

## CHAPTER II

### BENZENE, TOLUENE AND XYLENE

#### BENZENE

Benzene,  $C_6H_6$ , is a clear, colorless, highly flammable and volatile liquid with excellent solvent properties. The U.S. production alone of benzene in 1977 was 5.1 million tons (432). Although the use of benzene as a solvent has been suggested to be banned (407), benzene is still relatively frequently used as a solvent in chemical and industrial processes. Benzene is also indispensable as a starting material for chemical syntheses, especially in the plastics industry, and occurs as a natural component or intentional antiknock additive in motor fuels. Benzene is a constituent of so many commonly used products that it has been stated that it would be a rare individual who has not inhaled its fumes (155).

#### TOXICITY OF BENZENE

Acute effects of exposure to high concentrations of benzene vapors are mainly of central nervous system origin. Benzene is a CNS depressant, which may be easily demonstrated in experimental animals where the narcotic threshold is approximately 4000 ppm (250) with concentrations around 40000 ppm fatal to

life within half an hour (66). The same effect is observable in human exposure (51, 146, 165). An additional effect during acute exposure may be of cardiotoxic nature, manifesting itself as ventricular fibrillation, presumably due to sensitization of the myocardium to endogenous catecholamines. This effect has been reported both from animal experimentation (289) and from human occupational exposure (51). The incidence of acute toxicity of benzene is of course low in the occupational settings of today, but may be fatal in stressful situations.

The symptoms of acute and chronic benzene toxicity are completely different. Benzene itself is supposed to cause the acute effects whereas metabolites of benzene are considered as etiologic factors in chronic benzene toxicity. The chronic effects of benzene exposure have been the subject of innumerable both animal and human studies ever since the turn of the century when insidious injuries to the blood-forming tissues by benzene were recognized by Santesson (360) and Selling (387). A comprehensive review of the early cases and aspects of chronic benzene poisoning may be found in the work by Browning from 1965 (51), recently brought up to date by Goldstein (154) and Snyder and Kocsis (405). In spite of the numerous investigations, the mechanisms and pathogenesis of benzene induced injury to blood-forming tissues still remain obscure and nothing definite or conclusive can be said concerning the course and results of chronic benzene exposure.

The early results from studies of benzene hematotoxicity (360, 387), indicating a bone marrow depressant effect, have been confirmed by several later investigations of chronic

occupational benzene exposure. The most serious effect of such exposure is thus bone marrow aplasia with pancytopenia (51). During the initial stages the peripheral blood picture may show paradoxical alterations with decrease or increase of erythrocytes, leukocytes or thrombocytes (405), but as exposure continues cytopenia becomes obvious, sometimes beginning as unicellular but ending as an often irreversible and even fatal pancytopenia (154, 405). This disease is often described as aplastic anemia, denoting a combination of pancytopenia with an aplastic bone marrow. However, in cases where bone marrow smears have been examined together with peripheral blood samples, benzene induced pancytopenia has been found combined with both aplasia (9, 10, 261) and hyperplasia (9, 10, 18, 261) of the bone marrow. Pancytopenia with bone marrow hyperplasia may thus have been an overlooked phenomenon in studies of benzene hematotoxicity where the bone marrow has not been properly examined. This seemingly contradictory combination has been interpreted as a sign of preleukemia or true leukemia (405). The possible role of benzene in the induction of leukemia has been discussed both in early (51) and more recent studies (11, 12, 194, 465). It is now generally agreed that heavy benzene exposure may cause acute myeloblastic leukemia and perhaps also other both acute and chronic leukemias (154, 357). There are no cases reported in which leukemia began in the absence of previous pancytopenia and the onset of leukemia is often delayed considerably after cessation of benzene exposure (154).

