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Casarett & Doull's

Toxicology

The Basic Science of Poisons

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hepatocyte-labeling index, a measure of the proliferative response, differed between the two dosage regimens at comparable doses. Pereira (1994) reported essentially the same observation. This suggests that ingestion of $CHCl_3$ in small increments, similar to drinking water patterns of humans, fails to produce a sufficient amount of cytotoxic metabolite(s) per unit time to overwhelm detoxification and other protective mechanisms.

Potentiation of $CHCl_3$'s toxicity by CYP inducers and protection by GSH and P450 inhibitors suggest that a metabolite, presumably phosgene, is responsible for $CHCl_3$'s hepatorenal toxicity. Both target organs metabolize $CHCl_3$ to phosgene. There is evidence that CYP2E1 and CYP2B1/2 metabolically activate $CHCl_3$, but the former isoform is thought to catalyze $CHCl_3$ metabolism at a lower concentration than the latter (Nakajima *et al.*, 1995). By using an irreversible CYP2E1 inhibitor and CYP2E1-knockout mice, Constan *et al.* (1999) have demonstrated that metabolism of $CHCl_3$ by CYP2E1 is required for liver and kidney necrosis and cell proliferation. The electrophilic intermediate generated by $CHCl_3$'s metabolism (i.e., phosgene) is initially detoxified by covalently binding to cytosolic GSH. Once GSH is depleted, phosgene is free to covalently bind hepatic and renal proteins and lipids. Such binding damages membranes and other intracellular structures, leading to necrosis and subsequent reparative cellular proliferation. Sustained proliferation with repeated exposures promotes tumor formation in rodents by irreversibly "fixing" spontaneously altered DNA and clonally expanding initiated cells. The expression of certain genes, including *myc* and *fos*, is altered during regenerative cell proliferation in response to $CHCl_3$-induced cytotoxicity (Sprankle *et al.*, 1996; Kegelmeyer *et al.*, 1997). While the identity of phosgene's intracellular targets is largely unknown, Guastadisegni *et al.* (1999) have reported that phosgene reacts with phosphatidylethanolamine (PE). The adduct formed appears to consist of two PE moieties cross-linked at the amino head groups by the carbonyl moiety of phosgene. $CHCl_3$-modified PE preferentially accumulates on inner mitochondrial membranes, inducing ultrastructural modifications and inhibiting functions of the organelle. These researchers observed the induction of hepatic apoptosis and necrosis in $CHCl_3$-treated Sprague–Dawley rats and pointed out that apoptosis may be initiated by the release of regulatory factors normally sequestered in mitochondria, in particular Ca^{2+}. Evidence that Ca^{2+} perturbation plays a role in $CHCl_3$ toxicity comes from a report of Ca^{2+} mobilization in Madin–Darby canine kidney cells using Fura-2 as a Ca^{2+} probe. $CHCl_3$, albeit in millimolar concentrations, increased the cytosolic Ca^{2+} levels by releasing Ca^{2+} from multiple sites within the cell (Jan *et al.*, 2000).

There is no evidence of covalent binding of $CHCl_3$ metabolites to nucleic acids. There is binding to nuclear histone, which plays a key role in controlling DNA expression and might be a mechanism of $CHCl_3$'s carcinogenicity (Diaz and Castro, 1980; Fabrizi *et al.*, 2003). It has been hypothesized that the induction of oxidative stress and depletion of GSH by $CHCl_3$ may lead to indirect genotoxicity that could contribute to carcinogenicity. This hypothesis is supported by the small dose-dependent increase in M(1)dG adducts (malondialdehyde reacts with DNA to form adducts to deoxyguanosine), DNA strand breakage, and lipid peroxidation in $CHCl_3$-treated rat hepatocytes in the absence of any increase in DNA oxidation (Beddowes *et al.*, 2003). Such a mechanism would still be threshold dependent, given its reliance on the initial depletion of antioxidants.

