism. Additional studies will be necessary to fully explore the molecular regulatory mechanism and the functional implication associated with these differently regulated genes.

The authors did not indicate the context of how the mice were treated, whether these were pooled results, and when the samples were taken. It is assumed to be whole liver. There are several limitations for interpretations of the results such as those presented by Yang et al. (2007), which include the lack of phenotypic anchoring for the results. The authors have shown changes from whole liver and have listed changes in genes between wild-type and humanized mice on a PPAR -/- background that, in itself, will bring about changes in gene expression. The authors acknowledge difficulties in determining what their reported gene changes mean.

Yang et al. (2007) reported that "activation of PPARα alters hepatic miRNA expression (Shah et al., 2007)." They report that let-7C, a miRNA critical in cell growth and shown to target c-Myc, was inhibited by WY-14,643 treatment in wild-type mice and that the expression levels of both pri-let-7C and mature let-7C were significantly higher in hPPARα^{PAC} mice compared to wild-type mice. Treatment with WY-14,643 was reported to decrease the expression of Pri-let-7C and mature let-7C in wild-type mice but in hPPARα^{PAC} mice. The authors noted that:

in addition, the induction of *c-myc* by WY-14,643 treatment in wild type mice did not occur in WY-14,643 treated hPPARα^{PAC} mice. This is in agreement with the previous observation in liver-specific humanized PPARα (Shah et al., 2007) and further indicates the activation of human PPARα does not cause a change in hepatic miRNA and c-*myc* gene expression.

A qPCR analysis of pri-let-7C following 2 weeks of WY-14,632 treatment was reported for wild-type and hPPARα^{PAC} mice (n = 3–4). There appeared to be ~20 times more let-7C expression in hPPARα^{PAC} mice than control wild mice as a baseline. The gel given by the authors showed a very small difference in wild-type mice in let-7C northern blot analysis between a control wild-type and a WY-14,643-treated wild-type mouse. There appeared to be no difference in the hPPARα^{PAC} mice between control and WY-14,643 treatment and a larger stained area than the control wild-type mice. The relative c-Muc expression between the hPPARα^{PAC} mice and wild-type control mice did not correlate with changes in let-7C expression.

Thus, the amount of decrease by treatment with WY-14,632 in wild-type mice appeared to be extremely small compared to the much greater baseline expression in the hPPARα^{PAC} mice. The change brought by WY-14,632 treatment in wild-type mice was a small change compared to the 20-fold greater baseline expression in the hPPARα^{PAC} mice. The authors stated that the expression of the c-Myc regulator was higher in the hPPARα^{PAC} mice, indicating overregulation of cell division and an inability for hepatocytes to proliferate. However, their results showed that there was a greater difference in regulatory baseline function of the PPAR using this paradigm and this construct. Are these differences due to human PPAR or to the way PPAR was put back into PPAR -/- mouse and expected to function? If the experiment included mouse PPAR put back in this way on a null background, what would such an experiment show? Are these results representative of the PPAR or how it is now controlled and expressed? In addition, what would the study of other peroxisome proliferators besides WY-14,643 show in regard to changes in miRNA. Are these results reflective of a just the transient effect that is prolonged in a special case?

As discussed in Section E.3.1.2, there are issues with microarray data in addition to the newly emerging field of miRNA arrays, which include phenotypic anchoring and whether they are from whole liver or pooled samples. The results given in this report are for relative let-7C expression given and not absolute values. The changes in baseline let-7C expression between the wild-type and the hPPARα^{PAC} mice did not correlate with the magnitude of difference in northern blot analysis and did not correlate at all with c-Myc expression reported in this study. Thus, a direct correlation between the effect of let-7C expression and function and effects from WY-14,643 was not supported. The relative expression was reported, but the variation of baseline expression of the "PPAR controlled genes" was not. Given that one of the first figures reported a large difference between animals in expression of the human PPAR gene in the

transgenic animals, how did this difference affect the results given here as relative changes downstream?

Yang et al. (2007) concluded that the hPPARα^{PAC} mice represent the most relevant model for humans since, the tissue distribution of PPARα is similar to that observed in wild-type mice and the hPPARα in hPPARα^{PAC} mice is underregulation of its native promoter. Indeed upregulation of hepatic mPPARα in wild-type mice by fasting was mirrored by the hPPARα in hPPARα^{PAC} mice. However, there was no demonstration that the artificial chromosome that is replicating along with other DNA is controlled sterically by the same control since it is not on the mouse genome in the same place as the native PPAR. There is also not a demonstration of how stable the baseline of PPAR DNA expression is in this mouse model—does it vary as much or more than native PPAR between mice? The authors stated that:

> induction of PPARα target genes for fatty acid metabolism and a decrease in serum triglycerides by PP in hPPARα^{PAC} mice indicates that hPPARα is functional in the mouse environment with respects to regulation of fatty acid metabolism. This is in agreement with the liver-specific PPARα humanized mice that also exhibit these responses (Cheung et al., 2004). Indeed the DNA binding domain of hPPARα is 100% homologous with that of the mouse suggesting that both bind to the same PPRE binding site in the promoter region of target genes. Transfection of hPPAR into murine hepatocytes increased PPs induced peroxisome proliferation related effects (Macdonald et al., 1999). These results suggest that hPPARα and mPPARα do not differ in induction of target genes with known PPRE.

However, replacement with human PPAR in the Cheung et al. (Cheung et al., 2004) model is not sufficient to prevent the same types of toxicity as seen with PPAR knockouts on the hepatocytes such as steatosis.

Yang et al. (2007) note that:

> the increased LPL and decreased expression of apo C-III are proposed to explain the hypolipidemic effects of PPS (Auwerx et al., 1996). However, hPPARα^{PAC} mice treated with PP exhibit lowered serum triglycerides without alteration of the expression of LPL and apo C-III. This indicates the hypolipidemic effects in rodents are mediated via other molecular regulatory mechanisms. It is also suggested that the activation of PPARα by PPs stimulates hepatic fatty acid oxidation and thereby diminishing their incorporation into triglycerides and secretion of VLDL (Frøyland et al., 1997). Consistent with this idea, a robust induction of the genes encoding enzymes for fatty acid oxidation by PP in hPPARα^{PAC} mice were observed. Thus, the exact mechanism by which PPs exert their hypolipidemic effects needs reexamination.

However, the use of two different peroxisome proliferators (i.e., WY-14,643 and Fenofibrate) for two types of effects (peroxisomal and lipid) may be the cause of some paradoxes

here in terms of the mode of action for lipid effects. The baseline differences in the hPPARα^{PAC} mice for serum total triglycerides was not explored by these authors and the small number of animals used make conclusions difficult about the magnitude of difference. The differences in baseline expression for LPL are not discernable in the graphic representation of the results.

Yang et al. (2007) noted that:

> on the other hand, the difference in the affinity of ligands for the human and mouse PPARα receptor was proposed to account for the species difference. The ligand binding domain of hPPARα is 94% homologous with that of the mouse. *In vitro* transactivation assays have previously shown that WY has a higher affinity for rodent PPARα than human PPARα, while Fenofibrate has similar affinity for rodent and human PPARα (Shearer and Hoekstra, 2003; Sher et al., 1993). In the present study WY and Fenofibrate exhibit the same capacity to induce known PPARα target genes in the liver, kidney and heart in both wild-type and hPPARα^{PAC} mice.

The statement by the authors that Fenofibrate and WY-14,643 had the same affinity "as shown by this study" is not correct. The two treatments were not studied for the same enzymes or genes in the data reported in the study. Both WY-14,643 and Fenofibrate can induce PPARα targets, but it was not shown to the same extent. Yang et al. (2007) stated that:

> This is in agreement with the liver-specific PPARα humanized mice that also exhibit a similar capacity to induce PPARα target genes in liver by WY and Fenofibrate (Cheung et al., 2004). Thus, the ligand affinity difference between mouse and human PPARα may not be critical under the conditions of these studies.

Alternatively, these results could reflect that these studies were conducted with two different agonists with different affinities and responses due to receptor activation.

Finally, a useful comparison to make are the differences between wild-type mice, PPARα -/- mice that serve as the background for the transgenic human mouse models, and both transgenic models. The small and variable number of animals examined in these studies is readily apparent. The results of the Cheung et al. (2004) humanized mouse model and those reported for Yang et al. (2007) show differences in the study designs including PPARα agonists studied for particular effects and results reported for similar treatments (see Table E-18).

As shown above, the effect on the PPARα -/- by the knockout included decreased triglyceride levels and slightly increased liver weight. Although treatment with WY-14,643 and Fenofibrate were reported to decrease triglyceride levels in wild-type mice, paradoxically, so did knocking out the receptor. Exposures to WY-14,643 appeared to induce a slight increase and exposures to Fenofibrate induced a slight decrease in triglyceride levels in PPARα -/- mice, but the variability of response and small number of animals in the experiments limited the ability to discern a quantitative difference in the treatments.

In the study by Cheung et al. (2004), it appears that the insertion of humanized PPARα restored the baseline and treatment responses for triglyceride levels. Of note is that use of the same humanized mode in Ramdhan et al. (2010) showed accumulation of triglycerides in the liver of untreated mice. Overall, the results reported by Yang et al. (2007) appeared to show a lower level of triglycerides in control wild-type mice that was similar in magnitude to the treatment effect reported by Fenofibrate by Cheung et al. (2004). However, there also appeared to be restoration of this effect in the humanized mouse model of Yang et al. (2007).

In regard to DNA synthesis, both Cheung et al. (2004) and Yang et al. (2007) only gave results for WY-14,643 and for different durations of exposure, so they were not comparable. It appeared that ~60% of hepatocytes were labeled by 8 weeks of WY-14,643 treatment (Cheung et al., 2004) compared to ~20% after 2 weeks of exposure. Again, this highlights the difference between using WY-14,643 as a model for the PPARα as a class at times when almost all other PPARα agonists have ceased to increase DNA synthesis or have reductions in this parameter. The background changes due to the PPARα -/- knockout were not reported so that the effects of the knockout could not be ascertained. It appeared that insertion of humanized PPARα did not result in restoration of WY-14,643-induced DNA synthesis. The correlation with this parameter and any focal areas of necrosis were not discussed by the authors of the study.

Table E-18. Comparison between results for Yang et al. (2007) and Cheung et al. (2004)[a]

Effect	Wild type mice	PPAR -/- knockout mice	Humanized mice (liver only)	Humanized PAC mice
Triglycerides	**Cheung** **(n = 6–9)** Control 145 mg/mL 0.1% WY-14,643 (2 wks) 60 mg/mL 0.2% Fenofibrate (2 wks) 85 mg/mL **Yang** **(n = 4–6)** Control 95 mg/mL 0.1 % WY-14,643 (2wks) 55 mg/mL	**Cheung** **(n = 6–9)** Control 100 mg/mL 0.1% WY-14,643 (2 wks) 115 mg/mL 0.2% Fenofibrate (2 wks) 85 mg/mL	**Cheung** **(n = 6–9)** Control 175 mg/mL 0.1%WY-14,643 (2 wks) 60 mg/mL 0.2% Fenofibrate (2 wks) 85 mg/mL	**Yang** **(n = 4–6)** Control 120 mg/mL 0.1%WY-14,643 (2 wks) 75 mg/mL
BrdU incorporation	**Cheung** **(n = 5)** Control 1.6% 0.1% WY-14,643 (8 wks) 57.9% **Yang** **(n = 4–6)** Control 1.1% 0.1% WY-14,643 (2 wks) 21.8%	Not done	**Cheung** **(n = 5)** Control 1.6% 0.1% WY-14,643 (8 wks) 2.8%	**Yang** **(n = 4–6)** Control 0.8% 0.1% WY-14,643 (2 wks) 1.0%

Table E-18. Comparison between results for Yang et al. (2007) and Cheung et al. (2004) (continued)

Effect	Wild type mice	PPAR -/- knockout mice	Humanized mice (liver only)	Humanized PAC mice
Hepatomegaly[b] (% liver body weight ratio)	**Cheung (n = 5–9)** Control 4% 0.1% WY-14,643 (2 wks) 11% 0.2% Fenofibrate (2 wks) 8.5% **Yang (n = 4–6)** Control 4% 0.1% WY-14,643 (2 wks) 9%	**Cheung (n = 5–9)** Control 5% 0.1% WY-14,643 (2 wks) 5% 0.2% Fenofibrate (2 wks) 5.5%	**Cheung (n = 5–9)** Control 4.5% 0.1% WY-14,643 (2 wks) 7% 0.2% Fenofibrate (2 wks) 7%	**Yang (n = 4–6)** Control 4% 0.1% WY (2 wks) 6%

[a]The ages of the humanized knockout mice are not given for Cheung et al. (2004) but are 8–10 weeks for Yang et al. (2007).
[b]Percentages are approximate values extrapolated from figures for hepatomegaly.

In regard to hepatomegaly, Fenofibrate and WY-14,643 appeared to both give an increase in liver weight in the humanized mouse model of Cheung et al. (2004) with little effect in the knockout mouse. For Fenofibrate, there was little difference in liver weight gain in the wild-type mouse and that of the humanized mouse model of Cheung et al. (Cheung et al., 2004). However, Fenofibrate was not tested in the humanized mouse model of Yang et al. (2007). In that model, only WY-14,643 was used, but there was still an increase in liver weight. Thus, in terms of effects on liver weight gain and triglyceride levels, both models gave comparable results and appeared to indicate that insertion of humanized PPARα would restore some of the effects of the knockout. However, the results from both experiments highlight the need for adequate numbers of animals and other PPARα agonists to be tested besides WY-14,463 at such a high dose and certainly for longer periods of time to ascertain whether such manipulations will affect carcinogenicity.

The study by Ramdhan et al. (2010) is more definitive in regard to characterization of the effects of the knockout and insertion of human PPARα (see Section E.2.1.15). From this study, dysregulation by the knockout and by reinsertion of human PPARα at levels of >10-fold protein expression can be observed and include alterations in a number of gene and protein expression levels and an underlying background level of hepatic steatosis and triglyceride accumulation.

E.3.4.1.4. NF-κB Activation

NF-κB activation has also been proposed as a key event in the induction of liver cancer through PPARα activation. As discussed in Sections E.3.1.6 and E.3.3.3.3, activation of the NF-κB pathway is implicated in carcinogenesis, nonspecific for a particular mode of action for liver cancer, and is context-dependent on its effects. Its specific actions depend on the cell type and type of agent or signal that activates translocation of the complex. NF-κB is not only involved in biological processes other than tumor induction, but also exhibits some apparently contradictory behaviors (Perkins and Gilmore, 2006). Although many studies point to a tumor-promoting function of NF-κB subunits, evidence also exists for tumor suppressor functions. NF-κB actions are associated with TNF and JNK, among many other cell signaling systems and molecules, and have functions that alter proliferation and apoptosis. NF-κB activation reported in some studies may be associated with early Kupffer cell responses and be associative but not key events in the carcinogenic process. However, most assays look at total NF-κB expression in the whole liver and at the early periods of proliferation and apoptosis. The origin of the NF-κB is crucial as to its effect in the liver. For instance, hepatocyte specific deletion of IKKβ increased DEN-induced hepatocarcinogenesis, but a deletion of IKKβ in both hepatocytes and Kupffer cells, however, was reported to have the opposite effect (Maeda et al., 2005).

E.3.4.1.5. Phenotype as an Indicator of a PPARα Mode of Action

As discussed previously (see Sections E.3.1.5, and E.3.1.8), FAH precede both hepatocellular adenomas and carcinomas in rodents and in humans with chronic liver diseases that predispose them to HCCs. Striking similarities in specific changes of the cellular phenotype of preneoplastic FAH are emerging in experimental and human hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones, radiation, or viruses, or in animal models, by transgenic oncogenes or *Helicobacter hepaticus*. Several authors have noted that the detection of phenotypically similar FAH in various animal models and in humans prone to developing or bearing HCCs favors the extrapolation from data obtained in animals to humans (Bannasch et al., 2003; Su and Bannasch, 2003; Bannasch et al., 2001). In regard to phenotype by tincture, Caldwell and Keshava (2006) stated:

> In addition, the term "basophilic" in describing preneoplastic foci or tumors can be misleading. The different types of FAH have been related to three main preneoplastic hepatocellular lineages: 1) the glycogenotic-basophilic cell lineage, 2) its xenomorphic-tigroid cell variant, and 3) the amphophilic-basophilic cell lineage. Specific changes of the cellular phenotype of the first two lineages of FAHs are similar in experimental and human hepatocarcinogenesis, irrespective of whether they were elicited by DNA-reactive chemicals, hormones, radiation, viruses, transgenic oncogenes and local hyperinsulinism as described by the first two FAHs and this similarity favors extrapolation from data obtained in animals to humans (Bannasch et al., 2003; Su and Bannasch, 2003; Bannasch et al., 2001). In contrast, the amphophilic cell lineage of hepatocarcinogenesis has been observed mainly after exposure of rodents to peroxisome proliferators or to hepadnaviridae (Bannasch et al., 2001).
>
> Bannasch (1996) describes "amphophilic" FAH and tumors induced by peroxisome proliferators to maintain the phenotype as the foci progress to tumors. They are glycogen poor from the start with increased numbers of mitochondria, peroxisomes and ribosomes. The author further states that the "homogenous basophilic" descriptions by others of foci induced by WY are really amphophilic. Agents other than peroxisome proliferators can induce "acidophilic" or "eosinophilic" (due to increased smooth endoplasmic reticulum) or glycognotic foci which tend to progress to basophilic stages (due to increased ribosomes).
>
> Tumors and foci induced by peroxisome proliferators have been suggested to have a phenotype of increased mitochondrial proliferation and mitochondrial enzymes (thyromimetic rather than insulinomimetic) (2006).