The art and incidence of human benzene toxicity in relation to dose are difficult to determine due to non-existing

or insufficient knowledge of the exposure conditions. Much work has gone into the search of a suitable animal model for benzene hematotoxicity. In spite of the variety of animals and routes of administration used, well summarized by Leong (250), it has been concluded that there is quite a good correlation between dose, time of exposure and degree of toxicity (405). Leukopenias and other cytopenias are consistent findings in animal studies, regardless of species or route of administration. The use of animals in investigations of benzene hematotoxicity has been questioned repeatedly, since the animal blood picture may differ much from the human in the pre-aplastic stage (407). Although the animal bone marrow may be aplastic or hyperplastic (43, 279, 387), there has been a constant failure to reproduce the only animal study showing benzene leukemia (251) with the exception of some recently published preliminary results showing a low incidence of leukemias in chronically exposed mice and rats (404).

Although the morphological characteristics of chronic benzene hematotoxicity are relatively well known, the number of studies dealing with the mechanisms and molecular site of benzene toxicity are few. Several experiments indicate an effect of benzene on the early stages of hematopoiesis. The study by Lee *et al.* (245), measuring the appearance of  $^{59}\text{Fe}$  in circulating erythrocytes after a single subcutaneous injection of benzene to mice at different times prior to  $^{59}\text{Fe}$ -injection, indicates a selective damage to pronormoblasts and normoblasts. The investigations by Moeschlin and Speck (279) and Kissling and Speck (221) provide both a molecular site and cellular level of benzene toxicity in showing a decreased

uptake of  $^3\text{H}$ -thymidine into the DNA and a decreased uptake of  $^3\text{H}$ -thymidine and  $^3\text{H}$ -cytidine into the DNA and RNA of basophilic and polychromatophilic normoblasts and promyelocytes in the bone marrow of rabbits with benzene induced pancytopenia. Benzene was thus considered to inhibit nucleic acid synthesis in early hematopoietic cells. Decreased DNA synthesis is also displayed by cultured human leukocytes and HeLa cells incubated with  $2.2 \cdot 10^{-3}$  M benzene (230). Benzene administered subcutaneously to rats at a daily dosage of 1.0 ml/kg for 14 days caused a decrease in femoral marrow nucleic acid (144). Of great interest in the evaluation of benzene as a leukemogen and carcinogen is the ability of benzene to cause chromosomal aberrations, since these are associated with mutagenic effects which may develop into neoplasia (407). Several studies present statistically significant increases in both structural and numerical chromosome alterations in bone marrow or peripheral blood cells of occupationally exposed workers (132, 151, 217, 445). Chromosomal aberrations have also been induced by benzene in cultured human leukocytes (230), in rabbit bone marrow cells (220, 221) and in rat bone marrow cells after a single 2.0 ml/kg benzene injection (315). As discussed below, the hematological effects of benzene are believed to be caused by a benzene metabolite, presumably benzene oxide, which could become covalently bound to critical tissue macromolecules. Definite evidence for the formation of benzene oxide *in vivo* is, as yet, lacking, but irreversible binding of radioactivity from radio-labeled benzene to rat liver DNA *in vivo* has recently been demonstrated (259). Snyder *et al.* (406) have also shown irreversible binding of radioactivity in liver, brain, kidney,

spleen and fat in mice after subcutaneous injection of 2 ml/kg <sup>3</sup>H-benzene. It has also been suggested that benzene hemato-toxicity may in part be due to immunological effects of benzene (250, 405). Moreover, the combination of genetic host factors with chronic benzene exposure has been stressed in the etiology of leukemia (154).

Hematotoxicity is a unique effect of benzene, not shared by its simple alkyl derivatives (144, 475). Compared to the number of investigations devoted to the hematological effects of benzene, little attention has been paid to other benzene induced changes in the organism. Both acute and subacute exposures of experimental animals to benzene have resulted in histochemical changes in the spinal chord (209), the endocrine glands (189), the small intestine epithelium (208), the lung (418), the kidney (206, 207, 213) and the liver (203). The histochemical liver and kidney changes have also been related to ultrastructural alterations (204, 205, 210). Benzene is thus considered to disturb intracellular respiration and oxidative phosphorylation in the liver and kidney as evidenced by a decrease of mitochondrial enzyme activities in combination with a reduced number of mitochondria and mitochondrial degenerative changes and swelling. In addition there was a focal hypertrophy of the smooth endoplasmic reticulum (SER), the site of benzene biotransformation. The rough endoplasmic reticulum (RER) was poorly developed in both kidney and liver with an irregular distribution of ribosomes which could be associated with decreased protein synthesis as described by Tryfiates for rat liver (449). Furthermore, the liver and kidney cell nuclei show segregation, which is indicative of a disturbed RNA production,