Currently, the EPA classifies $CHCl_3$ as a probable human carcinogen (group B2), meaning there is sufficient evidence for carcinogenicity in animals and inadequate or no evidence in humans. Experimental evidence and the prevailing opinion that $CHCl_3$ is nongenotoxic, indicates that the relationship between $CHCl_3$ dose and tumor formation is nonlinear. The EPA has, as is called for in its new *Guidelines for Carcinogen Risk Assessment* (EPA, 2005), considered mode of action in the determination of $CHCl_3$'s cancer risk and relied on a nonlinear dose–response approach and the use of margin-of-exposure analysis. In doing so, the Agency concluded that the RfD for noncancer effects, based on the dog study of Heywood *et al.* (1979), was adequately protective for cancer by the oral route on the basis of cancer and noncancer effects having a common link through cytotoxicity. The wealth of mechanistic data available continues to inform the risk assessment for $CHCl_3$. For example, Constan *et al.* (2002) exercised a PBTK dosimetry model to compare hepatic responses in mice and humans to inhaled $CHCl_3$. They concluded that no safety factor was needed to account for interspecies differences in inhalation cancer risk. Additionally, Tan *et al.* (2003) have published a PBTK/TD model for $CHCl_3$ to describe the plausible mechanism linking the hepatic metabolism of $CHCl_3$ to hepatocellular killing and regenerative proliferation, thereby creating a predictive model that most accurately reflects the current science.

AROMATIC HYDROCARBONS

Benzene

Benzene produced commercially in the United States is derived primarily from petroleum. Benzene has been utilized as a general purpose solvent, but it is now used principally in the synthesis of other chemicals (ATSDR, 2006c). The percentage by volume of benzene in gasoline is 1–2%. Benzene plays an important role in unleaded gasoline due to its antiknock properties. Inhalation is the primary route of exposure in industrial and in everyday settings. Cigarette smoke is the major source of benzene in the home (Wallace, 1996). Smokers have benzene body burdens that are six to 10 times greater than those of nonsmokers. Passive smoke can be a significant source of benzene exposure to nonsmokers. Gasoline vapor emission and auto exhaust are the other key contributors to exposures of the general populace.

The most important adverse effect of benzene is hematopoietic toxicity. Chronic exposure to benzene can lead to bone marrow damage, which may be manifest initially as anemia, leukopenia, thrombocytopenia, or a combination of these. Bone marrow depression appears to be dose dependent in both laboratory animals and humans. Continued exposure may result in marrow aplasia and pancytopenia, an often fatal outcome. Survivors of aplastic anemia frequently exhibit a preneoplastic state, termed myelodysplasia, which may progress to myelogenous leukemia (Snyder, 2002; Bird *et al.*, 2005).

There is strong evidence from epidemiological studies that high-level benzene exposures result in an increased risk of acute myelogenous leukemia (AML) in humans (Bergsagel *et al.*, 1999; ATSDR, 2006a). Evidence of increased risks of other cancers in such populations is less compelling. Only AML incidence was significantly elevated in the largest cohort study to date, in which ~75,000 benzene-exposed workers in 12 cities in China were evaluated (Yin *et al.*, 1996). Marginal, nonsignificant increases were seen for lung cancer and chronic myelogenous leukemia. A subsequent study of some 250 Chinese shoe workers who inhaled low ($\leq$1 to $\geq$10 ppm) levels of benzene revealed dose-dependent hematotoxicity (Lan *et al.*, 2004). Exposures of 100 of these employees