Tumors from peroxisome proliferators in Kraupp-Grasl et al. (1990) and Grasl-Kraupp et al. (1993) for rat liver tumors were characterized as weakly basophilic with some eosinophilia and as similar to the description given by Bannasch et al. as amphophilic. However, a number of recent studies indicate that other "classic" peroxisome proliferators may

have a different phenotype than has been attributed to the class through studies of WY-14,643. A recent study of DEHP, another peroxisome proliferator assumed to induce liver tumors through activation of the PPARα receptor, reported the majority of liver FAH to be of the first two types after a lifetime of exposure to DEHP with a dose-related tendency for increased numbers of amphophilic FAHs in rats (Voss et al., 2005). As stated previously, the mode of action of DEHP-induced liver tumors in mice also appears not to be dependent on PPARα activation.

Michel et al. (2007) reported the phenotype of tumors and foci in rats treated with clofibric acid at a very large dose (5,000 ppm for 20 months) and noted that in controls, the first type of foci to appear was tigroid on day 264 and their incidence increased with time representing the most abundant type in this group. They reported no adenomas or carcinomas at up to 607 days after giving saline injection in the control animals.

DEN treatment was examined up to 377 days only, with tigroid, eosinophilic, and clear cell foci observed at that time. Clofibric acid was examined up to 607 days, with tigroid and clear cell foci reported to be the first to appear on day 264, but no other foci class. By day 377, there were tigroid, eosinophilic, and clear cell foci, but no basophilic foci reported with clofibric acid treatment and, although only a few animals were examined, 2/5 had adenomas but not carcinomas. By day 524, all types of foci were seen (including basophilic for the first time) and there were adenomas and carcinomas in 2/5 animals. By 607 days, a similar pattern was observed without adenomas, but 3/6 animals had carcinomas.

Although the number of animals examined was very small, these results indicate that clofibric acid was not inducing primarily "basophilic foci" as reported for peroxisome proliferators, but that the first foci are tigroid and clear cell foci. Basophilic foci did not appear until day 524 as similar to control values for foci development and distribution. However, unlike controls, clofibric acid induced eosinophilic and clear cell foci earlier. This is inconsistent with the phenotype ascribed to peroxisome proliferators as exemplified by WY-14,643.

In regard to GST-π and γ-transpeptidase (GGT), Rao et al. (1986) fed two male F344 rats a diet of 0.1% WY-14,643 for 19 months or three F344 rats 0.025% Ciprofibrate for 15–19 months and reported "altered areas,"(AA) "neoplastic nodules" (NN), and HCCs (HCC). For WY-14,643 treatment, 107 AA, 75 NN, and 5 HCC were noted, and for Ciprofibrate treatment, 107 AA, 27 NN, and 16 HCC were identified. In the WY-14,643-treated rats, HCC, and NN were both GGT and GST-π negative (96–100%) with 87% of AA was negative for both. In Ciprofibrate-treated rats, NN and HCC were negative for both markers (95%) but only 46% of AA were negative for both markers. Thus, a different pattern for tumor phenotype was reported for WY-14,643 and another peroxisome proliferator, Ciprofibrate, in this study as well.

In addition, GGT phenotype is reported not to be specific to weakly basophilic foci. GGT staining was reported to be negative in eosinophilic tumors after initiation and promotion.

Kraupp-Grasl et al. (1990) noted differences among PPARα agonists in their ability to promote tumors and suggested they not necessarily be considered a uniform group. Caldwell and Keshava (2006) suggested that the reports of a simple designation of "basophilic" is not enough to associate a foci as caused by peroxisome proliferators (Bannasch, 1996; Grasl-Kraupp et al., 1993; Kraupp-Grasl et al., 1990). Increased basophilia of tumors and increased numbers of carcinomas is consistent with the progressive basophilia described by Bannasch (1996), as many adenomas progress to carcinomas.

It should be noted that the amphophilic foci and tumors described by Bannasch et al. were primarily studied in rats. Morimura et al. (2006) noted that WY-14,643 induced diffusely basophilic tumors in mice and therefore, identified the WY-14,643 tumors in a way consistent with the descriptions of amphophilic tumors by Bannasch et al. The tumor induced by WY-14,643 in their humanized mouse was reported to be similar to those arising spontaneously in the mouse. However, the mouse response could differ from the rat, especially for PPARα agonists other than WY-14,643.

H-ras activation and mutation studies have attempted to assign a pattern to peroxisome proliferator-induced tumors as noted in Section E.2.4.4. However, also as noted in Section E.2.4.4, the genetic background of the mice used, the dose of carcinogen, and the stage of progression of "lesions" (i.e., foci vs. adenomas vs. carcinomas) may affect the number of activated H-ras containing tumors that develop. Fox et al. (1990) noted that tumors induced by Ciprofibrate (0.0125% diet, 2 years) had a much lower frequency of H-ras gene activation than those that arose spontaneously (2-year bioassays of control animals) or induced with the "genotoxic" carcinogen benzidine-2 HCl (120 ppm, drinking H_2O, 1 year) and that the Ciprofibrate-induced tumors were reported to be more eosinophilic as were the surrounding normal hepatocytes than spontaneously occurring tumors. Anna et al. (1994) also stated that mice treated with Ciprofibrate had a markedly lower frequency of tumors with activated H-ras but that the spectrum of mutations in tumors was similar those in "spontaneous tumors." Hegi et al. (1993) tested Ciprofibrate-induced tumors from Fox et al. (1990) in the NIH3T3 cotransfection-nude mouse tumorigenicity assay and concluded that ras protooncogene activation was not a frequent event in Ciprofibrate-induced tumors and that spontaneous tumors were not promoted with it.

Stanley et al. (1994) studied the effect of MCP, a peroxisome proliferator, in $B6C3F_1$ (relatively sensitive) and C57BL/10J (relatively resistant) mice for H-ras codon 61-point mutations in MCP-induced liver tumors (hepatocellular adenomas and carcinomas). In the $B6C3F_1$ mice, ~24% of MCP-induced tumors had codon 61 mutations, and for C57BL/10J mice, ~13%. The findings of an increased frequency of H-ras mutation in carcinomas compared to adenomas in both strains of mice is suggestive that these mutations were related to stage of progression. Thus, in mice, the phenotype of tumors did not appear to be readily distinguishable

from spontaneous tumors based on tincture for peroxisome proliferators other than WY-14,643, but did have more of a signature in terms of H-ras mutation and activation.

The expression of c-Jun has been used to discern TCE tumors from those of its metabolites. However, as pointed out by Caldwell and Keshava (2006), although Bull et al. (2004) have suggested that the negative expression of c-Jun in TCA-induced tumors may be consistent with a characteristic phenotype shown in general by peroxisome proliferators as a class, there is no supporting evidence of this. While increased mitochondrial proliferation and mitochondrial enzymes (thyromimetic rather than insulinomimetic) properties have been ascribed to peroxisome proliferator-induced tumors, the studies cited in Bull et al. (2004) have not examined TCA-induced tumors for these properties.

E.3.4.1.6. Human Relevance

In its framework for making conclusions about human relevance, the U.S. EPA Cancer Guidelines (U.S. EPA, 2005b) asks that critical similarities and differences between test animals and humans be identified. Humans possess PPARα at sufficient levels to mediate the human hypolipidemic response to peroxisome-proliferating fibrate drugs. Fenofibrate and Ciprofibrate induce treatment-related increases in liver weight, hypertrophy, numbers of peroxisomes, numbers of mitochondria, and smooth endoplasmic reticulum in cynomologous monkeys at 15 days of exposure (Hoivik et al., 2004). Given the species difference in the ability to respond to a mitogenic stimulus such as partial hepatectomy (see Section E.3.3), lack of hepatocellular DNA synthesis at this time point is not unexpected, and as Rusyn (2006) noted, examination at differing time point may produce differing results. It is therefore, generally acknowledged that "a point in the rat and mouse key events cascade where the pathway is biologically precluded in humans in principle cannot be identified" (Klaunig et al., 2003); NRC, 2006). Thus, from a qualitative standpoint, the effects described above are plausible in humans.

As for quantitative differences, there are two key issues. First, as stated in the Cancer Guidelines, when considering human relevance, "Any information suggesting quantitative differences between animals and humans is flagged for consideration in the dose-response assessment." Therefore, while Klaunig et al. (2003) and NRC (2006) go on to suggest that "this mode of action is not likely to occur in humans based on differences in several key steps when taking into consideration kinetic and dynamic factors," under the Cancer Guidelines, such "kinetic and dynamic factors" need to be made explicit in the dose-response assessment, and should not be part of the qualitative characterization of hazard. Second, the discussion above points to the lack of evidence supporting associations between the postulated events and carcinogenic potency. Thus, because interspecies differences in carcinogenicity do not appear to be associated with interspecies differences in postulated events, they do not provide reliable metrics with which to make inferences about relative human sensitivity.

E.3.4.2. Other TCE Metabolite Effects That May Contribute to its Hepatocarcinogenicity

While the focus of most studies of TCA has been its effects on peroxisomal proliferation, DCA has been investigated for a variety of effects that are also observed either in early stages of oncogenesis (glycogen deposition) or conditions that predispose patients to liver cancer. Some studies have examined microarray profiles in attempt to study the mode of action of TCE (see Section E.3.1.2 for caveats regarding such approaches). Caldwell and Keshava (2006) have provided a review of these studies, which is provided below.

E.3.4.2.1. DCA Effects and Glycogen Accumulation Correlations with Cancer

As noted previously, DCA administration has been reported to increase the observable amount of glycogen in mouse liver via light microscopy and, although to not be primarily responsible for DCA-induced liver mass increases, to increase whole-liver glycogen as much by 50% (Kato-Weinstein et al., 2001). Given that TCE and DCA tumor phenotypes indicate a role for DCA in TCE hepatocarcinogenicity (see Section E.2.4.4), Caldwell and Keshava (2006) described the correlations with effects induced by DCA that have been associated with hepatocarcinogenicity.

> A number of studies suggest DCA-induced liver cancer may be linked to its effects on the cytosolic enzyme glutathione (GST)-S-transferase-zeta. GST-zeta is also known as maleylacetoacetate isomerase and is part of the tyrosine catabolism pathway whose disruption in type 1 hereditary tyrosinemia has been linked to increased liver cancer risk in humans. GST-zeta metabolizes maleylacetoacetate (MAA) to fumarylacetoacetate (FAA) which displays apoptogenic, mutagenic, aneugenic, and mitogenic activities (Bergeron et al., 2003; Jorquera and Tanguay, 2001; Kim et al., 2000). Increased cancer risk has been suggested to result from FAA and MAA accumulation (Tanguay et al., 1996). Cornett et al. (1999) reported DCA exposure in rats increased accumulation of maleylacetone (a spontaneous decarboxylation product of MAA), suggesting MAA accumulation. Ammini et al. (2003) report depletion of the GST-zeta to be exclusively a post-transcriptional event with genetic ablation of GST-zeta causing FAA and MAA accumulation in mice. Schultz et al. (2002) report that elimination of DCA is controlled by liver metabolism via GST-zeta in mice, and that DCA also inhibits the enzyme (and thus its own elimination) with young mice being the most sensitive to this inhibition. On the other hand, older mice (60 weeks) had a decreased capacity to excrete and metabolize DCA in comparison with younger ones. The authors suggest that exogenous factors that deplete or reduce GST-zeta will decrease DCA elimination and may increase its carcinogenic potency. They also suggest that, due to suicide inactivation of GST-zeta, an assumption of linear kinetics can lead to an underestimation of the internal dose of DCA at high exposure rates. In humans, GST-zeta has been reported to be inhibited by DCA and to be polymorphic (Blackburn et al., 2001; Blackburn et al., 2000; Tzeng et al., 2000). Board et al. (2001) report one variant

to have significantly higher activity with DCA as a substrate than other GST zeta isoforms, which could affect DCA susceptibility.

Individuals with glycogen storage disease or with poorly controlled diabetes have excessive storage of glycogen in their livers (glycogenosis) and increased risk of liver cancer (Rake et al., 2002; Wideroff et al., 1997; Adami et al., 1996; La Vecchia et al., 1994). In an animal model where hepatocytes are exposed to a local hyperinsulinemia from transplanted islets of Langerhans and the remaining tissue is hypoinsulinemic, insulin induces alterations that resemble preneoplastic foci of altered hepatocytes (FAH) and develop into hepatocellular tumors in later stages of carcinogenesis (Evert et al., 2003). A number of studies have reported suppression of apoptosis, decreases in insulin, and glycogenosis in mice liver by DCA at levels that also induce liver tumors (Bull, 2004b; Bull et al., 2004; Lingohr et al., 2001). In isolated murine hepatocytes, Lingohr et al. (2002) reported DCA-induced glycogenosis was dose related, occurred at very low doses (10 μM), occurred without the presence of insulin, was not affected by insulin addition, was dependent on phosphatidylinositol 3-kinase (P13K) activity, and was not a result of decreased glycogen breakdown. The authors noted that PI3K is also known to regulate cell proliferation and apoptosis in hepatocytes, and that understanding these mechanisms may be important to understanding DCA-induced carcinogenesis. They also report insulin receptor (IR) protein levels decreased to 30% of controls in mice liver after up to 52 weeks of DCA treatment. Activation of the IR is also the principal pathway by which insulin stimulates glycogen synthetase (the rate limiting enzyme of glycogen biosynthesis). However, in DCA-induced liver tumors IR protein was elevated as well as mitogen-activated protein kinase (a downstream target protein of the IR) phosphorylation. DCA-induced tumors were glycogen poor (Lingohr et al., 2001). The authors suggest that normal hepatocytes down-regulate insulin-signaling proteins in response to the accumulation of liver glycogen caused by DCA and that the initiated cell population, which does not accumulate glycogen and is promoted by DCA treatment, responds differently from normal hepatocytes to the insulin-like effects of DCA.

Gene expression studies of DCA show a number of genes identified with cell growth, tissue remodeling, apoptosis, cancer progression, and xenobiotic metabolism to be altered in mice liver at high doses (2 g/L DCA) in drinking water (Thai et al., 2003, 2001). After 4 weeks, RNA expression was altered in 4 known genes (alpha-1 protease inhibitor, cytochrome B5, stearoyl-CoA desaturase and caboxylesterase) in two mice (Thai et al., 2001). Except for Co-A desaturase, a similar pattern of gene change was reported in DCA-induced tumors (10 tumors from 10 different mice) after 93 weeks. Using cDNA microarray in the same mice, Thai et al. (2003) identified 24 genes with altered expression, of which 15 were confirmed by Northern blot analysis after 4 weeks of exposure. Of the 15 genes, 14 revealed expression suppressed two- to fivefold and included: MHR 23A, cytochrome P450 (CYP), 2C29, CYP 3A11, serum paraoxonase/arylesterase 1, liver carboxylesterase, alpha-1 antitrypsin, ER p72, GST-pi 1, angiogenin, vitronectin precursor, cathepsin D, plasminogen precursor (contains angiostatin), prothrombin precursor and integrin alpha 3 precursor. An additional gene, CYP 2A4/5, had a twofold elevation in expression. After 93

weeks of treatment with 3.5 g/L DCA, Northern blot analyses of total RNA isolated from DCA-induced hepatocellular carcinomas showed similar alteration of expression (11 of 15). It was noted that peroxisome proliferator-activated receptor (PPAR)α and IR gene expression were not changed by DCA treatment. Genes involved in glycogen or lipid metabolism were not tested.

Although it has not been possible to determine directly whether DCA is produced from TCE at carcinogenic levels, there is indirect evidence that DCA is formed from TCE *in vivo* and contributes to liver tumor development. Pretreatment with either DCA or TCE inhibits GST-zeta while TCA pretreatment does not (Bull et al., 2004; Schultz et al., 2002). TCE treatment decreased V_{max} for DCA metabolism to 49% of control levels with a 1 g/kg TCE dose resembling effects those of 0.05 g/L DCA (Schultz et al., 2002).

E.3.4.2.2. Genetic Profiling Data for TCE: Gene Expression and Methylation Status Studies

Caldwell and Keshava (2006) and Keshava and Caldwell (2006) reported on both genetic expression studies and studies of changes in methylation status induced by TCE and its metabolites (see Sections E.4.1.3 and E.3.3.5) as well as differences and difficulties in the patterns of gene expression between differing PPARα agonists. In Section E.4.3, the effects of co-exposures of DCA, TCA, and chloroform on methylation status are discussed. In particular are concerns for the interpretation of studies that employ pooling of data as well as interpretation of "snapshots in time of multiple gene changes."