also shown to occur in bone marrow cells (221). Hypertrophy of the liver SER together with loss of RER has also been reported by Saito *et al.* (358) after 14 days administration of 1.1 g/kg benzene daily to rats. This was taken as a sign of liver injury. Benzene is not considered a serious hepatotoxic agent although early reports describe centrilobular liver necrosis in animals treated with benzene (51). Centrilobular liver necrosis has also been found in cases of chronic human benzene poisoning (18, 261) but has been interpreted as secondary to hemato-poietic injury (51). However, the studies by Jonek *et al.* (203, 205) showing enzymatic and ultrastructural liver changes as well as the study by Wirtschafter and Cronyn (473) reporting signs of cytoplasmic degeneration and leakage of hepatocellular enzymes into serum after a single subcutaneous benzene injection, are suggestive of benzene hepatotoxicity. Noteworthy are also the recent reports of irreversible binding of a benzene metabolite to mouse liver (406) and to rat liver DNA (259) although this was not discussed as being connected with hepatic injury.

#### ABSORPTION, DISTRIBUTION, BIOTRANSFORMATION AND ELIMINATION OF BENZENE

Benzene exerts a high vapor pressure at room temperature (Appendix 1) and occupational contact with benzene takes place almost exclusively by inhalation. Benzene is rapidly absorbed not only by inhalation but also by ingestion and injection (146). The amount of benzene reported as retained by animals and humans during inhalation varies considerably. In men exposed to an atmosphere of 0.34 mg benzene/l for 5 hours

about 46% of the benzene inhaled is retained (51). Srbová *et al.* (416) reported a retention of 52-70% in humans inhaling 47-100 ppm for 2 hours, Nomiyama and Nomiyama (294) an average retention of 30.2% at inhalation of 52-62 ppm for 4 hours. In humans, values up to 80% have been reported (416). Dogs retain 61-80% when exposed to 0.4-0.6 µg benzene/ml (116). Absorption is very rapid initially, but equilibrium between the concentration in the inspired air and in the blood is attained slowly. Schrenk *et al.* (373), exposing dogs to 500 or 900 ppm continuously, found equilibrium after 4 days and a linear relationship between the concentration in the inspired air and the equilibrium blood concentration. After cessation of exposure, a considerable amount of the retained benzene is eliminated unchanged in the expiratory air. Elimination is very rapid during the first hours but has been stated to require several days to be complete (373). The amount of benzene exhaled varies from 16.4 to 41.6% in 5 to 7 hours in the investigation by Srbová *et al.* (416). As little as 12% of the benzene retained by humans has been found in the expiratory air (51, 188). Rabbits given 0.25-1 g benzene/kg orally exhaled 34.0-64.3% of the dose unchanged in 16-70 hours, most of it during the first 12 hours (307, 310). Of 880 mg/kg benzene administered subcutaneously to mice, 71.5% was exhaled and the exhalation was complete in 4-6 hours (20). The time course of elimination of benzene in the expired air by humans has been subject to mathematical treatment by Nomiyama and Nomiyama (295), who were able to fit their experimental data to a three-compartment pharmacokinetic model describing the initial very rapid exhalation of benzene followed by a slow second phase and an

even slower third phase. A similar three-compartment model was constructed by Sato *et al.* (363) for benzene in exhaled air and blood of men after inhalation of 25 ppm for 2 hours. The three-exponential decay curves were taken to reflect the transfer of benzene between tissue groups in relation to their blood supply and fat content, stressing the importance of fatty tissues in causing the last very slow part of the benzene elimination curve.