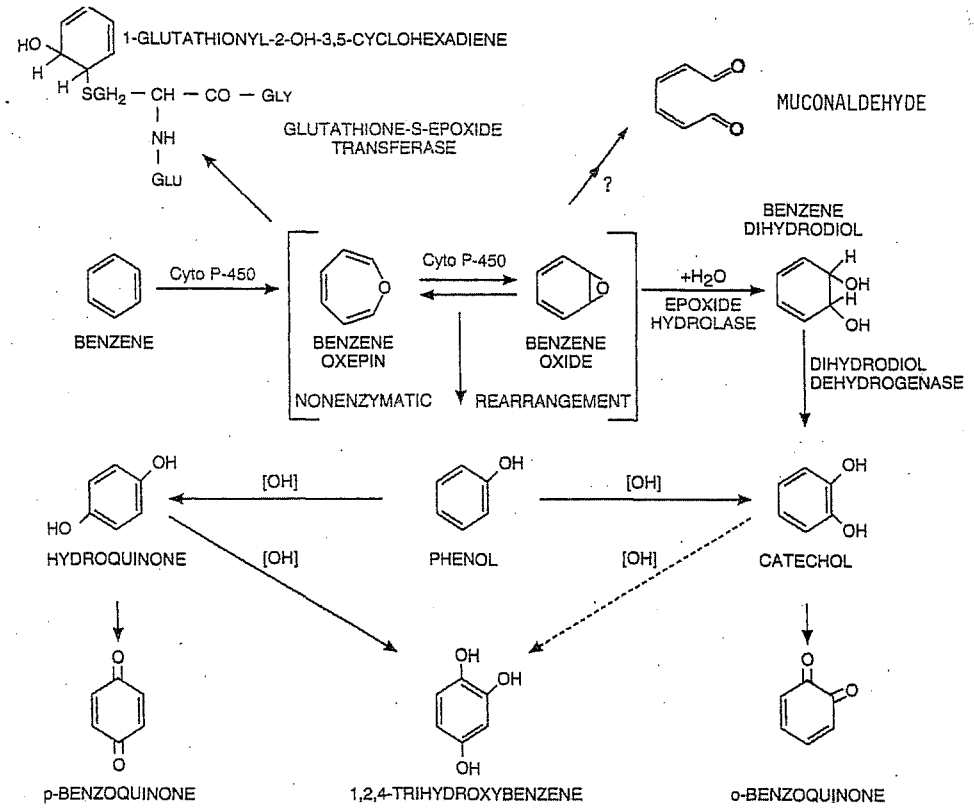

Figure 24-9. Biotransformation of benzene.

A question mark leads from the oxepin-oxide compartment to muconaldehyde because the substrate for the ring opening has yet to be identified. The dotted line leading to 1,2,4-trihydroxybenzene (1,2,4-T) indicates that it is not clear what the relative contributions of hydroquinone and catechol are to 1,2,4-T. [Modified and used with permission of Rangan and Snyder (1997).]

to ≤1 ppm reduced mature white cell and platelet counts, as well as myeloid progenitor cell colony formation. The progenitors were more sensitive to benzene than mature white cells. Polymorphisms in myeloperoxidase (an enzyme that metabolizes benzene to toxic quinones and free radicals) and NAD(P)H:quinone oxidoreductase (an enzyme that protects against these moieties) conferred increased susceptibility to white cell decreases. Turtletaub and Mani (2003) found that formation of DNA and protein adducts in mouse liver and bone marrow was dose dependent over a range of benzene doses, the lowest of which was 5 μg/kg.

Types of cancer other than AML have been tentatively attributed to benzene. Some investigations of persons exposed to engine exhausts have reported a significant association with multiple myeloma. Bezabeth *et al.* (1996) and Bergsagel *et al.* (1999) have concluded, however, that there is no scientific evidence to support a causal relationship between benzene exposure and multiple myeloma. Increased incidences of malignant lymphomas and a variety of solid tumors were found in male and female B6C3F1 mice dosed orally with high doses of benzene for up to 103 weeks (Huff *et al.*, 1989). Male and female F-344 rats in this bioassay exhibited excesses of Zymbal gland, skin, and oral cavity carcinomas. Thus, benzene is clearly an animal and human carcinogen, but major species differences exist.