For the Laughter et al. (2004) study in particular, it is not clear whether transcription arrays were performed on pooled data (no data on variability between individual animals were provided and the methodology section of the report is not transparently written in this regard). The issue of phenotypic anchoring also arises as data on percent liver/body weight indicates significant variability within TCE treatment groups, especially in PPARα-null mice. For studies of gene expression using microarrays, Bartosiewicz et al. (2001) used a screening analysis of 148 genes for xenobiotic-metabolizing enzymes, DNA repair enzymes, heat shock proteins, cytokines, and housekeeping gene expression patterns in the liver in response TCE. The TCE-induced gene induction was reported to be highly selective; only Hsp 25 and 86 and Cyp2a were upregulated at the highest dose tested. Collier et al. (2003) reported differentially expressed mRNA transcripts in embryonic hearts from Sprague-Dawley rats exposed to TCE, with sequences downregulated with TCE exposure appearing to be those associated with cellular housekeeping, cell adhesion, and developmental processes. TCE was reported to induce upregulated expression of numerous stress-response and homeostatic genes.

Laughter et al. (2004) reported transcription profiles using macroarrays containing approximately 1,200 genes in response to TCE exposure. Forty-three genes were reported to be significantly altered in the TCE-treated wild-type mice and 67 genes were significantly altered in the TCE-treated PPARα knockout mice. Out of the 43 genes expressed in wild-type mice

upon TCE exposure, 40 genes were reported by the authors to be dependent on PPARα and included genes for CYP4a12, epidermal growth factor receptor, and additional genes involved in cell growth. However, the interpretation of this information is difficult because in general, PPARα knockout mice have been reported to be more sensitive to a number of hepatotoxins, partly because of defects in the ability to effectively repair tissue damage in the liver (Shankar et al., 2003; Mehendale, 2000) and because a comparison of gene expression profiles between controls (wild-type and PPARα knockout) were not reported.

As stated previously, knockout mice in this study also responded to TCE exposure with increased liver weight, had increased background liver weights, and had higher baseline levels of hepatocyte proliferation than wild-type mice. Nakajima et al. (2000) reported that the number of peroxisomes in hepatocytes increased by twofold in wild-type mice but not in PPARα knockout mice. However, TCE induced increased liver weight in both male and female wild-type and knockout mice, suggesting hepatic effects independent of PPARα activation. Ramdhan et al. (2010) also reported increased liver weight after TCE exposure in male wild type, PPARα-null, and PPARα humanized mice to a similar extent.

In regards to toxicity, after 3 weeks of TCE treatment (0–1,500 mg/kg via gavage), Laughter et al. (2004) reported toxicity at the 1,500 mg/kg level in the knockout mice that was not observed in the wild-type mice—all knockout mice were moribund and had to be removed from the study. Differences in experimental protocol made comparisons between TCE effects and those of its metabolites difficult in this study (see Section E.2.1.13). After short-term inhalation exposure, Ramdhan et al. (2010) reported increased TCE induction of toxicity in PPARα-null and humanized mice in terms of hepatic steatosis and minimal levels of necrosis.

As reported by Voss et al. (2006), dose-, time course-, species-, and strain-related differences should be considered in interpreting gene array data. The comparison of differing PPARα agonists presented in Keshava and Caldwell (2006) illustrate the pleiotropic and varying liver responses of the PPARα receptor to various agonists, but did imply that these responses were responsible for carcinogenesis.

As discussed in Section E.3.3.5 and in Caldwell and Keshava (2006),

> Aberrant DNA methylation has emerged in recent years as a common hallmark of all types of cancers, with hypermethylation of the promoter region of specific tumor suppressor genes and DNA repair genes leading to their silencing (an effect similar to their mutation) and genomic hypomethylation (Pereira et al., 2004a; Ballestar and Esteller, 2002; Berger and Daxenbichler, 2002; Rhee et al., 2002; Herman et al., 1998). Whether DNA methylation is a consequence or cause of cancer is a long-standing issue (Ballestar and Esteller, 2002). Fraga et al. (2005; 2004) reported global loss of monoacetylation and trimethylation of histone H4 as a common hallmark of human tumor cells; they suggested, however, that genomewide loss of 5-methylcytosine (associated with the acquisition of a transformed phenotype) exists not as a static predefined value throughout the

process of carcinogenesis but rather as a dynamic parameter (i.e., decreases are seen early and become more marked in later stages).

Although little is known about how it occurs, a hypothesis has also been proposed that that the toxicity of TCE and its metabolites may arise from its effects on DNA methylation status. In regard to methylation studies, many are co-exposure studies as they have been conducted in initiated animals, and as stated above, some are very limited in regard to the reporting and conduct of the study.

Caldwell and Keshava (2006) reviewed the body of work regarding TCE, DCA, and TCA for this issue. Methionine status has been noted to affect the emergence of liver tumors. As noted by Counts et al. (1996), a choline/methionine-deficient diet for 12 months did not increase liver tumor formation in C3H/HeN mice, but was tumorigenic to $B6C3F_1$ mice. Tao et al. (2000) and Pereira et al. (2004a) have studied the effects of excess methionine in the diet to see if it has the opposite effects as a deficiency (i.e., and reduction in a carcinogenic response rather than enhancement). As noted above for Tao et al. (2000), the administration of excess methionine in the diet is not without effect. The data of Tao et al. (2000) suggested that percent liver/body weight ratios are affected by short-term methionine exposure (300 mg/kg) in female $B6C3F_1$ mice.

Pereira et al. (2004a) reported that very high levels of methionine supplementation to an AIN-760A diet affected the number of foci and adenomas after 44 weeks of co-exposure to 3.2.g/L DCA. While the highest concentration of methionine (8.0 g/kg) was reported to decrease both the number of DCA-induce foci and adenomas, the lower level of methionine co-exposure (4.0 g/kg) increased the incidence of foci. Co-exposure of methionine (4.0 or 8.0 g/kg) with 3.2 g/L DCA was reported to decrease by ~25% DCA-induced glycogen accumulation and increase mortality, but not to have much of an effect on peroxisome enzyme activity (which was not elevated by >33% over control for DCA exposure alone).

Methionine treatment alone at the 8 g/kg level was reported to increase liver weight, decrease lauroyl-CoA activity, and increase DNA methylation. The authors suggested that their data indicate that methionine treatment slowed the progression of foci to tumors. Given that increasing hypomethylation is associated with tumor progression, decreased hypomethylation from large doses of methionine are consistent with a slowing of progression. Whether these results would be similar for lower concentrations of DCA and lower concentrations of methionine that were administered to mice for longer durations of exposure cannot be ascertained from these data. It is possible that in a longer-term study, the number of tumors would be similar. Whether methionine treatment co-exposure had an effect on the phenotype of foci and tumors was not presented by the authors in this study. Such data would have been valuable to discern if methionine co-exposure at the 4.0 mg/kg level that resulted in an increase in DCA-induced foci also resulted in foci of a differing phenotype or a more heterogeneous

composition than DCA treatment alone. Finally, a decrease in tumor progression by methionine supplementation is not shown to be a specific event for the mode of action for DCA-induced liver carcinogenicity.

Tao et al. (2000) reported that 7 days of gavage dosing of TCE (1,000 mg/kg in corn oil), TCA (500 mg/kg, neutralized aqueous solution), and DCA (500 mg/kg, neutralized aqueous solution) in 8-week-old female B6C3F$_1$ mice resulted in not only increased liver weight but also increased hypomethylation of the promoter regions of c-Jun and c-Myc genes in whole-liver DNA (data shown for 1–2 mice per treatment). Treatment with methionine was reported to abrogate this response only at a 300 mg/kg i.p. dose with 0–100 mg/kg doses of methionine having no effect. Ge et al. (2001b) reported DCA- and TCA-induced DNA hypomethylation and cell proliferation in the liver of female mice at 500 mg/kg and decreased methylation of the c-Myc promoter region in liver, kidney, and urinary bladder. However, increased "cell proliferation" preceded hypomethylation. Ge et al. (2002) also reported hypomethylation of the c-Myc gene in the liver after exposure to the peroxisome proliferators 2,4-dichlorophenoxyacetic acid (2,4-D)(1,680 ppm), dibutyl phthalate (20,000 ppm), Gemfibrozil (8,000 ppm), and WY-14,643 (50–500 ppm, with no effect at 5 or 10 ppm) after 6 days in the diet. Caldwell and Keshava (2006) concluded that hypomethylation did not appear to be a chemical-specific effect at these concentrations. As noted in Section E.3.3.5, chemical exposure to a number of differing carcinogens have been reported to lead to progressive loss of DNA methylation.

Caldwell and Keshava (2006) also noted similar changes in methylation after initiation and treatment with DCA or TCA.

> After initiation by N-methyl-N-nitrosourea (25 mg/kg) and exposure to 20 mmL/L DCA or TCA (46 weeks), Tao et al. (2004a) report similar hypomethylation of total mouse liver DNA by DCA and TCA with tumor DNA showing greater hypomethylation. A similar effect was noted for region-2 (DMR-2) of the insulin-like growth factor-II (IGF-II) gene. The authors suggest that hypomethylation of total liver DNA and the IGF-II gene found in non-tumorous liver tissue would appear to be the result of a more prolonged activity and not cell proliferation, while hypomethylation of tumors could be an intrinsic property of the tumors. Over expression of IGF-II gene in liver tumors and preneoplastic foci has been shown in both animal models of hepatocarcinogenesis and humans, and may enhance tumor growth, acting via the over-expressed IGF-I receptor (Scharf et al., 2001; Werner and Le Roith, 2000). IGF-I is the major mediator of the effects of the growth hormone; it thus has a strong influence on cell proliferation and differentiation and is a potent inhibitor of apoptosis (Fürstenberger and Senn, 2002). Normally, expression of IGF-II in liver is greater during the fetal period than the adult, but is over-expressed in human hepatocarcinomas due to activation of fetal promoters (Scharf et al., 2001) and loss of imprinting (Khandwala et al., 2000). Takeda et al. (1996) report IGF-II expression in the liver is monoallelic (maternally imprinted) in the fetal period is relaxed during the postnatal period,

(resulting in biallelic expression), and is imbalanced in human hepatocarcinomas (leading to restoration of monoallelic IG-II expression).

However, Bull (2004b) and Bull et al. (2004) have recently suggested that hypomethylation and peroxisome proliferation occur at higher exposure levels than those that induce liver tumors for TCE and its metabolites. They reported that a direct comparison in the no-effect level or low-effect level for induction of liver tumors in the mouse and several other endpoints shows that, for TCA, liver tumors occur at lower concentrations than peroxisome proliferation in vivo, but that PPARα activation occurs at a lower dose than either tumor formation or peroxisome proliferation. A similar comparison for DCA shows that liver tumor formation occurs at a much lower exposure level than peroxisome proliferation, PPARα activation, or hypomethylation. In addition, they reported that these chemicals are effective as carcinogens at doses that do not produce cytotoxicity.

E.3.4.2.3. Oxidative Stress

Several studies have attempted to study the possible effects of "oxidative stress" and DNA damage resulting from TCE exposures. The effects of induction of metabolism by TCE, as well as through co-exposure to ethanol, have been hypothesized in itself to increase levels of "oxidative stress" as a common effect for both exposures (see Section E.4.3.4). Oxidative stress has been hypothesized to be the mode of action for peroxisome proliferators as well, but has been found to be correlated with neither cell proliferation nor carcinogenic potency of peroxisome proliferators (see Section E.3.4.1.1). As a mode of action, it is not defined or specific, as the term "oxidative stress" is implicated as part of the pathophysiologic events in a multitude of disease processes and is part of the normal physiologic function of the cell and cell signaling.

In regard to measures of oxidative stress, Rusyn (2006) noted that although an overwhelming number of studies draw a conclusion between chemical exposure, DNA damage, and cancer based on detection of 8-OHdG, a highly mutagenic lesion, in DNA isolated from organs of in vivo treated animals, a concern exists as to whether increases in 8-OHdG represent damage to genomic DNA, a confounding contamination with mitochondrial DNA, or an experimental artifact. As described in Section E.2.2.8, the study by Channel et al. (1998) demonstrated that corn oil as vehicle had significant effects on measures of "oxidative stress" such as TBARS. Also as noted previously (see Sections E.2.1.1 and E.2.2.11), studies of TCE that employ the i.p. route of administration can be affected by inflammatory reactions resulting from routes of administration and subsequent toxicity that can involve oxygen radical formation from inflammatory cells.

The issues with interpretation of the Channel et al. (1998) study of TCE administered via corn oil gavage to mice have already been discussed in Section E.2.2.8. The TBARS results

indicated suppression of TBARS with increasing time of exposure to corn oil alone with data presented in such a way for 8-OHdG and total free radical changes that the pattern of corn oil administration was obscured. It was not apparent from that study that TCE exposure induced oxidative damage in the liver.

Toraason et al. (1999) measured 8-OHdG and a "free radical-catalyzed isomer of arachidonic acid and marker of oxidative damage to cell membranes, 8-Epi-prostaglandin F2α (8epiPGF)," excretion in the urine and TBARS (as an assessment of malondialdehyde and marker of lipid peroxidation) in the liver and kidney of male Fischer rats (150–200 g) exposed to single 0, 100, 500, or 1,000 mg/kg TCE i.p. injections in Alkamuls vehicle (n = 6/group). Two sequential urine samples were collected 12 hours after injection and animals were sacrificed at 24 hours with DNA collected from liver tissues and TBARS measured in liver homogenates. The mean body weights of the rats were reported to vary by 13%, but the liver weights varied by 44% after the single treatments of TCE. In contrast to the large volume of the literature that reports TCE-induced increases in liver weight, the 500 and 1,000 mg/kg exposed rats were reported to have reduced liver weight by 44% in comparison to the control values.

Using this paradigm, 500 mg/kg TCE was reported to induce stage II anesthesia and a 1,000 mg/kg TCE to induce Level III or IV (absence of reflex response) anesthesia and burgundy colored urine with 2/6 rats at 24 hours comatose and hypothermic. The animals were sacrificed before they could die and the authors suggested that they would not have survived another 24 hours. Thus, using this paradigm, there was significant toxicity and additional issues related to route of exposure. Urine volume declined significantly during the first 12 hours of treatment and while water consumption was not measured, it was suggested by the authors to be decreased due to the moribundity of the rats. Given that this study examined urinary markers of "oxidative stress," the effects on urine volume and water consumption, as well as the profound toxicity induced by this exposure paradigm, limit the interpretation of the study.

The authors noted that because both using volume and creatinine excretion were affected by experimental treatment, urinary excretion of 8-OHdG changed significantly based on the mode of data expression. Excretion of 8epiPGF was reported to be no different from controls at 12–24 hours and was decreased 24 hours after TCE exposure at the two highest levels. Excretion of 8-OHdG was reported to not be affected by any exposure level of TCE and, if expressed on the basis of 24-hours, decreased. TBARS concentration per g of liver was reported to be increased at the 500 and 1,000 mg/kg TCE exposure levels (~2–3–fold). The effects of decreased liver size in the treated animals for this measure in comparison to control animals, was not discussed by the authors. For 8-OHdG measures in the liver and lymphocytes, the authors reported that "cost prohibited analysis of all of the tissues samples" so that a subset of animals was examined exhibiting the highest TBARS levels. The number of animals used for this determination was not given nor were the data reported, except for 500 mg/kg TCE exposure level. TCE was reported to increase 8-OHdG/dG in liver DNA relative to controls to about the same extent in

lymphocytes from blood and liver (approximately twofold) with the results for liver reported to be significant. The issues of bias in selection of the data for this analysis, as well as the issues already stated for this paradigm limit interpretation of these data, while the authors suggest that evidence of oxidative damage was equivocal.

DCA and TCA have also been investigated using similar measures. Larson and Bull (1992b) exposed male B6C3F$_1$ mice (26 ± 3 g [SD]) to a single dose of 0, 100, 300, 1,000, or 2,000 mg/kg-day TCA or 0, 100, 300, or 1,000 mg/kg-day DCA in distilled water by gavage (n = 4). F344 rats (237 ± 4 g) received a single oral dose of 0, 100, or 1,000 mg/kg DCA or TCA (n = 4 or 5) TBARS was measured from liver homogenates and assumed to be malondialdehyde. The authors stated that a preliminary experiment had shown that maximal TBARS was increased 6 hours after a dose of DCA and 9 hours after a dose of TCA in mice (data shown) and that by 24 hours, TBARS concentrations had declined to control values (data not shown). However, time-course information in rats was not presented and the same times used for both species (i.e., 6- and 9-hour time periods after administration of DCA and TCA) for examination of TBARS activity. A dose of 100 mg/kg DCA (rats or mice) or TCA (mice) did not elevate TBARS concentrations over that of control liver, with this concentration of TCA not examined in rats.

For TCA, there was a slight dose-related increase in TBARS over control values starting at 300 mg/kg in mice (i.e., 1.68-, 2.02-, and 2.70-fold of control for 300, 1,000, and 2,000 mg/kg TCA). For DCA, there were similar increases over control for both the 300 and 1,000 mg/kg dose levels in mice (i.e., 3.22- and 3.45-fold of control, respectively).

For rats, the 1,000 and 2,000 mg/kg levels of TCA were reported to show a statistically significant increase in TBARS over control (i.e., 1.67- and 2.50-fold, respectively) with the 300 and 1,000 mg/kg level of DCA showing similar increases, but with only the 300 mg/kg-induced change statistically significant different than control values (i.e., 3- and 2-fold of control, respectively). Of note is the report that the induction of TBARS in mice is transient and had subsided within 24 hours of a single dose of DCA or TCA, that the response in mice appeared to be slightly greater with DCA than TCA at similar doses, and that for DCA, there was similar TBARS induction between rats and mice at similar dose levels.