Distribution studies of benzene in animals are few. Fabre (120) administered benzene to rabbits by inhalation of 1 mg/l for 185 days 6 h/day or 5 mg/l for 15 days 6 h/day. The animals were killed on the 4th or the 5th day, respectively, after the last exposure. The highest concentrations of benzene were found (in decreasing order) in the fur, spleen, cerebellum, bone marrow and in the fur, adrenals, brain and spleen, respectively. Body fat was not examined. The most extensive distribution study found in the literature is the study by Schrenk *et al.* (373) who exposed dogs to varying concentrations of benzene (250-1320 ppm) continuously or during 2-8 hours a day for up to 278 days. Due to the varying exposure conditions, the data are difficult to judge, but a constant finding was the large amount of benzene present in the bone marrow and in body fat. Parke and Williams (310), using  $^{14}\text{C}$ -benzene, distinguished between the part of the radioactivity present as benzene or benzene metabolites in various organs of rabbits after administration of 0.34-0.4 g/kg orally. After 2 days the highest level of radioactivity was found in involuntary muscle (9.3% of the dose) and fat (2.6% of the dose), all in the form of metabolites. Some benzene was found in the blood.

After 7 days the amount of radioactivity remaining in the organs was negligible. The recent distribution study by Andrews *et al.* (20) also compares the distribution of benzene versus its metabolites. Mice were given 880 mg/kg  $^3\text{H}$ -benzene subcutaneously and the concentration of benzene (estimated as toluene-extractable radioactivity) and benzene metabolites (estimated as water-soluble radioactivity) were followed with time in the fat, liver, spleen, blood and bone marrow. Benzene was rapidly absorbed and reached its maximal concentration values in the tissues within 3 hours. The uptake in fat was slowest but reached the highest values. High levels of benzene were also found in the bone marrow. Maximum levels of benzene metabolites were found in the bone marrow with hepatic levels considerably lower. Metabolites were also found in fat. Fatal cases of human benzene poisoning showed high brain and fat concentrations (181, 472).

The oxidation of benzene to phenol, excreted in the urine, in the dog and in man was first observed in 1867 (374). Our knowledge today of benzene biotransformation (Fig. II:1), reviewed by Snyder and Kocsis (405) and Rusch *et al.* (355), originates from the work by Williams and co-workers, initiated in 1949. Rabbits dosed orally with benzene excreted sulfate and glucuronic acid conjugates of phenol, catechol, quinol and hydroxyquinol in the urine (323, 324). Later investigations showed the excretion in urine of about 1% of an 0.5-1 g/kg oral dose of benzene to rabbits as phenylmercapturic acid (308) and scission of the aromatic ring to trans-trans-muconic acid accounting for about 0.5% of an oral 0.5 g/kg dose to rabbits (309). Studies with  $^{14}\text{C}$ -labeled benzene (310, 311)