It is essential to understand the metabolism of benzene in order to address its mechanisms of toxicity. The initial metabolic step (Fig. 24-9), oxidation of benzene to an epoxide (i.e., benzene oxide), is catalyzed primarily by hepatic CYP2E1. Valentine *et al.* (1996) demonstrated that benzene-treated transgenic CYP2E1 knockout mice had relatively low levels of all benzene metabolites in their urine. A 5-day, 200-ppm benzene inhalation regimen produced severe genotoxicity and cytotoxicity in wild-type B6C3F1 mice, but no adverse effects in the knockout mice. Benzene oxide, which is in equilibrium with its oxepin form, is further metabolized by three pathways: (1) conjugation with GSH to form a premercapturic acid, which is converted to phenyl-mercapturic acid; (2) rearrangement nonenzymatically to form phenol; and (3) hydration by epoxide hydrolase to benzene dihydrodiol, which in turn can be oxidized by dihydrodiol dehydrogenase to catechol. If phenol is hydroxylated in the *ortho* position, more catechol will be formed. Catechol can be converted to *o*-benzoquinone. If benzene is hydroxylated in the *para* position, *p*-hydroquinone is formed. It can be oxidized to *p*-benzoquinone. The *o*- and *p*-benzoquinones are believed to be among the ultimate toxic metabolites of benzene. Another potentially toxic metabolite, muconaldehyde, may arise from ring opening of benzene oxide. Muconaldehyde undergoes a series of reactions that ultimately lead to *t,t*-muconic acid, an end metabolite found in the urine (Golding and Watson, 1999; Snyder, 2004).

Metabolism of benzene occurs primarily in the liver, though metabolism in bone marrow is believed by many authorities to play the key role in myelotoxicity. Levels of DNA and protein adducts were significantly higher in bone marrow than liver of benzene-dosed mice (Turtletaub and Mani, 2003). It has been generally

accepted that phenolic conjugates are formed in the liver and transported via the blood to the bone marrow, where they are hydrolyzed and oxidized to quinones. Researchers have been unable to reproduce benzene toxicity, however, by giving individual phenolic metabolites to animals. The quinones are believed to be too reactive to be transported in the bloodstream. Lindstrom *et al.* (1997) have reported that benzene oxide has an estimated half-life of 7.9 minutes in rat blood, and thus may be able to travel from the liver to the bone marrow. Ring-opened metabolites of muconaldehyde, which are less reactive, could be transported to bone marrow and reoxidized. Bernauer *et al.* (1999) measured activity and levels of CYP2E1 in the liver and bone marrow of five strains of mice of varying sensitivity to benzene. CYP2E1 amounts and activities were considerably greater in the liver than the marrow, but no interstrain differences were seen in either tissue. It is likely that non-CYP enzymes (e.g., peroxidase, myeloperoxidase, and cyclooxygenase) play an important role in generation of semiquinones and quinones in bone marrow (Ross *et al.*, 1996). Snyder (2004) concluded that benzene hematopoietic toxicity and leukemogenesis are primarily a function of the bone marrow, a site remote from the liver where most benzene metabolism occurs.

Benzene's metabolic pathways appear to be qualitatively, though not quantitatively similar in species studied to date (Henderson, 1996). Mice have a greater overall capacity to metabolize benzene than do rats or primates. Mani *et al.* (1999) reported good correlation between protein–DNA adduct levels in bone marrow and susceptibility to benzene genotoxicity and carcinogenesis in F-344 rats and three strains of mice. The B6C3F1 mouse shows the highest adduct levels and is the most sensitive of the animals tested. Powley and Carlson (1999) reported similar findings upon measurement of benzene metabolism in mouse, rat, rabbit, and human lung and liver microsomes. A paucity of information is available on the ability of human bone marrow to metabolically activate benzene.