A study by Austin et al. (1996) appears to a follow-up publication of the preliminary experiment cited in Larson and Bull (1992b). Male B6C3F$_1$ mice (8 weeks old) were treated with single doses of DCA or TCA in buffered solution (300 mg/kg) with liver examined for 8-OHdG. The authors stated that in order to conserve animals, controls were not employed at each time point. For DCA, the time course of 8-OHdG was studied at 0, 4, 6, and 8 hours after administration, and for TCA, at 0, 6, 8, and 10 hours after of a 300 mg/kg dose (n = 6). There was a statistically significant increase over controls in 8-OHdG for the 4- and 6-hour time points for DCA (~1.4- and 1.5-fold of control, respectively) but not at 8 hours in mice. For TCA, there was a statistically significant increase in 8-OHdG at 8 and 10 hours for TCA (~1.4- and 1.3-fold of control, respectively).

The results for PCO and liver weight for Parrish et al. (1996) are discussed in Section E.2.3.2.3 for male B6C3F$_1$ mice exposed to TCA or DCA (0, 0.01, 0.5, and 2.0 g/L) for 3 or 10 weeks (n = 6). The study focused on an examination of the relationship with measures of peroxisome proliferation and oxidative stress. The dose-related increase in PCO activity at 21 days (~1.5-, 2.2-, and ~4.1-fold of control, for 0.1, 0.5, and 2.g/L TCA) was reported not to be increased similarly for DCA. Only the 2.0 g/L dose of DCA was reported to induce a statistically significant increase at 21 days of exposure of PCO activity over control (~1.8-fold of control). After 71 days of treatment, TCA induced dose-related increases in PCO activities that were approximately twice the magnitude as that reported at 21 days (i.e., ~9-fold greater at 2.0 g/L level). Treatments with DCA at the 0.1 and 0.5 g/L exposure levels produced statistically significant increase in PCO activity of ~1.5- and 2.5-fold of control, respectively. The administration of 1.25 g/L clofibric acid in drinking water, used as a positive control, gave ~6–7-fold of control PCO activity at 21 and 71 days exposure.

Parrish et al. (1996) reported that laurate hydroxylase activity was reported to be elevated significantly only by TCA at 21 days and to approximately the same extent (~1.4–1.6-fold of control) increased at all doses tested. At 71 days, both the 0.5 and 2.0 g/L TCA exposures induced a statistically significant increase in laurate hydroxylase activity (i.e., 1.6- and 2.5-fold of control, respectively) with no change reported after DCA exposure. The actual data rather than percent of control values were reported for laurate hydroxylase activity with the control values varying 1.7-fold between 21- and 71-day experiments. Levels of 8-OHdG in isolated liver nuclei were reported to not be altered from 0.1, 0.5, or 2.0 g/L TCA or DCA after 21 days of exposure and this negative result was reported to remain even when treatments were extended to 71 days of treatment.

The authors noted that the level of 8-OHdG increased in control mice with age (i.e., approximately twofold increase between 71- and 21-day control mice). Clofibric acid was also reported not to induce a statistically significant increase of 8-OHdG at 21 days, but to produce an increase (~1.4-fold of control) at 71 days. Thus, the increases in PCO activity noted for DCA and TCA were not associated with 8-OHdG levels (which were unchanged) and, also, not with changes in laurate hydrolase activity observed after either DCA or TCA exposure. Of note is the variability in both baseline levels of PCO and laurate hydrolase activity. Also of note is that the authors report taking steps to minimize artifactual responses for their 8-OHdG determinations. The authors concluded that their data do not support an increase in steady-state oxidative damage to be associated with TCA initiation of cancer and that extension of treatment to time periods sufficient to insure peroxisome proliferation failed to elevate 8-OHdG in hepatic DNA. The increased 8-OHdG at 10 weeks after Clofibrate administration but lack of 8-OHdG elevation at similar levels of PCO induction were also noted by the authors to suggest that peroxisome proliferative properties of TCA were not linked to oxidative stress or carcinogenic response.

As noted above for the study of Leakey et al. (2003b) (see Section E.2.5), hepatic malondialdehyde concentration in ad-libitum-fed and dietary-controlled mice did not change with CH exposure at 15 months, but the dietary-controlled groups were all approximately half that of the ad-libitum-fed mice. Thus, while overall increased tumors observed in the ad libitum diet correlated with increased malondialdehyde concentration, there was no association between CH dose and malondialdehyde induction for either diet.

E.4. EFFECTS OF CO-EXPOSURES ON MODE OF ACTION—INTERNAL AND EXTERNAL EXPOSURES TO MIXTURES INCLUDING ALCOHOL

Caldwell et al. (2008b) published a review of the issues and studies involved with the effects of co-exposures to TCE metabolites that could be considered internal (i.e., an internal co-exposure for the liver) and co-exposures to metabolites and other commonly occurring chemicals that are present in the environment. As they stated:

> Human exposure to a pollutant rarely occurs in isolation. EPA's Cumulative Exposure project and subsequent National Air Toxics Assessment have demonstrated that environmental exposure to a number of pollutants, classified as potential human carcinogens, is widespread [U.S. EPA, 2006;(Woodruff et al., 1998)]. Interactions between carcinogens in chemical mixtures found in the environment have been a concern for several decades. Furthermore, how these interactions affect the mode of action (MOA) by which these chemicals operate and how such effects may modulate carcinogenic risk is of concern as well. Thus, an understanding of the MOA(s) of a pollutant can help elucidate its potential carcinogenic risk to humans, and can also help identify susceptible subpopulations through their intrinsic factors (e.g., age, gender, and genetic polymorphisms of key metabolic and clearance pathways) and extrinsic factors (e.g. co-exposures to environmental contaminants, ethanol consumption, and pharmaceutical use). Trichloroethylene (TCE) can be a useful example for detailing the difficulties and opportunities for investigating such issues because, for TCE, there is both internal exposure to a "chemical mixture" of multiple carcinogenic metabolites (Chiu et al., 2006a; Chiu et al., 2006b) and co-exposures from environmental contamination of TCE metabolites, and from pollutants that share common metabolites, metabolic pathways, MOAs, and targets of toxicity with TCE.
>
> Typically, ground water or contaminated waste sites can have a large number of pollutants that vary in regard to information available to support the characterization of their potential hazard, and that have differing MOAs and targets. For example, Veeramachaneni et al. (2001) reported reproductive effects in male rabbits, resulting from exposure to drinking water containing concentrations of chemicals typical of ground water near hazardous waste sites. The drinking water exposure mixture contained arsenic, chromium, lead, benzene, chloroform, phenol, and TCE. Even at 45 weeks after the last exposure, mating desire/ability, sperm quality, and Leydig cell function were subnormal. However, while the exposure levels are relevant to human

environmental exposures, design of this study precludes a conclusion as to which individual toxicant, or combination of the seven toxicants, caused the effects. Thus, this study exemplifies he problems associated with studying a multi-mixture milieu. Studies of the interactions of TCE metabolites or common co-exposures that report the interactions of 2 or 3 chemicals at one time are easier to interpret.

Since EPA published its 2001 draft assessment, several approaches have been reported that include examination of tumor phenotype, gene expression, and development of physiologically-based pharmacokinetic (PBPK) models to assess possible effects of co-exposure. They attempt to predict whether such co-exposures would produce additivity of response or if co-exposure would change the nature of responses induced by TCE or its metabolites. In addition, new studies on co-exposure to DBA may help identify a co-exposure of concern. These studies may give potential insights into possible MOAs and modulators of TCE toxicity. More recent information on the toxicity of individual metabolites of TCE (Caldwell and Keshava, 2006) may be helpful in trying to identify which are responsible for TCE toxicity, but may also identify the effects of environmental co-exposures.

Recently, EPA sought advice from the National Academy of Sciences (NAS) (Chiu et al., 2006a) with the NAS charge questions including the following. (1) What TCE metabolites, or combinations of metabolites, may be plausibly involved in the toxicity of TCE? (2) What chemical co-exposures may plausibly modulate TCE toxicity? (3) What can be concluded about the potential for common drinking water contaminants such as other solvents and/or haloacetates to modulate TCE toxicity? (4) What can be concluded about the potential for ethanol consumption to modulate TCE toxicity? Thus, the understanding of the effects of co-exposure, in the context of MOA, is an important element in understanding the risk of a potential human carcinogen.

U.S. EPA's draft TCE risk assessment (U.S. EPA, 2001) identified several factors involving co-exposure to TCE metabolites, environmental contaminants, and ethanol that could lead to differential sensitivity to TCE toxicity. Research needs identified there, as well as in previous reviews (Bull, 2000; Pastino et al., 2000), included further elucidation of the interaction of TCA and DCA in TCE-induced liver tumors and a better understanding of the functional relationships among risk factors. The complexity of TCE's potential interactions with chemical co-exposures from either common environmental co-contaminants or common behaviors such as alcohol consumption mirrors the complexity of the metabolism and the actions of TCE metabolites. Thus, TCE presents a good case study for further exploration of the effects of co-exposure on MOA.

The following sections first reiterate the findings of Bull et al. (2002) in regard to simple co-exposures of DCA and TCA that can be experienced as an internal co-exposure after TCE exposure. A number of studies have examined the effects of TCE or its metabolites after previous exposure to presumably genotoxic carcinogen to not only determine the effect of the co-exposure on liver carcinogenicity but also to use such paradigms to distinguish between the

effects of TCA and DCA. Finally, not only is TCE a common co-exposure with its own metabolites, but is also a common co-exposure with other solvents, and the brominated analogues of TCA and DCA. The available literature is examined for potential similarities in target and effects that may cause additional concern. The effects of ethanol on TCE toxicity is examined as well as the potential pharmacokinetic modulation of risk using recently published reports of PBPK models that may be useful in predicting co-exposure effects.

E.4.1. Internal Co-exposures to TCE Metabolites: Modulation of Toxicity and Implications for TCE Mode of Action

Exposure to TCE will produce oxidative metabolites in the liver as an internal co-exposure. As stated above, the phenotypic analysis of TCE-induced tumors have similarities to combinations of DCA and TCA and in some reports to resemble more closely DCA-induced tumors in the mouse. Results from Bull et al. (2002) are presented in Section E.2.2.22 for the treatment of mice to differing concentrations of DCA and TCA in combination and the resemblance of tumor phenotype to that of TCE. In regard to cancer dose-response, the most consistent treatment-related increase in response occurred with combinations of exposure to DCA and TCA that appeared to increase lesion multiplicity when compared to effects from individual chemicals separately. Bull et al. (2002) presented results for "selected" lesions examined for pathology characterization that suggest co-exposure of 0.5 g/L DCA with either 0.5 or 2 g/L TCA had a greater-than-additive effect on the total number of hyperplastic nodules. In addition, co-exposure to 0.1 g/L DCA and 2 g/L TCA was reported to have a greater-than-additive effect on the total number of adenomas, but not carcinomas, induced. The random selection of lesions for the determination of potential treatment-related effects on incidence and multiplicity, rather than characterization of all lesions, increases the uncertainty in this finding.

E.4.2. Initiation Studies as Co-exposures

There is a body of literature that has focused on the effects of TCE and its metabolites after rats or mice have been exposed to "mutagenic" agents to "initiate" hepatocarcinogenesis. Given that most of these "initiating agents" have many effects that are not only mutagenic but also epigenetic, that the dose and exposure paradigm modify these effects, that "initiators" can increased tumor responses alone, and that the tumors that arise from these protocols are reflective of simultaneous actions of both "initiator" and "promoter," paradigms that first expose rats or mice to a "mutagen" and then to other carcinogenic agents can be described as a co-exposure protocols.

As stated previously, DEN and *N*-nitrosomorpholine have been reported to increase differing populations of mature hepatocytes with DEN not only being a mutagen but also being able to induce concurrent hepatocyte regeneration at a high dose. Thus, the effects of the TCE

or its metabolites are hard to discern from the effects of the "initiating" agent in terms of mode of action.

As demonstrated in the studies of Pereira et al. (1997) below, the gender also determines the nature of the tumor response using these protocols. In addition, when the endpoint for examination is tumor phenotype the consequences of tumor progression are hard to discern from the mode of action of the agents using paradigms of differing concentrations, different durations of exposure, lesions counted as "tumors" to include different stages of tumor progression (foci to carcinoma), and highly variable and low numbers of animals examined. However, differences in phenotype of tumors resulting from such co-exposures, like the co-exposure studies cited above for just TCE metabolites, can help determine that exposure to TCE metabolites results in differing actions as demonstrated by differing effects in the presence of cocarcinogens. As stated above, Kraupp-Grasl et al. (1990) use the same approach and note differences among PPARα agonists in their ability to promote tumors suggest that they should not necessarily be considered a uniform group.

E.4.2.1. Herren-Freund et al. (1987)

The results of TCE exposure alone were reported previously (Section E.2.2.17) for this study. This study's focus was on the effect of TCE, TCA, DCA, and phenobarbital on hepatocarcinogenicity in male $B6C3F_1$ mice after "initiation" at 15 days with 2.5 or 10 μg/g body weight of ethylnitrosourea (ENU) and then subsequent exposure to TCE and other chemicals in drinking water begging at 4 weeks of age (an age when the liver is already undergoing rapid growth). DCA and TCA were given in buffered solutions and sodium chloride was given in the water of control animals. The experiment was reported to be terminated at 61 weeks because the "mice started to exhibit evidence of tumors." Concentrations of TCE were 0, 3 and 40 mg/L, of DCA and TCA 0, 2 and 5 g/L, and of phenobarbital 0 and 500 mg/L. The number of animals examined in each group ranged from 16 to 32. ENU alone in this paradigm was reported to induce statistically significant increases in adenomas and HCCs (39% incidence of adenomas and 39% incidence of carcinomas vs. 9 and 0% for controls) at the 10 μg/g dose (n = 23), but not at 2.5 μg/g dose (n = 22).

The effects of high doses of DCA and TCA alone have already been discussed for other studies, as well as the lack of statistical power using a paradigm with so few and variable numbers of animals, the limitations of an abbreviated duration of exposure that does not allow for full expression of a carcinogenic response, and problems of volatilization of TCE in drinking water. DCA and TCA treatments at these levels (5 g/L) were reported to increase adenomas and carcinomas irrespective of ENU pretreatment and to approximately the same extent with and without ENU. TCE at the highest dose was reported to increase the number of animals with adenomas (37 vs. 9% in control) and carcinomas (37 vs. 0% in controls) but only the number of adenomas/animal was statistically significant as the number of animals examined was only 19 in

the TCE group. Phenobarbital was reported to have no effect on ENU tumor induction using this paradigm.

E.4.2.2. Parnell et al. (1986)

This study used a rat liver foci bioassay (GGT) for hepatic foci after at 3 and 6 month using protocols that included partial hepatectomy, DEN (10 mg/kg) or TCA (1,500 ppm in drinking water) treatment, and then promotion with 5,000 ppm TCA (i.e., 5 g/L) for 10, 20, or 30 days and phenobarbital (500 ppm) in male Sprague-Dawley rats (5–6 weeks old at partial hepatectomy). The number of animals per group ranged from 4 to 6. PCO activities were given for various protocols involving partial hepatectomy, DEN, TCA, and phenobarbital treatments, but there were no control values given that did not have a least one of these treatments.

Overall, it appeared that there was a slight decrease of PCO activity in rats treated with partial hepatectomy/DEN/phenobarbital treatments and a slight increase over other treatments for rats treated with partial hepatectomy/DEN/5,000 ppm TCA or just TCA from 2 weeks to 6 months of sampling. In regard to GGT-positive foci, the partial hepatectomy/DEN/ phenobarbital group (n = 6) was reported to have more positive foci at 3 or 6 months than rats "initiated" with TCA and phenobarbitol after partial hepatectomy or partial hepatectomy/ phenobarbital treatment alone (2.05 foci/cm^2 vs. ~0.05–0.10 foci/cm^2 for all other groups). The number of GGT-positive foci in rats without any treatment were not studied or presented by the authors. For "promotion" protocols, the number of GGT-positive foci induced by the partial hepatectomy/DEN/phenobarbital protocol at 3 and 6 months, appeared to be reduced when phenobarbital exposure was replaced by TCA co-exposure, but there was no dose-response between the 50, 500, and 5,000 ppm. However, TCA treatment along with partial hepatectomy and DEN treatment did increase the levels of foci (means of 0.71–0.39 foci/cm^2 at 3 months and 1.83–2.45 foci/cm^2 at 6 months) over treatment of just partial hepatectomy and DEN (0.05 ± 0.20 foci/cm^2 at 3 months and 0.30 ± 0.39 foci/cm^2 at 6 months).

For the TCA animals treated only with 5,000 ppm TCA, the number of GGT-positive foci at 3 months was 0.23 ± 0.16 foci/cm^2 and at 6 months 0.03 ± 0.32 foci/cm^2 with no values for untreated animals presented. For the positive control (partial hepatectomy/DEN/ phenobarbital), the number of GGT-positive foci increased from 3 to 6 months (1.65 ± 0.23 foci/cm^2 and at 6 months 7.61 ± 0.72 foci/cm^2). The authors concluded that:

> although TCA is reported to cause hepatic peroxisomal stimulation in rats and mice, the results of this study indicate that it is unlikely TCA's effects are related to the promoting ability seen here. The minimal stimulation of, 10 to 20% over controls of peroxisomal associated, PCO activity in TCA exposed rats was seen only at the 5000 ppm level and only within the promotion protocol. This finding is in contrast to the promoting activity seen at all three concentrations of TCA.