Factors that alter the metabolism of benzene have the potential to influence the hematopoietic toxicity and carcinogenicity of the VOC. Pretreatment of rats with ethanol, a CYP2E1 inducer, enhanced metabolism of benzene and potentiated its acute and subacute myelotoxicity in rats (Nakajima *et al.*, 1985) and mice (Marrubini *et al.*, 2003). Phenobarbital had a negligible effect. Pretreatment of male B6C3F1 mice with acetone, another CYP2E1 inducer, increased benzene oxidation by about five-fold (Kenyon *et al.*, 1996). Pretreatment with diethyldithiocarbamate, a CYP2E1 inhibitor, completely abolished benzene oxidation. Co-exposure of F-344 rats to gasoline and benzene resulted in competitive metabolic inhibition (Travis *et al.*, 1992). Metabolism of benzene by hepatic microsomes from 10 human donors was directly proportional to CYP2E1 activity in the samples (Seaton *et al.*, 1994). Thus, it would be anticipated that CYP2E1 polymorphisms may influence susceptibility to benzene toxicity. Male mice have consistently been found to be more sensitive than females to genotoxic effects of benzene. PBTK analysis of data from gas uptake experiments with B6C3F1 mice of both sexes revealed that the optimized maximum rate of benzene metabolism was twice as high in males (Kenyon *et al.*, 1996). It is not known whether there is a sex-dependent difference in benzene metabolism in humans. Sato *et al.* (1975) saw a more rapid rate of pulmonary elimination of benzene in men than in women following inhalation of 25 ppm for 2 hours. Benzene was retained longer in the females due to their higher body fat content. Unfortunately, most epidemiology studies of benzene-exposed workers have not provided a gender comparison. Li *et al.* (1994) did not see a statistically significant difference in cancer mortality between male and female workers exposed to benzene, though risks tended to be somewhat higher for males.

There are a number of cell populations in the bone marrow that may serve as targets for benzene metabolites. Benzene exposure in vivo results in inhibited growth and development of pluripotential bone marrow stem cells. More mature precursors, such as stromal cells and erythroid and myeloid colony-forming units, are also affected. Trush *et al.* (1996) point out that hydroquinone-induced inhibition of interleukin (IL)-1 synthesis by stromal macrophages results in altered differentiation of myeloid and lymphoid cells that are normally active in immune responses. These investigators also note that killing of stromal macrophages and fibroblasts could result in such a pronounced reduction of cytokines and growth factors that immature and committed hematopoietic progenitors would die from apoptosis. The erythroid series is more susceptible than the myeloid series to benzene-induced cytotoxicity. Immature myeloid cells can proliferate when the development of erythroid cells is restricted and acquire neoplastic characteristics upon dedifferentiation. The end result is AML (Golding and Watson, 1999).

Investigations of benzene toxicity/leukemogenesis have uncovered a variety of potential mechanisms (Golding and Watson, 1999; Snyder, 2002; Bird *et al.*, 2005). As mentioned before, experimental evidence indicates that the complementary actions of benzene and several of its metabolites are required for myelotoxicity. It has been recognized for 20 years that a number of benzene metabolites can bind covalently to GSH, proteins, DNA, and RNA. This can result in disruption of the functional hematopoietic microenvironment by inhibition of enzymes, destruction of certain cell populations, and alteration of the growth of other cell types. Covalent binding of hydroquinones to spindle-fiber proteins will inhibit cell replication (Smith, 1996). In vitro studies have established that reactive benzene metabolites bind covalently to DNA of several tissues of different species. Through the use of accelerator mass spectrophotometry, Creek *et al.* (1997) have demonstrated DNA binding in mice exposed to extremely low ^{14}C-benzene levels. The binding was dose dependent over a wide range of doses. DNA adduct levels were so low, however, that this mechanism alone may be insufficient to fully account for leukemogenic effects.

It appears likely that oxidative stress contributes to benzene toxicity. As the bone marrow is rich in peroxidase activity, phenolic metabolites of benzene can be activated there to reactive quinone derivatives. Ross *et al.* (1996) discovered that myeloperoxidase present in murine and human progenitor cells could bioactivate hydroquinone to *p*-benzoquinone. Electron spin resonance experiments by Hiraku and Kawanishi (1996) revealed the formation of a semiquinone radical in *p*-benzoquinone-treated HL-60 cells (a human myeloid cell line). This suggested that reactive oxygen moieties (e.g., O_2- and H_2O_2) are produced via the formation of the semiquinone radicals. These active oxygen species can cause DNA strand breaks or fragmentation, leading to cell mutation or apoptosis, respectively. Ross *et al.* (1996), however, pointed out that quinones can also inhibit proteases involved in induction of apoptosis. These authors noted that modulation of apoptosis may lead to aberrant hematopoiesis and neoplastic progression.

A number of biomarkers of exposure to benzene have been developed and carefully evaluated. Concentrations of the parent compound in exhaled breath parallel blood concentrations. Clayton *et al.* (1999) reported a high correlation between the extent of smoking-related activities and levels of benzene in exhaled breath of humans. Urinary excretion of a variety of benzene metabolites (i.e., phenol, catechol, hydroquinone, 1,2,4-trihydroxybenzene,

S-phenylmercapturic acid, and *t,t*-muconic acid) have been shown to be correlated with benzene exposure in occupational settings. Phenol, catechol, and hydroquinone, however, are neither sensitive nor specific biomarkers, because relatively high levels are found in nonexposed individuals (Medeiros *et al.*, 1997). Similarly, *t,t*-muconic acid is not specific, because it is a metabolite of sorbic acid, a common food additive. Boogaard and van Sittert (1996) conclude that *S*-phenylmercapturic acid, an end product of the conjugation of benzene oxide and GSH, is a suitable urinary biomarker for low-level benzene exposure because of its specificity and relatively long half-life. Adducts to hemoglobin and cysteine groups of proteins have been demonstrated in rodents, but not in humans. DNA damage has been detected in benzene-exposed workers (Liu *et al.*, 1996; Andreoli *et al.*, 1997), though such measures have not yet found widespread acceptance as biomarkers of effect.

A number of PBTK models have been developed to aid in predicting risks of myelotoxicity and leukemia posed to humans by benzene. Medinsky *et al.* (1989) published one of the first such models for benzene. They assumed that benzene metabolism followed Michaelis–Menten kinetics and occurred via benzene oxide. B6C3F1 mice, which are more sensitive than F-344 rats to benzene myelotoxicity, were predicted to metabolize two to three times more inhaled benzene. This and other PBTK models do not accurately simulate some laboratory data sets. Modeling benzene metabolism and disposition is difficult because of its inherent complexity and variability. In light of intricate dose–response relationships, Medinsky *et al.* (1996) emphasized the importance of considering competitive metabolic interactions between benzene and its metabolites, as well as the balance between enzymatic activation and inactivation processes. Cole *et al.* (2001) employed in vitro metabolic parameters for several pathways to construct a PBTK model that simulated most tissue dosimetry data sets for benzene-exposed mice quite well. Recently, Yokley *et al.* (2006) described a PBTK model that simulated tissue doses of benzene, benzene oxide, phenol, and hydroquinone in humans exposed orally and by inhalation. A Markov Chain Monte Carlo statistical technique was used to assess dissimilarities in population distributions of key model parameters. Variability in metabolic parameters and certain physiological parameters (e.g., organ weight) had to be inputed to accurately predict the range of human values.

Toluene

Toluene is present in paints, lacquers, thinners, cleaning agents, glues, and many other products. Toluene is also used in the production of other chemicals. Gasoline, which contains 5–7% toluene by weight, is the largest source of atmospheric emissions and exposure of the general populace (ATSDR, 2000). Inhalation is the primary route of exposure, though skin contact occurs frequently. Toluene is a favorite of solvent abusers, who intentionally inhale high concentrations to achieve a euphoric effect (Filley *et al.*, 2004). Large amounts of toluene enter the environment each year by volatilization. Relatively small amounts are released in industrial wastewater. Toluene is frequently found in water, soil, and air at hazardous waste sites (Fay and Mumtaz, 1996).