E.4.2.3. Pereira and Phelps (1996)

The results for mice that were not "initiated" by exposure to MNU, but exposed to DCA or TCA, are discussed in Section E.2.3.2. However, differences in responses after initiation are useful for showing differences between single and co-exposures as well as differences between DCA and TCA effects. On day 15 of age, female $B6C3F_1$ mice received an i.p. injection of MNU (25 mg/kg) and at 7 weeks of age, received DCA (2.0, 6.67, or 20 mmol/L), TCA (2.0, 6.67 mmol, or 20 mmol/L) or sodium chloride continuously for 31 or 51 weeks of exposure. The number of animals studied ranged from 6 to 10 in 31-week groups and from 6 to 39 in the 52-week groups. There was a "recovery group" in which mice received either 20 mmol/L DCA (2.58 g/L DCA) (n = 12) or TCA (3.27 g/L TCA) (n = 11) for 31 weeks and then switched to saline for 21 weeks until sacrifice at 52 weeks. Strengths of the study included the reporting of hepatocellular lesions as either foci, adenomas, or carcinomas and the presentation of incidence and multiplicity of each separately reported for the treatment paradigms. Limitations included the low and variable number of animals in the treatment groups.

MNU was reported to not "significantly" induce foci or altered hepatocytes, adenomas, or carcinomas at 31 (n = 10) or 51 weeks (n = 39). However, MNU did increase the incidence and number/mouse of foci, adenomas, and carcinomas at the 52-week sacrifice time in comparison to saline controls, albeit at lower levels than observed in DCA or TCA cotreatments groups (e.g., 10 vs. 0% foci, 17.5 vs. 2.5% adenomas, and 10 vs. 0% incidence of carcinomas at 52 weeks for MNU-treated mice vs. saline control). Co-exposure of DCA (20.0 mmol/L) for 52 weeks in MNU-treated mice increased the number of foci and hepatocellular adenomas with the authors reporting "the yield of total lesions/mouse increased as a second order function of the concentration of DCA (correlation coefficients ≥0.998)." TCA co-exposure in MNU-treated mice was reported not to result in a significant difference in yield of foci or altered hepatocytes with either continuous 52- or 31-week exposure, but exposures to 20.0 or 6.67 mmol/L TCA did result in increased yield of liver tumors with both exposure protocols (see below).

For TCA treatment in MNU-treated mice, the incidences of foci were similar (12.5 vs. 18.2%), but the number of foci/mouse was ~3-fold greater in the cessation protocol than with continuous exposure. The incidence of adenomas was reported to be the same (~66%) as well as the number of adenomas/animal between continuous and cessation exposures. For carcinomas, there was a greater incidence for mice with continuous TCA exposure (83 vs. 36%) as well as a greater number of carcinomas/mouse (~4-fold) than for those initiated mice with cessation of TCA exposure. As noted above, the number of animals treated with TCA was low and variable (e.g., 23 mice studied at 52 weeks 20.0 mmol/L TCA, and 6 mice at 52 weeks 6.67 mmol/L TCA), limiting the ability to discern a statistically significant effect in regard to dose-response. The concentration-response relationship for tumors/mouse after 31 and 51 weeks was reported to be best represented by linear progression.

A comparison of results for animals treated with MNU and 20.0 mmol/L DCA or TCA for 31 weeks and sacrificed at 31 weeks and those that were treated with MNU and DCA or TCA for 31 weeks and then sacrificed at 52 weeks is limited by the number of animals exposed (n = 10 for 31-week sacrifice DCA or TCA, n = 12 for DCA recovery group, and n = 11 for TCA recovery group). No carcinoma data were reported for animals exposed at 31 weeks and sacrificed at 31 weeks, making comparisons with recovery groups impossible for this parameter and thus, determinations about progression from adenomas to carcinomas. For the MNU- and DCA-treated animals, the incidence or number of animals reported to have foci at 31 weeks was reported to be 80% but 38.5% for in the recovery group. For adenomas, the incidence was reported to be 50% for DCA-treated animals at 31 weeks and 46.2% for the recovery group. For MNU- and TCA-treated animals, the incidence of foci at 31 weeks was reported to the 20 and 18.2% for the recovery group. For adenomas, the incidence was reported to be 60% for the TCA-treated animals at 31 weeks and 63.6% for the recovery group. Thus, this limited data set shows a decrease in incidence of foci for the MNU and DCA-treated recovery group but no change in incidence of foci for TCA or for adenomas for DCA or TCA treatment between those sacrificed at 31 weeks and those sacrificed 21 weeks later.

In regard to multiplicity, the number of foci/mouse was reported to be 2.80 ± 0.20 for the 31-week DCA group and 0.46 ± 0.18 for the recovery group (mean ± SEM). The number of adenomas/mouse was reported to be 1.80 ± 0.83 for the 31-week group and 0.69 ± 0.26 for the recovery group. Thus, both the number of foci and adenomas per mouse was reported to be decreased after the recovery period for MNU- and DCA-treated mice. Given that the number of animals with foci was decreased by half, the concurrent decrease in foci/mouse is not surprising. For TCA treatments, the numbers of foci/mouse were reported to be 0.20 ± 0.13 for the 31-week group and 0.45 ± 0.31 for the recovery group. The number of adenomas/mouse for TCA-treatment groups was reported to be 1.30 ± 0.45 for the 31-week group and 0.91 ± 0.28 for the recovery group. For the MNU- and TCA-treated mice, the numbers of foci/mouse were reported to be increased and the number of adenomas/mouse reported to be slightly lower. Because carcinoma data are not presented for the 31-week group, it is impossible to determine whether the TCA adenomas regressed to foci or the TCA adenomas progressed to carcinomas and more foci apparent with increased time.

For the comparison of the numbers of foci, adenomas, or carcinomas per mouse that were reported for the mice exposed at 31 weeks and sacrificed and those exposed for 52 weeks, issues arise as to the impact of such few animals studied at 31 weeks, and the differing incidences of lesions reported for these mice on tumor multiplicity estimates. The number of animals studied who treated with MNU and 20.0 mmol/L DCA or TCA for 31 weeks and then sacrificed was n = 10, while the number of animals exposed to 20.0 mmol/L DCA or TCA for 52 weeks was 24 for the DCA group and 23 for the TCA group. The number of animals treated at lower concentrations of DCA or TCA were even lower at the 31-week sacrifice (e.g., n = 6

for MNU and 6.67 mmol/L DCA at 31 weeks) and also for the 52-week durations of exposure (e.g., n = 6 for MNU and 6.6.7 mmol/L TCA).

At 31 weeks, 80% of the animals were reported to have foci and 50% to have foci after 52 weeks of exposure to 20.0 mmol/L DCA and MNU treatment. Thus, similar to the "recovery" experiment, the number of animals with foci decreased even with continuous exposure between 31 and 52 weeks. For adenomas, 20.0 mmol DCA exposure for 31 weeks was reported to induce adenomas in 50% of mice and after 52 weeks of exposure to induce adenomas in 73% of mice. For TCA, the number of animals with foci was reported to be 20% at 31 weeks and 12% at 52 weeks after exposure to 20.0 mmol/L TCA after MNU treatment and similar to the incidence of foci reported for the TCA-recovery group. For 20.0 mmol TCA, adenomas reported in 60% of mice after 31 weeks and in 67% of mice after 52 weeks of exposure and also similar to the incidence of adenomas reported for the TCA-recovery group.

In regard to multiplicity, the number of foci/mouse was decreased from 2.80 ± 0.20 to 1.46 ± 0.48 between 31 and 52 weeks of 20.0 mmol DCA in MNU exposed mice. The number of adenomas/mouse was reported to be increased from 1.80 ± 0.83 to 3.62 ± 0.70 between 31 and 52 weeks of 20.0 mmol DCA and MNU exposed mice. For 20.0 mmol/L TCA, the number of foci/mouse was 0.20 ± 0.13 and 0.13 ± 0.7 for 31- and 52-week exposures. The number of adenomas/mouse was reported to be 1.30 ± 0.45 and 1.29 ± 0.24 for 31- and 52-week exposures. Thus, by only looking at foci and adenoma multiplicity data, there would not appear to be a change between 31 and 52 weeks.

However, during progression, a shift may occur such that foci become adenomas with time and adenomas become carcinomas with time. For carcinomas, there were no data reported for 31-week exposure in MNU and DCA- or TCA-treated mice. However, at 52 weeks, 20.0 mmol DCA was reported to induce carcinomas in 19.2% of mice and 20.0 mmol TCA to induce carcinomas in 83% of mice. The corresponding numbers of carcinomas/mouse was 0.23 ± 0.10 for 20.0 mmol/L DCA treatment and 2.79 ± 0.48 for 20.0 mmol/L TCA treatment at 52 weeks in MNU treated mice. Thus, although fewer than 20% of MNU-treated mice were reported to have foci at 20.0 mmol TCA, by 52 weeks, almost all had carcinomas with ~67% also having adenomas. For DCA, many more mice had foci at 31 weeks (80%) than for TCA and by 52 weeks ~70% had adenoma with only ~20% reported to have carcinomas. The incidence data are suggestive that as these high doses of DCA and TCA, TCA was more efficient inducing progression of a carcinogenic response than DCA in MNU-treated mice.

The authors interpreted the decrease in foci and adenomas between animals treated with MNU and 20.0 mmol/L DCA for 31 weeks and sacrificed and those sacrificed 21 weeks later to indicate that these lesions were dependent on continued exposure. However, the total number of lesions cannot be ascertained because carcinoma data were not reported for 31-week exposures. Carcinomas were reported in the recovery group at 52 weeks (0.15 ± 0.10 carcinomas/mouse in 15.4% of animals). Of note is that not only did the number of foci/mouse

and incidence decrease between the 31-week group and the recovery group, but also between 31 and 52 weeks of continuous exposure for the MNU and 20.0 mmol/L DCA treated groups. Although derived from very few animals, the 6.67 mmol/L DCA group reported no change for foci/mouse but a decrease in the incidence of foci between 31- and 52 weeks of exposure in MNU treated mice (i.e., 0.67 ± 0.18 foci/mouse in 50% of the animals at 31 weeks and 0.50 ± 0.34 foci/mouse in 20% of mice treated for 52 weeks). The numbers of foci/mouse for both MNU-treated and untreated control mice were reported to be decreased between 31 and 51 weeks as well.

As noted in Section E.3.1.8, the number of "nodules" in humans, which may be analogous to foci and adenomas, can spontaneously regress with time rather than becoming HCCs. Also, as tumors get larger with progression, the number of tumors/mouse can decrease due to coalescence of tumors and difficulty distinguishing between them. While data are suggestive of a decrease in the number of adenomas/mouse after cessation of DCA exposure, the incidence data are similar between the 31-week exposure and recovery groups.

Of note is that the number of carcinomas/mouse and the incidence of carcinomas was reported to be similar between the MNU-treated mice exposed continuously to 20.0 mmol/L DCA for 52 weeks and those that were treated for 31 weeks and then sacrificed at 52 weeks. Also of note is that, although incidences and multiplicities of foci and adenomas were reported to be relatively low in the 2.0 mmol/L DCA exposure groups, at 52 weeks, 40% of the mice tested had carcinomas with 0.70 ± 0.40 carcinomas/mouse. This was a greater percentage of animals with carcinomas and multiplicity than that reported for the highest dose of DCA. This result suggests that the effects in regard to tumor progression, and specifically for carcinoma induction, differ between the lowest and highest doses used in this experiment. However, the low numbers of animals examined for the lower doses, 31-week exposures, and in the recovery group decrease the confidence in the results of this study in regard to the effects of cessation of exposure on tumor progression.

In regard to tumor phenotype, in MNU-treated female mice that were not also exposed to either DCA or TCA, all four foci and 86.7% of 15 adenomas were reported to be basophilic and 13.3% eosinophilic at the end of the 52-week study. However, when MNU-treated female mice were also exposed to DCA, the number eosinophilic foci and tumors increased with increasing dose after 52 weeks of continuous exposure. At the 20.0 mmol/L level, all 38 foci examined were eosinophilic and 99% of the tumors (almost all adenomas) were eosinophilic. At the 2.0 mmol/L DCA exposure, there were no foci examined but about five of nine tumors examined (~2:1 carcinoma:adenoma ratio) were basophilic and the other four were eosinophilic.

For TCA co-exposure in MNU-treated mice, the 20 mmol/L TCA treatment was reported to give results of one of the three foci examined to be basophilic and two that were eosinophilic. For the 98 tumors examined (~2:1 carcinoma/adenoma ratio), 71.4% were

reported to be basophilic and 28.6% were eosinophilic. At the 2.0 mmol/L TCA exposure level, the two foci examined were reported to be basophilic, while the six tumors (all adenomas) were reported to be 50% eosinophilic and 50% basophilic. Thus, after 52 weeks, female mice treated with MNU and a high dose of DCA had eosinophilic foci and adenomas and those treated with the high dose of TCA had a mixture of basophilic and eosinophilic foci and tumors with a 3:1 ratio of tumors (mostly carcinomas) being basophilic. At the lower doses of either DCA or TCA, the tumors tended to be mostly carcinomas for DCA and adenomas for TCA, but both were ~50% basophilic and 50% eosinophilic. The tumors observed from MNU treatment alone were all adenomas and mostly 87% basophilic. Thus, not only did treatment concentrations of DCA and TCA give a different result for tumor multiplicity and incidence, but also for tumor phenotype in MNU treated female mice. Eosinophilic foci and tumors were reported to be consistently GST-π positive while basophilic lesions "did not contain GST-π, except for a few scattered cells or very small area comprising less than 5% of the tumor."

Thus, exposure to either DCA or TCA increased the incidence and number of animals with lesions (foci, adenomas, or carcinomas) in MNU-treated vs. nontreated mice (see Section E.2.3.2). These results suggest that the pattern of foci, adenoma and carcinoma incidence, multiplicity, and progression appeared to differ between TCA and DCA in MNU-treated female mice. However, the low and variable number of animals used in this study, make quantitative inferences between DCA and TCA exposures in "initiated" animals, problematic.

E.4.2.4. Tao et al. (2000)

The source of liver tumors for this analysis was reported to be the study of Pereira and Phelps (1996). Samples of liver "tumors" and "noninvolved" liver were homogenized for protein expression for c-Jun and c-Myc and therefore, contained homogeneous cell types for study. The term "liver tumors" was not defined, so it cannot be ascertained as to whether the lesions studied were altered foci, hepatocellular adenomas, or carcinomas. Liver tissues were reported to be frozen prior to study which raises issues of m-RNA quality. Although this study reports that there were no MNU-induced "tumors," the original paper of Pereira and Phelps (1996) reports that there were 4 foci and 15 adenomas in MNU-only treated mice. The authors reported no difference in c-Jun and c-Myc m-RNA from DCA or TCA-induced tumors from mice "initiated" with MNU. DNA methyltransferase was reported to be decreased in noninvolved liver in MNU-only treated mice in comparison to that from TCA- and DCA-treated mice. For a comparison between noninvolved liver and tumors, tumors were reported to have a greater level than did noninvolved liver.

E.4.2.5. Latendresse and Pereira (1997)

This study used the tumors from Pereira and Phelps (1996), except for the MNU-treated only groups and those groups treated with either DCA or TCA but not MNU initiation, to further study various biomarkers. The omissions were cited as to be due to insufficient tissue. For immunohistochemical evaluation of the molecular biomarkers other than GST-π, liver specimens from seven MNU/20.0 mmol DCA- (i.e., 2.58 g/L DCA) treated and six MNU/20.0 mmol TCA- (i.e., 3.27 g/L TCA) treated female mice randomly selected. For GST-π, the number of animals from which lesion specimens were derived, was 24 MNU/DCA-treated and 23 MNU/TCA-treated mice.

The DCA-treated mice were reported to have 1–9 lesions/mouse and TCA-treated mice had 1–3 lesions/mouse. The number of lesions examined for each biomarker varied greatly. For TCA-induced foci, no foci were examined for any biomarker except 3 lesions for GST-π, while for DCA, 12–15 foci were examined for each biomarker and 38 lesions were examined for GST-π. Similarly for TCA-induced adenomas, there were 8–10 lesions examined for all biomarkers with 32 lesions examined GST-π, while for DCA, there were 12 lesions for all biomarkers with 94 lesions examined for GST-π. Finally, for TCA-induced carcinomas, there were 3–4 lesions examined per group with 64 lesions examined for GST-π, while for DCA-induced carcinomas, there were no lesions examined for any biomarker except 3 examined for GST-π. The biomarkers used were: GST-π, TGF-α, TGF-β, c-Jun, c-Fos, c-Myc, cytochrome oxidase CYP2E1, and cytochrome oxidase CYP4A1.

MNU/DCA treatment was reported to produce "predominantly eosinophilic lesions" with:

> in general, the hepatocytes of DCA-promoted foci and tumors were less pleomorphic and uniformly larger and had more distinctive cell borders than the hepatocytes in lesions caused by TCA. Parenchymal hepatocytes of DCA-promoted mice were uniformly hypertrophied, with prominent cell borders, and the cytoplasm was markedly vacuolated, which was morphologically consistent with the previous description of glycogen deposition in these lesions. In contrast, TCA-promoted proliferative lesions tended to be basophilic, as previously reported, and were composed of hepatocytes with less distinct cell borders, slight cytoplasmic vacuolization, and greater variability in nuclear size and cellular size.

The hepatocytes of altered foci and hepatocellular adenomas from MNU-treated female mice also treated with DCA were reported to stain positively for TGF-α, c-Jun, c-Myc, CYP2E1, CYP4A1, and GST-π. The authors do not present the data for foci and adenomas separately, but as an aggregate, and as the number of lesions with <50% cells stained or the number of lesions with >50% cells stained either "minimally to mildly" or "moderately to densely" stained.

Because no carcinomas for DCA were examined and especially because no foci for TCA analyses were included in the aggregates, it is difficult to compare the profile between TCA and DCA exposure in initiated animals and to separate these results from the effects of differences in tumor progression. Thus, any differences seen in these biomarkers due to progression from foci to adenoma in DCA-induced lesions or from progression of adenoma to carcinoma in TCA-induce lesions, was lost. If the results for adenomas had been reported separately, there would have been a common stage of progression from which to compare the DCA and TCA effects on initiated female mice liver tumors. For DCA-induced "lesions" (~50% foci and ~50% adenomas), most lesions had >50% cells staining with moderate to dense levels for TGF-α, and CYP2E1, CYP4A1, and GST-π and most lesions had <50% cells staining for even minimally to mild staining for TGF-β and c-Fos. For c-Jun and c-Myc, the aggregate DCA-induced "lesions" were heterogeneous in the amount of cells and the intensity of cell staining for these biomarkers in MNU-treated female mice.

For the TCA "lesions" (~60% adenomas and ~30% carcinomas) the authors note that:

> in general, the hepatocytes of tumors promoted by TCA demonstrated variable immunostaining. With the exception of c-Jun, greater than 50% of the hepatocytes in TCA lesions were essentially negative or stained only minimally to mildly for the protein biomarkers studies. In some instances, particularly in TCA-promoted tumors, there was regional staining variability within the lesions, including immunoreactivity for c-Jun and c-Myc proteins, consistent with clonal expansion or tumor progression.

As stated above, the term "lesion" refers to foci and adenomas for DCA, but for adenomas and carcinomas for TCA, making inferences as to differences in the actions of the two compounds through the comparisons of biomarkers confounded by the effects of tumor progression. The largest differences in patterns between TCA induced "lesions" and those by DCA appeared to be TGF-α (with no lesions having >50% cells stained mildly or moderately/densely for TCA-induced lesions), CYP2E1 (with few lesions having >50% stained moderately/densely for TCA-induced lesions), CYP4A1 (with no lesions having >50% stained mildly or moderately/densely for TCA-induced lesions), and GST-π (with all lesions having <50% cells stained even mildly for TCA-induced lesions). However, as shown by these data, while the "lesions" induced by TCA and DCA had some commonalities within each treatment, there was heterogeneity of lesions produced by both treatments in female mice already exposed to MNU. Overall, the tumor biomarker pattern suggests differences in the effects of DCA and TCA through differences in tumor phenotype they induce as co-exposures with MNU treated female mice.

The authors noted that nonlesion parenchymal hepatocytes in DCA-treated initiated mice stained mostly negative for CYP2E1 and CYP4A1, while in TCA-treated mice, staining patterns

in parenchymal nonlesions hepatocytes were centrilobular for CYP2E1 and panlobular for CYP4A1 (a pattern for CYP4A1 that is opposite of that found in the TCA-induced lesions).

E.4.2.6. Pereira et al. (1997)

This study used a similar paradigm as that of Pereira and Phelps (1996) to study co-exposures of TCA and DCA to female $B6C3F_1$ mice already exposed to MNU. At 15 days, the mice received 25 mg/kg MNU and starting at 6 weeks of age neutralized solutions of either 0, 7.8, 15.6, or 25.0 mmol/L DCA (n = 30 for control and 25 mmol/L DCA and n = 20 for 7.8 and 15.6 mmol/L DCA), 6.0 or 25.0 mmol/L TCA (n = 30 for 25.0 mmol/L TCA and n = 20 for 6.0 TCA), or combinations of DCA and TCA that included 25.0 mmol/L TCA + 15.6 mmol/L DCA (n = 20), 7.8 mmol/L DCA + 6.0 mmol/L TCA (n = 25), 15.6 mmol/L DCA + 6.0 mmol/L TCA (45), and 25.0 mmol/L DCA + 6.mmol/L TCA (n = 25). The corresponding concentrations of DCA and TCA in g/L are 25 mmol = 3.23 g/L, 15.6 mmol = 2.01 g/L and 7.8 mmol = 1.01 g/L DCA and 25 mmol = 4.09 g/L, and 6.0 mmol = 0.98 g/L TCA. Accordingly, the number of animals at the beginning of the study varied between 20 and 45. At terminal sacrifice (after 44 weeks of exposure), the numbers of animals examined were less with the lowest number examined to be 17 mice in the 7.8 mmol/L DCA group and the largest to be 42 mice in the 15.6 mmol/L DCA + 6.0 mmol/L TCA exposed group.

The authors reported that only a total of eight HCCs were found in the study (i.e., 25.0 mmol/L DCA induced three carcinomas, 7.8 mmol DCA + 6.0 mmol TCA induced one carcinoma, and 25.0 mmol/L TCA induced four carcinomas). Thus, they presented data for foci/mouse, adenomas/mouse, and their sum of both as "total lesions." The incidences of lesions (i.e., how many mice in the groups had lesions) were not reported. The shortened duration of exposure (i.e., 44 weeks), the omission of carcinomas from total "lesion" counts (precluding consideration of progression of adenomas to carcinomas), the lack of reporting of tumor incidences between groups, and the variable and low numbers of animals examined in each group make quantitative inferences regarding additivity of these treatments difficult. MNU-treated mice did have a neoplastic response, albeit low using this paradigm.

For mice that were only exposed to MNU (n = 30 at terminal sacrifice), the mean numbers of foci, adenomas, and "lesions" per mouse were 0.21, 0.07, and 0.28, respectively. No data were given for mice without MNU treatment but few lesions would be expected in controls. Pereira and Phelps (1996) reported that saline-only treatment in 40 female mice for 51 weeks resulted in 0% foci, 0.03 adenomas/mouse in 2.5% of mice, and 0% carcinomas. In general, it appeared that the numbers of foci, adenomas, and the combination of both reported as "lesions" per mouse that would have been predicted by the addition of multiplicities given for DCA, TCA, and MNU treatments alone, were similar to those observed as co-exposure treatments. The largest numbers of foci and adenomas/mouse were reported for the 25.0 mmol/L DCA and 6.0 mmol/L TCA treatments in MNU-treated mice (mean of

6.57 “lesions”/mouse) with the lowest number reported for 7.8 mmol/L DCA and 6 mmol/L TCA (mean of 1.16 “lesions”/mouse).

The authors reported that the foci of altered hepatocytes were predominantly eosinophilic in DCA-treated female mice initiated with MNU, while those observed after MNU and TCA treatment were basophilic. MNU treatment alone induced four basophilic and two eosinophilic foci, and two basophilic adenomas. MNU and DCA treatment was reported to produce only eosinophilic foci and adenomas at the 25.0 mmol/L DCA exposure level. At the 7.8 mmol/L DCA level of treatment in MNU-treated mice, two foci were basophilic, four were eosinophilic, and the one adenoma observed was reported to be eosinophilic. Thus, the concentration of exposure appeared to alter the tincture of the foci observed after MNU and DCA exposure using this paradigm. In this study, MNU and TCA treatment was reported to induce foci and adenomas that were all basophilic at both 25.0 mmol/L TCA and 6.0 mmol/L TCA exposures. After 7.8 mmol/L DCA + 6.0 mmol/L TCA exposure, 2/23 foci were basophilic and 21/23 foci were reported to be eosinophilic, while all four adenomas reported for this group were eosinophilic.

Irrespective of treatment, eosinophilic foci for were reported to be GST-π positive and basophilic foci to be GST-π negative. An exception was the four carcinomas in the group treated with 25 mmol/L TCA, which were reported to be predominantly basophilic but contained small areas of GST-π positive hepatocytes.

It should be noted that the increased dose (up to 3.23 g/L DCA and 4/09 g/L TCA) raises issues of toxicity and effects on water consumption, as other studies have noted toxicity at highly doses of DCA and TCA. The use of an abbreviated duration of exposure in the study raises issues of sensitivity of the bioassay at the lower doses used in the experiment. In particular, was enough time provided to observe the full development of a tumor response? Finally, a question arises as to what can be concluded from the low numbers of foci examined in the study and the effect of such low numbers on the ability to discern differences in these foci by treatment. As with Pereira and Phelps (1996), there appeared to be a difference the nature of the response induced by co-exposure of MNU to relatively high vs. low DCA concentrations. Of note is that while this experiment reported no HCCs at the lowest dose of DCA at 44 weeks (7.8 mmol DCA), Pereira and Phelps (1996) reported that in nine mice treated with MNU and 2.0 mmol DCA for 52 weeks, there were no foci, but 20% of mice had adenomas (0.20 adenomas/mouse) and 40% of mice had carcinomas (0.70 carcinomas/mouse).

These results suggest that DCA co-exposure affects TCA-induced lesions. The authors concluded that mixtures of DCA and TCA appear to be at least additive and likely synergistic and similar to the pathogenesis of DCA.

E.4.2.7. Tao et al. (1998)

The focus of this study was an examination of tumors resulting from MNU and DCA or TCA exposure in mice with the source of tumors was reported to be the study of Pereira et al. (1997). Thus, similar concerns discussed above for that study paradigm are applicable to the results of this study. The authors stated that there were also two recovery groups in which exposure was terminated 1 week prior to euthanization at week 44. The Pereira et al. (1997) study does not report a cessation group in the study. In this study, the number of animals treated in the cessation group, the incidences of tumors in the mice, and the number of tumors examined were not reported. Another group of female $B6C3F_1$ mice (7–8 weeks old) were reported to not be administered MNU but given 25 mmol/L DCA (3.23 g/L DCA), 25 mmol TCA (4.09 g/L TCA), or control drinking water for 11 days (n = 7).

Hepatocellular adenomas in DCA-treated mice and adenomas and carcinomas in TCA-treated mice were reported to be analyzed for percent-5-methylcytosine in the DNA of tumor tissues. The levels of 5-methylcytosine in liver DNA of mice administered DCA or TCA for 11 days were reported to be reduced in comparison to control tissues (reduced to ~36% of control for DCA and ~41% of control for TCA with the control value reported to be ~3.5% of DNA methylated). The number of animals examined was reported to be 7–10 animals per group.

For control liver from mice that had received MNU but not DCA or TCA, and noninvolved liver after 44 weeks of exposure to either, the levels of 5-methylcytosine were similar and not different from the ~3.5% of DNA methylated in untreated mice in the 11-day experiment. Thus, initial decreases in methylated DNA shown by exposure to DCA or TCA alone for 11 days, were not observed in "noninvolved" liver of animals exposed to either DCA or TCA and MNU.

In regard to tumor tissues, the level of 5-methylcytosine in DNA of hepatocellular adenomas receiving DCA and MNU was reported to be decreased by 36% in comparison to noninvolved liver from the same animals. When exposure to DCA was terminated for 1 week prior to sacrifice, the level of 5-methylcytosine in the adenomas was reported to be higher and no longer differed statistically from the noninvolved liver from the same animal or liver from control animals only administered MNU. The number of samples was reported to be 9–16 samples without identification as to how many samples were used for each tumor analysis or how many animals provided the samples (i.e., were most of the adenomas from on animal?)

For TCA, the 5-methylcytosine level was reported to be reduced by 40% in hepatocellular adenomas and 51% reduction in HCCs in comparison to noninvolved liver from the same animals. These levels were also reported to be less than that the control animals administered only MNU.

Termination of exposure to TCA 1 week prior to sacrifice was reported to not produce a statistically significant change in the level of 5-methylcytosine in either adenomas or

carcinomas. The levels of 5-methylcytosine were reported to be lower in carcinomas than adenomas (~20% reduction) and to be lower in the "recovery" carcinomas than continuous carcinomas (~25%), but were not reported as statistically significant. The results are reported to have been derived from 8 to 16 "samples each." Again, information on the number of animals with tumors, whether the tumors were from primarily from one animal, and which DNA results are from 8 vs. 16 samples, was not provided by the authors.

Given that Pereira et al. (1997), the source for material of this study, reported that treatment of MNU and 25.0 mmol/L TCA treatment for 44 weeks induced only four carcinomas, a question arises as to how many carcinomas were used for the 44-week 5-methylcytosine results in this study for carcinomas (i.e., how can 8–16 samples arise from four carcinomas?). In addition, a question arises as to whether there was a difference in tumor-response in those animals with and without 1 week of cessation of exposure, which cannot be discerned from this report. The use of highly variable number of samples between analysis groups and lack of information as to how many tumors were analyzed adds uncertainty to the validity of these findings. There did not appear to be a difference in methylation activity from short-term exposure to either DCA or TCA alone in whole-liver DNA extracts. However, the authors conclude that the difference in methylation status between tumors resulting from MNU and DCA or TCA exposures supports differences in the action between DCA and TCA.

E.4.2.8. Stauber et al. (1998)

In this study, 5–8-week-old male B6C3F$_1$ mice were used for isolation of primary hepatocytes, which were subsequently isolated and cultured in DCA or TCA. In a separate experiment, 0.5 g/L DCA was given to mice as pretreatment for 2 weeks prior to isolation. The authors note that an indication of an "initiated cell" is anchorage-independent growth. DCA and TCA solutions were neutralized before use. The primary hepatocytes from three mice per concentration were cultured for 10 days with DCA or TCA colonies (eight cells or more) determined in quadruplicate. The levels of DCA used were 0, 0.2, 0.5, and 2.0 mM DCA or TCA. At concentrations of ≥0.5 mM, DCA and TCA both induced an increase in the number of colonies that was statistically significant and increased with dose, with DCA giving a slightly greater effect. The authors noted that concentrations >2.0 mM were cytotoxic, but did not show data on toxicity for this study.

Of great interest is the time-course experiment from this study in which the number of colonies from DCA treatment in vitro peaked by 10 days and did not change through days 15–25 at the highest dose. For the lower concentrations of DCA, increased time in culture induced similar peak levels of colony formation by days 20–25 as that reached by 10 days at the higher dose. Therefore, the number of colonies formed was independent of dose if the cells were treated long enough in vitro. The number of colonies that formed in control hepatocyte cultures also increased with time but at a lower rate than those treated with DCA (2.0 mM DCA gave

approximately twofold of control by 25 days of exposure to hepatocytes in culture). However, the level reached by cells untreated in tissue culture alone by 20 days was similar to the level induced by 0.5 mM DCA by 10 days of exposure. This finding raises the issue of what these "colonies" represent, as tissue culture conditions alone transform these cells to what the authors suggest is an "initiated" state. TCA exposure was not tested with time to see if it had a similar effect to DCA.

At 10 days, colonies were tested for c-Jun expression with the authors noting that "colonies promoted by DCA were primarily c-Jun positive in contrast to TCA promoted colonies that were predominantly c-Jun negative." For colonies that arose spontaneously from tissue culture conditions, 10/13 (76.9%) were reported to be c-Jun+, those treated with DCA 28/34 (82.3%) were c-Jun+, and those treated with TCA 5/22 (22.7%) were c-Jun+. These data show heterogeneity in cell in colonies, although more were c-Jun+ with DCA than TCA. The number of colonies reported in the c-Jun labeling results represent sums between experiments and thus, present total numbers of the control and the of colonies derived from doses of DCA and TCA at 0.2–2.0 mM at 10 days. Thus, changes in colony c-Jun+ labeling due to increasing dose cannot be determined.

The authors reported that with time (24, 48, 72, and 96 hours) of culture conditioning, the number of c-Jun+ colonies was increased in untreated controls. DCA treatment was reported to delay the increase in c-Jun+ expression induced by tissue culture conditions alone in untreated controls. TCA treatment was reported to not affect the increasing c-Jun+ expression that increased with time in tissue culture. In this instance, tissue culture environment alone was shown to transform cells and can be viewed as a "co-exposure." DCA pretreatment in vivo was reported to increase the number of colonies after plating, which reached a plateau at 0.10 mM and gave changes as at low a concentration of 0.02mM DCA administered in vitro. The background level of colony formation varied between controls (i.e., twofold different in pretreatment experiments and nonpretreatment experiments). Therefore, although the number of colonies was greater for pretreatment with DCA, the magnitude of difference over the control level was the same after DCA treatment in vitro with and without pretreatment.

The authors presented a comparison of "tumors" from Stauber and Bull (1997) and state that DCA tumors were analyzed after 38 weeks of treatment but that TCA tumors were analyzed after 52 weeks. They note that 97.5% of DCA-induced "tumors" were c-Jun+, while none of the TCA-induced "tumors" were c-Jun+. The concentrations used to give tumors in vivo for comparison with in vitro results were not reported. What was considered to be "tumors" from the earlier report for this analysis was also not noted. Stauber and Bull (1997) reported results for combination of foci and tumors raising issues as to what was examined in this report. The authors stated that because of such short time, no control tumors results were given. The short and variable time of duration of exposure increases the possibility of differences between the in

vivo data resulting from differences in tumor progression as well as a decreased ability by the shortened time of observation for full expression of the tumor response.