Toluene TK has been thoroughly characterized in humans and laboratory animals. Toluene is well absorbed from the lungs and GI tract. It rapidly accumulates in and affects the brain, due to that organ's high rate of blood perfusion and relatively high lipid content. Toluene subsequently is deposited in other tissues according to their lipid content, with adipose tissue attaining the highest levels. Toluene is well metabolized, but a portion is exhaled unchanged. Hepatic P450s catalyze metabolism of toluene primarily to benzyl alcohol and lesser amounts of cresols. Benzyl alcohol is converted by ADH and aldehyde dehydrogenase (ALDH) to benzoic acid, which is primarily conjugated with glycine and eliminated in the urine as hippuric acid. Nakajima *et al.* (1992a) reported that CYP2E1 and CYP2C11 are primarily responsible for catalyzing the initial hydroxylation step in rat liver at low and high toluene levels, respectively. CYP2E1 is most active at low doses in humans (Nakajima *et al.*, 1997). Benignus *et al.* (1998) attempted to use a PBTK model to relate toluene blood levels to behavioral effects in rats and humans. It appeared that humans were more sensitive to increases in blood levels, but more rat data and much more human data are needed for model validation.

The CNS is the primary target organ of toluene and other alkylbenzenes. Manifestations of acute exposure range from slight dizziness and headache to unconsciousness, respiratory depression, and death. Occupational inhalation exposure guidelines are established to prevent significant decrements in psychomotor functions. Acute encephalopathic effects are rapidly reversible upon cessation of exposure (Fig. 24-3), and are not associated with neuroimaging changes (Filley *et al.*, 2004). Subtle neurologic effects have been described in some groups of occupationally exposed individuals. Exposure to ~100-ppm toluene for years may result in subclinical effects, as evidenced by altered brainstem auditory-evoked potentials (Abbate *et al.*, 1993) and changes in visual-evoked potentials (Vrca *et al.*, 1995). Foo *et al.* (1990) reported good correlation between toluene exposure and poor scores on neurobehavioral tests of 30 female rotogravure workers. Severe neurotoxicity is often diagnosed in persons who have abused toluene for a prolonged period. A relatively specific neurobehavioral profile is manifest including inattention, apathy, memory dysfunction, diminished visuospatial skills, frontal lobe dysfunction, and psychiatric status (Ron, 1986; Filley *et al.*, 2004). Magnetic resonance imaging reveals ventricular enlargement, cerebral atrophy and white matter hyperintensity, a characteristic profile termed toluene leukoencephalopathy. Such changes represent severe, chronic myelintoxicity. More advanced neuroimaging techniques may be able to reveal mild, subtle changes associated with early alterations in myelin of workers as well as toluene abusers (Caldemeyer *et al.*, 1996; Filley *et al.*, 2004). Rat pups subjected to high doses of toluene on days 6–19 of gestation exhibited a significant reduction in myelination per forebrain cell when they were 21 days old (Gospe and Zhou, 1998).

There is limited information on mechanisms by which toluene and similar solvents produced acute or residual CNS effects (Balster, 1998). The Meyer–Overton theory of partitioning of the lipophilic parent compounds into membrane lipids has been widely accepted for a century. It has been proposed that the presence of solvent molecules in cholesterol-filled interstices between phospholipids and sphingolipids changes membrane fluidity, thereby altering intercellular communication and normal ion movements (Engelke *et al.*, 1996). Such a process is reversible. An alternate hypothesis is that toluene partitions into hydrophobic regions of proteins and interacts with them, thereby altering membrane-bound enzyme activity and/or receptor specificity in a reversible manner (Balster, 1998). Other evidence suggests that toluene and other VOCs may act acutely by enhancing $GABA_A$ receptor function (Mihic *et al.*, 1994), attenuating NMDA receptor-stimulated calcium flux (Cruz *et al.*, 1998), and/or activating dopamingeric systems (von Euler, 1994). The mechanism of chronic toluene neurotoxicity is unknown. Toluene is known to be deposited in brain areas with the highest