E.4.3. Co-exposures of Haloacetates and Other Solvents

As noted by Caldwell et al. (2008b), drinking water exposure data suggest that co-exposure of TCE and its haloacetic acid metabolites, TCA and DCA, is not an uncommon event, as DCA and TCA are the two most abundant haloacetates in most water supplies (Boorman, 1999; Weisel et al., 1999). Dibromoacetic acid (DBA) concentrations have also been reported to range up to approximately 20 μg/L in finished water and distribution systems (U.S. EPA, 2002a). Caldwell et al. (2008b) have also noted that co-exposure in different media also occurs with solvents like perchloroethylene (PERC) that may share some modes of action, targets of toxicity, and common metabolites that can, therefore, potentially affect TCE health risk (Wu and Schaum, 2000). Some of the information contained in the following sections has been excerpted from the discussions by Caldwell et al. (2008b) regarding the implications for the risk of TCE exposure as modulated by co-exposures to haloacetates and other solvents that have been studied and reported in the literature.

E.4.3.1. Carbon tetrachloride, DCA, TCA: Implications for Mode of Action from Co-exposures

Studies of specific combinations of TCE and chemicals colocated in contaminated areas have been reported by Caldwell et al. (2008b). For carbon tetrachloride:

> Pretreatment with TCE in drinking water at levels as low as 15 mM for three days has been reported to increase susceptibility to liver damage to subsequent exposure to a single IP injection of 1 mM/kg carbon tetrachloride (CCl_4) in Fischer 344 rats (Steup et al., 1991). Potential mechanistic explanations for this observation included altered metabolism, decreased hepatic repair capability, decreased detoxification ability, or combination of one or more of the above activities. Simultaneous administration of an oral dose of TCE (0.5ml/kg) has also been reported to increase the liver injury induced by an oral dose of 0.05 ml/kg CCl_4 (Steup et al., 1993). The authors suggested that TCE appeared to impair the regenerative activity in the liver, thus leading to increased damage when CCl_4 is given in combination with TCE.

As discussed in Section E.4.2, initiation studies are in themselves a co-exposure. The study of Bull et al. (2004) is included here as it not only used a co-exposure of vinyl carbamate with TCE metabolites, but also used carbon tetrachloride as a co-exposure. The rationale for this approach was that co-exposure of TCE (and therefore, to its metabolites) and carbon tetrachloride are likely to occur as they are commonly found together at contaminated sites.

Bull et al. (2004) hypothesized that modification of tumor growth rates is an indication of promotion rather than effects on tumor number, and that by studying tumor growth rates, they could classify carcinogens by their modes of action. $B6C3F_1$ male mice were initiated with vinyl carbamate (3 mg/kg) at 2 weeks of age and then treated with DCA, TCA, or carbon tetrachloride (0.1, 0.5, or 2.0 g/L for DCA and TCA; 50, 100 or 500 mg/kg carbon tetrachloride in 5% Alkamuls via gavage) in pair-wise combinations of the three for 18–36 weeks. The exposure level of carbon tetrachloride to 5, 20, and 50 mg/kg was reported to be reduced at week 24 due to toxicity for carbon tetrachloride. The number of mice in each group was reported to be 10 with the study divided into five segments. There were evidently differences between treatment segments as the authors state that "because of some significant quantitative differences in results that were obtained with replicate experiments treated in different time frames, the simultaneous controls have been used in the analysis and presentation of these data."

As with Bull et al. (2002), the interpretation of the results of the study is limited by a low number of animals per group, short duration time of exposure, and limited examination and reporting of results. For example, a sample of 100/8,000 lesions identified in the study was examined to verify that the general descriptor of neoplastic and nonneoplastic lesion was correctly labeled with "tumors" describing a combination of hyperplastic nodules, adenomas, and carcinomas. No incidence data were reported by the authors, but general lesion growth information included mean lesion volume and multiplicity of lesions (numbers of lesions/mouse). Using these reported indices, there appeared to be differences in treatment-related effects.

As discussed in Caldwell et al. (2008b):

> Each treatment was examined alone and then in differing combinations with each other. Mice initiated with vinyl-carbamate, but without further exposure to the other toxicants, were reported to have a few lesions that were of small size during the examination period (20–36 weeks). At 30 weeks of CCl4 exposure, there was a dose-related response reported for multiplicity but mean lesion size was smaller at the highest dose in initiated animals. At 36 weeks, DCA exposure was reported to increase multiplicity at the two highest exposure levels and increased lesion size at all levels compared to initiated-only animals. However, at a similar level of induction, multiplicity and mean size of those lesions resulting from DCA treatment were reported to be much smaller in comparison with CCl4 treatment (i.e., a 20-fold difference for lesion volume). At 36 weeks, treatments with the same concentration of TCA or DCA induced similar multiplicity, but the mean lesion volume was reported to be approximately 4-fold greater in tumors induced by DCA as compared to TCA, and in animals treated with DCA multiplicity had reached a plateau by 24 weeks rather than 36 for those treated with TCA.

Thus, using multiplicity of lesions and lesion volume as indicators of differences in mode of action, exposure to carbon tetrachloride, DCA, and TCA appeared to produce distinct differences in results in animals previously treated with vinyl carbamate.

As discussed in Caldwell et al. (2008b):

> Simultaneous coexposure of differing combinations of CCl_4, DCA, and TCA were reported to give more complex results between 24 and 36 weeks of observation but to show that coexposure had effects on lesion multiplicity and volume in initiated animals. At 36 weeks, TCA coexposure appeared to reduce the lesion volume of either DCA- or CCl_4-induced lesions after vinyl carbamate treatment. Similarly, DCA coexposure was reported to reduce the lesion volume of either TCA- or CCl_4-induced lesions when each was given alone after vinyl carbamate treatment. With regard to multiplicity, TCA coexposure was reported to reduce DCA-induced multiplicity only at the lowest dose of TCA while coexposure with DCA increased multiplicity of CCl_4-induced lesions at all exposure levels. At 24 weeks, there appeared to be little effect on mean lesion volume by any of the coexposures but DCA coexposure decreased multiplicity of TCA-induced lesions (up to 3-fold) while TCA treatment slightly increased the number of CCl_4-induced multiplicity (1.6-fold). This study confirms that short duration of exposure to all three of these chemicals can cause lesions in already exposed to vinyl carbamate, and suggests that combinations of these agents differentially influence lesion number and growth rates. The authors have interpreted their results to indicate differences in MOA between such treatments. However, the limitations of the study limit conclusions regarding how such coexposure may be able to affect toxicity and tumor induction and what the MOA is for each of these agents. This is especially true at lower and more environmentally relevant concentrations given for longer durations to uninitiated animals.

E.4.3.2. Chloroform, DCA, and TCA Coexposures: Changes in Methylation Status

In Section E.3.4.2.2, information on the effects of TCE and its metabolites was presented in regard to effects on methylation status. After 7 days of gavage dosing, TCE, TCA, and DCA were reported to increased hypomethylation of the promoter regions of c-Jun and c-Myc genes in mouse whole-liver DNA; however, Caldwell and Keshava (2006) concluded that hypomethylation did not appear to be a chemical-specific effect at the concentration used. Bull et al. (2004) suggested that hypomethylation occurs at higher exposure levels than those that induce liver tumors for TCE and its metabolites. Along with studies of methylation changes induced by a exposure to a single agent, Pereira et al. (2001) have attempted to examine the effects on methylation changes from co-exposures. This study was also reviewed by Caldwell et al. (2008b).

Pereira et al. (2001) hypothesized that changes in the methylation status of DNA can be a key event for the mode of action for DCA- and TCA-induced liver carcinogenicity through changes in gene regulation, and that chloroform ($CHCl_3$) co-exposure may result in modification of DNA methylation. As discussed in Caldwell et al. (2008b),

> After 17 days of exposure of exposure to $CHCl_3$ (0, 400, 800, 1,600 mg/L, n = 6 mice per treatment group) female $B6C3F_1$ mice were coexposed to DCA or TCA (500 mg/kg) during the last 5 days of exposure to chloroform. As noted by

> Caldwell et al. (2008b), Pereira et al. (2001) reported the effects of hypomethylation of the promoter region of *c-Myc* gene and on expression of its mRNA in the whole livers of female $B6C3F_1$ mice and thus, these results represent composite changes in DNA methylation status from a variety of cell types and for hepatocytes lumped from differing parts of the liver lobule. When given alone, DCA, TCA, and to a lesser extent, the highest concentration of $CHCl_3$ (1,600 mg/L), was reported to decrease methylation of the *c-myc* promoter region. Coadministration of $CHCl_3$ (at 800 and 1,600 mg/L) was reported to decrease DCA-induced hypomethylation while exposure to $CHCl_3$ had no effect on TCA-induced hypomethylation. Treatment with DCA, TCA, and, to a lesser extent $CHCl_3$, was reported to increase levels of *c-myc* mRNA. While expression of *c-myc* mRNA was increased by DCA or TCA treatment, increasing coexposures to $CHCl_3$ were reported to attenuate the actions of DCA but not TCA. Thus, differences in methylation status and expression of the *c-myc* gene induced by DCA or TCA exposure was reported to be differentially modulated by coexposure to $CHCl_3$. The authors suggest these differences support differing actions by DCA and TCA. However, whether these changes represent key events in the induction of liver cancer is a matter of debate, especially as a "snapshot in time" approach for such a nonspecific endpoint.

In a co-exposure study in which an "initiating agent" was used as a co-exposure along with other co-exposure, Pereira et al. (2001) treated male and female 15-day-old $B6C3F_1$ mice with MNU (a cause of liver and kidney tumors) and then, starting at 5 weeks of age, treated them with DCA (3.2 g/L) or TCA (4.0 g/L) along with co-exposure to $CHCl_3$ (0, 800, or 1,600 mg/L) for 36 weeks. Mice were reported to be examined for evidence of promotion of liver and kidney tumors. The numbers of animals in the exposure groups were highly variable, ranging from 25 (female-initiated mice exposed to DCA) to 6 (female-initiated mice exposed to DCA and 1,600 mg/L $CHCl_3$), thus limiting the power of the study to ascertain treatment-related changes. However, unlike Bull et al. (2004), all liver tissues were examined with incidences of foci, adenomas, carcinomas, and both adenoma and carcinoma reported separately for treatment groups. Multiplicity for a combination of adenomas and carcinomas were reported as well as the tincture of foci and tumors.

Although as noted by Caldwell et al. (2008b):

> [T]he statistical power of the study to detect change was very low, an examination of the pattern of tumors induced by coexposure to MNU and TCE metabolites in female mice suggested that: (1) DCA exposure increased the incidence of adenomas but not carcinomas; (2) TCA increased incidence of carcinomas with little change in adenoma incidence; (3) coexposure to 800 and 1,600 mg/L of $CHCl_3$ decreased adenoma incidence by DCA treatment but not TCA; and (4) $CHCl_3$ coexposure decreased multiplicity of TCA-induced tumors and foci, but not for DCA.

Caldwell et al. (2008b) also note that this study suggests:

> [A] gender-related effect on tumor induction from this study with: (1) adenoma incidences similar in male and female mice treated with DCA, but carcinoma incidence greater in males; (2) adenoma and carcinoma incidences greater in males than females treated with TCA; (3) tumor multiplicity similar in both genders for DCA treatments, but lower in females mice for TCA; and (4) less of an inhibitory effect by $CHCl_3$ on adenoma incidence from DCA exposure in male mice.

Pereira et al. (2001) also described the tinctural characteristics of the specific lesions induced by their co-exposure treatments. Both foci and tumors induced by DCA exposure in "initiated" mice were reported to be over 95% eosinophilic in females, while in males, 89% of the foci were eosinophilic and 91% of tumors were basophilic. Thus, not only was there a gender-related difference in the incidences of tumors and foci but also foci and tumor phenotype. $CHCl_3$ co-exposure was reported to change the DCA-induced foci from primarily eosinophilic to basophilic (i.e., 11 vs. 75% basophilic) in male mice coexposed to MNU. In male and female mice, TCA-induced tumors and foci were basophilic with no effect of $CHCl_3$ on phenotype in MNU treated mice.

E.4.3.3. Co-exposures to Brominated Haloacetates: Implications for Common Modes of Action and Background Additivity to Toxicity

As noted by Caldwell et al. (2008b), along with chlorinated haloacetates and other solvents, "co-exposures with TCE and brominated haloacetates may occur through drinking water. These compounds may affect TCE toxicity in a similar fashion to their chlorinated counterparts. As bromide concentrations increase, brominated haloacetates increase in the water supply."

Kato-Weinstein et al. (2001) administered dibromoacetate (DBA), bromochloroacetate (BCA), bromodichloroacetate (BDCA), TCA, and DCA in drinking water at concentrations of 0.2–3 g/L for 12 weeks to $B6C3F_1$ male mice. The focus of the study was to determine the similarity in action between the brominated and chlorinated haloacetates. Each of the haloacetates, given individually, were reported to increase liver/body weight ratios in a dose-dependent manner.

The dihaloactates, DCA, BCA, and DBA, caused liver glycogen accumulation both by chemical measurements in liver homogenates and in ethanol-fixed liver sections (to preserved glycogen) stained with PAS. For DCA, a maximal level of glycogen increase was observed at 4 weeks of exposure at a 2 g/L exposure concentration. They report a 1.60-fold of control percent liver/body weight and 1.50-fold of control glycogen content after 8 weeks of exposure to 2 g/L DCA in male $B6C3F_1$ mice. The baseline level of glycogen content (~60 mg/g) and the increase in glycogen after DCA exposure was consistent with the results reported by Pereira et al. (2004a). The percent liver/body weight data for control mice was for animals sacrifice at

20 weeks of age. The 4–12-week exposure to DCA were staggered so that all animals would be 20 weeks of age at sacrifice. Thus, the animals were at differing ages at the beginning of DCA treatments between the groups.

However, as with Pereira et al. (2004a), the ~10% increase in liver mass that the glycogen increases represent are lower than the total increase in liver mass reported for DCA exposure. The authors noted possible contamination of BCA with small percentages of DCA and DBA in their studies (i.e., 84% BCA, 6% DCA and 8% DBA). The trihaloacetates (TCA and low concentrations of BDCA) were reported to produce slight decreases in liver glycogen content, especially in the central lobular region in cells that tended to accumulate glycogen in control animals. These effects on liver glycogen were reported at the lowest dose examined (i.e., 0.3 g/L). At the highest concentration, BDCA was reported to induce a pattern of glycogen distribution similar to that of DCA in mice.

All dihaloacetates were reported to reduce serum insulin levels at high concentrations. Conversely, trihaloacetates were reported to have no significant effects on serum insulin levels. For the study of peroxisome proliferation and DNA synthesis, mice were treated with BCA, DBA, and BDCA for 2, 4, or 26 weeks. The effects on DNA synthesis were small for all brominated haloacetates with only DBA reported to show a significant increase in DNA synthesis at 2 and 4 weeks but not at 26 weeks (the increase in DNA synthesis was threefold of the highest control level). Of note is the highly variable level of DNA synthesis reported for control values that varied to a much higher degree (~3–6-fold variation within control groups at the same time points) than did treatment-related changes. DBA was the only brominated haloacetate that was reported to consistently increased PCO activity as a percentage of control values (actual values and variability between controls were not reported) with a 2–3-fold increase in PCO activity at 0.3–3.0 g/L DBA. DBA-induced PCO activity increases were reported to be limited to 2–4 weeks of treatment in contrast to TCA, which the authors reported to increase PCO activity consistently over time.

Tao et al. (2004a) reported DNA methylation, glycogen accumulation, and peroxisome proliferation after exposure of female $B6C3F_1$ mice and male F344 rats exposed to 1 or 2 g/L DBA in drinking water for 2–28 days. DBA was reported to induce dose-dependent DNA hypomethylation in whole mouse and rat liver after 7 days of exposure with suppression sustained for the 28-day exposure period. The expression of mRNA for c-Myc in mice and rats and mRNA expression of the IGF-II gene in female mice were reported to be increased during the same period. Both rats and mice were reported to exhibit increased glycogen with mice having increased levels at 2 day and rats at 4 days. DBA was reported to cause an increase in lauroyl-CoA oxidase activity (a marker of peroxisome proliferation) in both mice (after 7 days) and rats (after 4 days) that was sustained for 28 days.

Methylation changes reported here for DBA exposure in rats and mice are consistent with those reported for TCA and DCA by Pereira et al. (2001) in mice. The pattern of glycogen

accumulation was also similar to that reported for DCA by Kato-Weinstein et al. (2001) and suggests that the brominated analogues of TCE metabolites exhibited similar actions as their chlorinated counterparts. In regard to peroxisomal enzyme activities, Kato-Weinstein et al. (2001) reported PCO activity to be limited to 2–4 weeks with Tao et al. (2004b) reporting lauroyl-CoA oxidase activity to be sustained for the lengths of the study (28 days) for DBA.

As noted by Caldwell et al. (2008b):

> "given the similarity of DCA and DBA effects, it is plausible that DBA exposure also induces liver cancer. Melnick (2008) reported the results of DBA exposure to F344/N rats and $B6C3F_1$ mice exposed to DBA for 3 months or 2 years in drinking water (0, 0.05, 0.5, or 1.0 g/L DBA for 2 years). Neoplasms at multiple sites were reported in both species exposed to DBA for 2 years with no effects on survival and little effect on mean body weight in either species. Similar to TCE, DCA and TCA, the liver was reported to be a target of DBA exposure. After 3-months of exposure, there were dose-related increases in hepatocellular vacuolization and liver weight reported in rats and mice described as 'glycogen-like.'"

The authors report that the major neoplastic effects of DBA in rats were induction of malignant mesotheliomas in males and increased incidence of mononuclear cell leukemia in males and females. For mice, the major neoplastic effect of DBA exposure was reported to be the increased incidence of hepatocellular adenomas and carcinomas at all exposure levels.

In addition to these liver tumors, hepatoblastomas were also reported to be increased in all exposure groups of male mice and exceeded historical control rates. The incidence of alveolar/bronchiolar adenoma and carcinoma was reported to be increased in the 0.5 g/L group of male mice along with marginal increases in alveolar hyperplasia in DBA-treated groups. The authors reported that the increases in hepatocellular neoplasms were not associated with hepatocellular necrosis or regenerative hyperplasia and concluded that an early increase in hepatocyte proliferation was not likely involved in the mode of action for DBA because no increases in hepatocyte DNA labeling index were observed in mice exposed for 26 days and the slight increase that occurred in male F344 rats was not accompanied by an increase in liver tumor response.

As noted by Caldwell et al. (2008b),

> [T]he results of Kato-Weinstein et al. (2001), Tao et al. (2004b), and Melnick et al. (2008) are generally consistent for DBA and show a number of activities that may be common to TCE metabolites (i.e., brominated and chlorinated haloacetate analogues generally have similar effects on liver glycogen accumulation, serum insulin levels, peroxisome proliferation, hepatocyte DNA synthesis, DNA methylation status, and hepatocarcinogenicity). It is therefore, plausible that these effects may be additive in situations of coexposure. However, as noted by (Melnick et al., 2008), methylation status, events associated with PPARα

agonism, hepatocellular necrosis, and regenerative hyperplasia are not established as key events in the MOA of these agents, and the MOAs for DCA- and DBA-induced liver tumors are unknown.

E.4.3.4. Co-exposures to Ethanol: Common Targets and Modes of Action

As noted in the U.S. EPA's draft TCE assessment (U.S. EPA, 2001), alcohol consumption is a common co-exposure that has been noted to affect TCE toxicity with TCE exposure cited as potentially increasing the toxicity of methanol and ethanol, not only by altering their metabolism to aldehydes, but also by altering their detoxification (e.g., similar to the "alcohol flush" reported for those who have an inactive aldehyde dehydrogenase allele). As noted by Caldwell et al. (2008b) "chemical co-exposures from both the environment and behaviors such as alcohol consumption may have effects that overlap with TCE in terms of active agents, pharmacokinetics, pharmacodynamics, and/or target tissue toxicity." Caldwell et al. (2008b) also noted:

> In their review of solvent risk (including TCE), Brautbar and Williams (2002) suggest that laboratory testing that is commonly used by clinicians to detect liver toxicity may not be sensitive enough to detect early liver hepatotoxicity from industrial solvents and that the final clinical assessment of hepatotoxicity and industrial solvents must take into account synergism with medications, drugs of use and abuse, alcohol, age-dependent toxicity, and nutrition. Although many of these factors may be important, the focus in this section is on the effects of ethanol. Contemporary literature reports effects similar to those of TCE's and previous reports indicate ethanol consumption impacts TCE toxicity in humans, affects the pharmacokinetics and toxicity of TCE in rats, and is also a risk factor for cancer.
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> The association between malignant tumors of the upper gastrointestinal tract and liver and ethanol consumption is based largely on epidemiological evidence, and thought to be causally related (Bradford et al., 2005; Badger et al., 2003). Studies of the mechanisms of ethanol carcinogenicity have suggested the importance of its metabolism, including induction of CYP2E1 associated increases in production of reactive oxygen species and enhanced activation of a variety of pro-carcinogens, alteration of retinol and retinoic acid metabolism, and the actions of the metabolite acetaldehyde (Badger et al., 2003). While ethanol is primarily metabolized by alcohol dehydrogenase, it undergoes simultaneous oxidation to acetate by hepatic P450s, primarily CYP2E1. Both chronic ethanol consumption as well as TCE treatment induces CYP2E1 (U.S. EPA, 2001). Oneta et al. (2002) report that even at moderate chronic ethanol consumption, hepatic CYP2E1 is induced in humans, which they suggest, may be of importance in the pathogenesis of alcoholic liver disease; of ethanol, drug, and vitamin A interactions; and in alcohol-associated carcinogenesis. Induction of CYP2E1 can cause oxidative stress to the liver from nicotinamide dinucleotide phosphate (NADPH)-dependent reduction of dioxygen to reactive products even in the absence of substrate, and subsequent apoptosis (Badger et al., 2003).

Bradford et al. (2005) suggest that CYP2E1, and not NADPH oxidase, is required for ethanol-induced oxidative DNA damage to rodent liver but that NADPH oxidase-derived oxidants are critical for the development of ethanol-induced liver injury.

There is increasing evidence that acetaldehyde, which is toxic, mutagenic, and carcinogenic, rather than alcohol is responsible for its carcinogenicity (Badger et al., 2003). Mitochondrial aldehyde dehydrogenase (ALDH2) disposes of acetaldehyde generated by the oxidation of ethanol, and ALDH2 inactivity through mutation or polymorphism has been linked to esophageal cancer in humans (everyday drinkers and alcoholics) (Badger et al., 2003). For instance, increased esophageal cancer risk was reported for patients with the ALDH3*1 polymorphism as well as increased acetaldehyde in their saliva. TCE exposure has also been reported to induce a similar alcohol flush in humans which may be linked to its ability to decrease ALDH activities at relatively low concentrations and thus conferring a similar status to individuals with inactive ALDH2 allele (Wang et al., 1999). Whether the MOA for the buildup of acetaldehyde after ethanol and TCE co-exposure is: (1) the induction of CYP2E1 by TCE resulting in increased metabolism to acetaldehyde; (2) inhibition of ALDH and thus reduced clearance of acetaldehyde, or (3) a number of other actions are unknown. Crabb et al. (2001) reported 20–30% reductions in ALDH2 protein level by PPARα agonists (Clofibrate treatment in rats and WY treatment in both wild and PPARα-null mice). This could be another pathway for TCE-induced effects on ethanol metabolism. It is an intriguing possibility that the reported association between the increased risk of human esophageal cancer and TCE exposure (Scott and Chiu, 2006) could be related to TCE effects on mitochondrial ALDH, given a similar association of this endpoint with ethanol consumption or ALDH polymorphism.

Finally, ethanol ingestion may have significant effects on TCE pharmacokinetics. Baraona et al. (2002a; 2002b) reported that chronic, but not acute, ethanol administration increased the hepatotoxicity of peroxynitrite, a potent oxidant and nitrating agent, by enhancing concomitant production of nitric oxide and superoxide. They also reported that nitric oxide mediated the stimulatory effects of ethanol on blood flow. Ethanol markedly enhanced portal blood flow (2-fold increase), with no changes in the hepatic, splenic, or pancreatic arterial blood flows in rats.

E.4.3.5. Co-exposure Effects on Pharmacokinetics: Predictions Using PBPK Models

Along with experimental evidence that has focused on chronic and acute experiments using rodents, the potential pharmacokinetic modulation of risk has also been recently published reports using PBPK models that may be useful in predicting co-exposure effects. Caldwell et al. (2008b) also examined and discussed these approaches and noted:

An important issue for prediction of the effects and relationship on MOAs by co-exposure is the degree to which modulation of TCE toxicity by other agents can be quantified. Pharmacokinetics or the absorption, distribution, metabolism, and

elimination of an agent, can be affected by internal and external co-exposure. Such information can help to identify the chemical species that may be causally associated with observed toxic responses, the MOA, and ultimately identify potentially sensitive subpopulations for an effect such as carcinogenicity.

Physiologically based pharmacokinetic (PBPK) models are often used to estimate and predict the toxicologically relevant dose of foreign compounds in the body and have been developed to predict effects on pharmacokinetics that are additive or less or greater than additive. One of the first such models was developed for TCE (Andersen et al., 1987b). Given that TCE, PERC, and methyl chloroform (MC) are often found together in contaminated groundwater, Dobrev et al. (2001) attempted to investigate the pharmacokinetic interactions among the three solvents to calculate defined "interaction thresholds" for effects on metabolism and expected toxicity. Their null hypothesis was defined as competitive metabolic inhibition being the predominant result for TCE given in combination with other solvents. Gas uptake inhalation studies were used to test different inhibition mechanisms. A PBPK model was developed using the gas uptake data to test multiple mechanisms of inhibitory interactions (i.e., competitive, noncompetitive, or uncompetitive) with the authors reporting competitive inhibition of TCE metabolism by MC and PERC in simulations of pharmacokinetics in the rat. Occupational exposures to chemical mixtures of the three solvents within their Threshold Limit Value (TLV)/TWA limits were predicted to result in a significant increase (22%) in TCE blood levels compared with single exposures.

Dobrev et al. (2002) extended this work to humans by developing an interactive human PBPK model to explore the general pharmacokinetic profile of two common biomarkers of exposure, peak TCE blood levels, and total amount of TCE metabolites generated in rats and humans. Increases in the TCE blood levels were predicted to lead to higher availability of the parent compound for GSH conjugation, a metabolic pathway that may be associated with kidney toxicity/carcinogenicity. A fractional change in TCE blood concentration of 15% for combined TLV exposures of the three chemicals (25/50/350 ppm of PERC/TCE/MC) resulted in a predicted 27% increase of the S-(1, 2-dichlorovinyl)-L-cysteine (DCVC) metabolites, indicating a nonlinear risk increase due to combined exposures to TCE. Binary combinations of the solvents produced GST-mediated metabolite levels almost twice as high as the expected rates of increase in peak blood levels of the parent compound. The authors suggested that using parent compound peak blood levels (a less sensitive biomarker) would result in two to three times higher (i.e., less conservative) estimates of potentially safe exposure levels. In regard to the detection of metabolic inhibition by PERC and MC, the simulations showed TCE blood concentrations to be the more sensitive dose-metric in rats, but the total of TCE metabolites to be the more sensitive dose measure in humans. Finally, interaction thresholds were predicted to be occurring at lower levels in humans than rats.

Thrall and Poet (2000) investigated the pharmacokinetic impact of low-dose co-exposures to toluene and TCE in male F344 rats *in vivo* using a real-time breath

analysis system coupled with PBPK modeling. The authors report that, using the binary mixture to compare the measured exhaled breath levels from high- and low-dose exposures with the predicted levels under various metabolic interaction simulations (competitive, noncompetitive, or uncompetitive inhibition), the optimized competitive metabolic interaction description yielded an interaction parameter Ki value closest to the Michaelis-Menten affinity parameter (K_M) of the inhibitor solvent. This result suggested that competitive inhibition is the most plausible type of metabolic interaction between these two solvents.

Isaacs et al. (2004) have reported gas uptake co-exposure data for $CHCl_3$ and TCE. The question as to whether it is possible to use inhalation data in combination with PBPK modeling to distinguish between different metabolic interactions was addressed using sensitivity analysis theory. Recommendations were made for design of optimal experiments aimed at determining the type of inhibition mechanisms resulting from a binary co-exposure protocol. This paper also examined the dual nature of inhibition of each chemical in the pair to each other, which is that TCE and $CHCl_3$ were predicted to interact in a competitive manner. Even though as stated by Dobrev et al. (2001), other solvents inhibit TCE metabolism, it is also possible to quantify the synergistic interaction that TCE has on other solvents, using techniques such as gas uptake inhalation exposures.

Haddad et al. (2000) has developed a theoretical approach to predict the maximum impact that a mixture consisting of co-exposure to dichloromethane, benzene, TCE, toluene, PERC, ethylbenzene, m-, p-, and o-xylene, and styrene would have on venous blood concentration due to metabolic interactions in Sprague-Dawley rats. Two sets of experimental co-exposures were conducted. The first study evaluated the change in venous blood concentration after a 4 hour constant inhalation exposure to the 10 chemical mixtures. This experiment was designed to examine metabolic inhibition for this complex mixture. The second study was designed to study the impact of possible enzyme induction by using the same inhalation co-exposure after a 3 day pretreatment with the same 10 chemical mixture. The resulting venous concentration measurements for TCE from the first study were consistent with metabolic inhibition theory. The 10-chemical mixture was the most complex co-exposure used in this study. The authors stated that as mixture complexity increased, the resulting parent compound concentration time courses changed less, an observation which is consistent with metabolic inhibition. For the pretreatment study, the authors found a systematic decrease in venous concentration (due to higher metabolic clearance) for all chemicals except PERC. Overall, these studies suggest a complex metabolic interaction between TCE and other solvents.

A PBPK model for TCE including all its metabolites and their interactions can be considered a mixtures model where all metabolites have a common starting point in the liver. An integrated approach taking into account TCE metabolites and their metabolic inhibition and interactions among each other is suggested in Chiu et al. (2006b).

E.5. POTENTIALLY SUSCEPTIBLE LIFE STAGES AND CONDITIONS THAT MAY ALTER RISK OF LIVER TOXICITY AND CANCER

As described in Sections E.1.2 and E.3.1, there are a number of conditions that are associated with increased risk of liver cancer and toxicity that include age, use of a number of prescription medications including fibrates and statins, disease state (e.g., diabetes, NALD, viral infections), and exposure to external environmental contaminants that have an effect on TCE toxicity and targets. Obviously, epigenetic and genetic factors play a role in determining the risk to the individual. In terms of liver cancer, there is general consensus that despite the associations that have been made with etiological factors and the risk of liver cancer, the mechanism is still unknown. The mode of action of TCE toxicity is also unknown, but exposure to TCE and its metabolites have shown in rodent models to induce liver cancer and in a fashion that is not consistent with only a hypothesized mode of action of PPARα receptor activation that is in need of revision. However, multiple TCE metabolites have been shown to also induce liver cancer with varying effects on the liver that have also been associated with early stages of neoplasia (glycogen storage) or other actions associated with risk of hepatocarcinogenicity. The growing epidemic of obesity has been suggested to increase the risk of liver cancer and may reasonably increase the target population for TCE effects on the liver.

Lifestyle factors such as ethanol ingestion have not only been shown to increase liver cancer risk in those who already have fatty liver, but also to increase the toxicity of TCE. However, as noted by Caldwell et al. (2008b), while there is evidence to suggest that TCE exposure may increase the risk of liver toxicity and cancer, there are no data to support a quantitative estimate of how co-exposures may modulate that risk.

> These findings can also serve to alert the risk manager to the possibility that multiple internal and external exposures to TCE that may act via differing MOAs for the production of liver effects. This information suggests a possible lack of "zero" background exposures and can help identify potential susceptible populations.
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> Background levels of haloacetates in drinking water may add to the cumulative exposure an individual receives via the metabolism of TCE. The brominated haloacetates apparently share some common effects and pathways with their chlorinated counterparts. Thus, concurrent exposure of TCE, its metabolites, and other haloacetates may pose an additive response as well as an additive dose. However, personal exposures are difficult to ascertain and the effects of such co-exposures on toxicity are hard to quantify. EPA's guidance on cumulative risk assessments directs "each office to take into account cumulative risk issues in scoping and planning major risk assessments and to consider a broader scope that integrates multiple sources, effects, pathways, stressors, and populations for cumulative risk analyses in all cases for which relevant data are available" [U.S. EPA, 1997]. Widespread exposure to possible background levels of TCE metabolites or co-contaminants and other extrinsic factors have the potential to

affect TCE toxicity. However, the available data for co-exposures on TCE toxicity appears inadequate for quantifying these effects, particularly at environmental levels of contamination and exposure. Thus, the risk manager and assessor are going to be limited by not having information regarding either (1) the type of exposure data necessary to assess the magnitude of co-exposures that may affect toxicity, or (2) the potential quantitative impacts of these co-exposures that would enable specific adjustments to risk. Nonetheless, the risk manager should be aware that qualitatively a case can be made that extrinsic factors may affect TCE toxicity.

E.6. UNCERTAINTY AND VARIABILITY

Along with general conclusions about the coherence of data that enable conclusions about effects on the liver shown through experimental studies of TCE, there have also been extensive discussions throughout this report regarding the specific limitations of experimental studies whose design was limited by small and varying groups of animals and variability in control responses as well as reporting deficiencies. Section E.3.1.5 has brought forward the uncertainty in the mode of action for liver cancer in general. The consistency of different animal models with human HCC is described in Section E.3.3, with Section E.3.1.2 providing a discussion of the promise and limitations of emerging technologies to study the modes of action of liver cancer in general and for TCE specifically. Issues regarding the target cell for HCC and the complexities of studying the mode of action for a heterogeneous disease are described in Sections E.3.1.4 and E.3.1.8, respectively. Finally, the uncertainty regarding key events in how activation of the PPARα receptor my lead to hepatocarcinogenesis and the problems with extrapolation of results using the common paradigm to study them (exposure to high levels of WY-14,643 in abbreviated bioassays in knockout mice) are outlined in Section E.3.4.1. As such uncertainties are identified, future research can focus on resolving them.