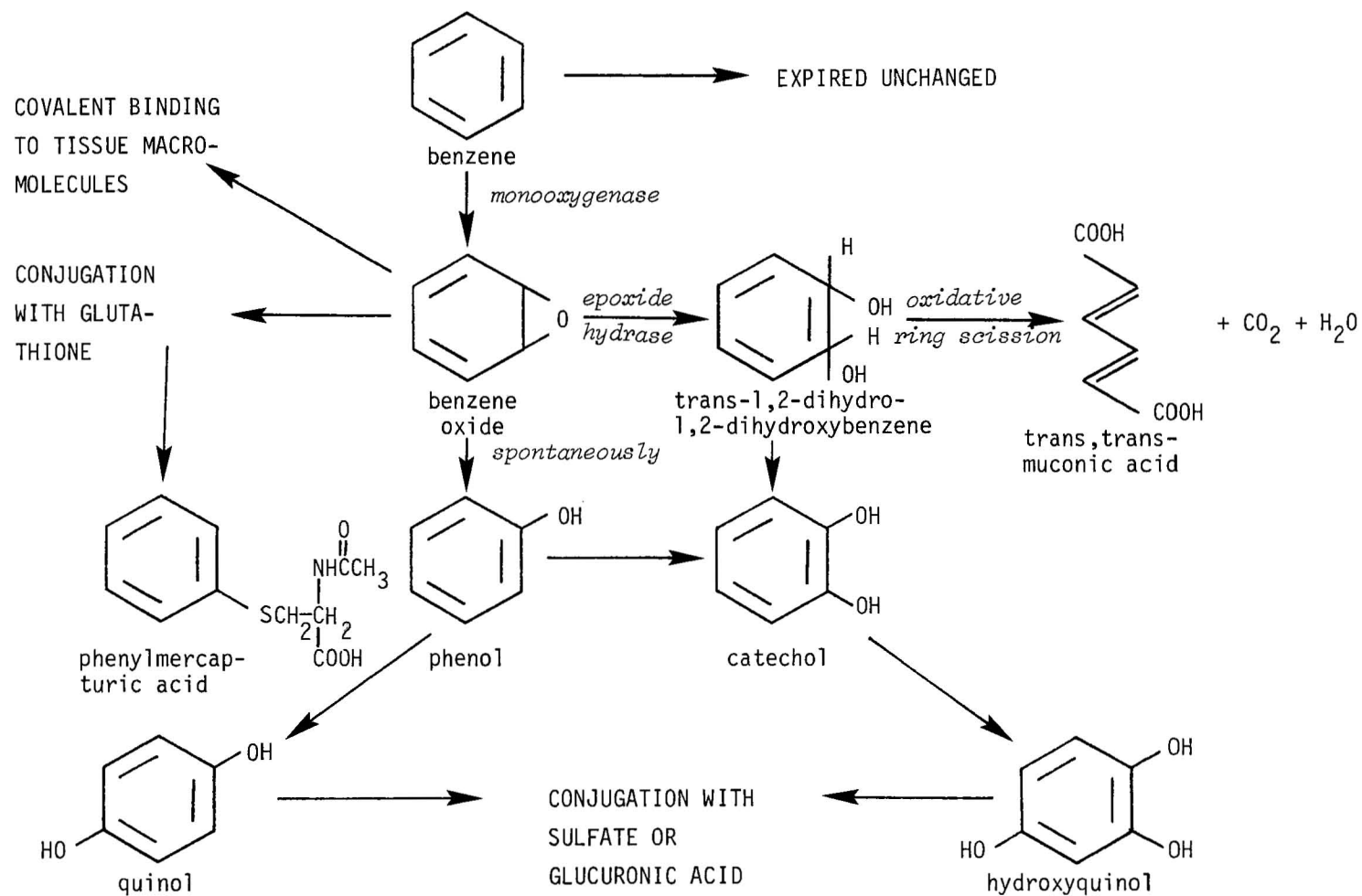


Fig.II:1. The biotransformation of benzene.

confirmed the earlier results. In 2-3 days 80-90% of an oral dose of 0.34-0.5 g/kg  $^{14}\text{C}$ -benzene to rabbits could be recovered as follows. About 45% of the dose was eliminated in the expired air, 43% as unchanged benzene and 1.5% as  $^{14}\text{CO}_2$ . The  $\text{CO}_2$  excretion began about 20 hours after dosing. The urine contained about 35% of the administered radioactivity, 23% as phenol, 4.8% as quinol, 2.2% as catechol and 0.3% as hydroxyquinol. These phenolic metabolites were conjugated with sulfate or glucuronic acid. 0.5% of the urinary radioactivity could be accounted for as phenylmercapturic acid and 1.3% as trans-trans-muconic acid. 5-10% of the administered dose remained in the tissues. In an investigation by Abou-el-makarem *et al.* (2), 0.8% of an intraperitoneal dose of 50 mg/kg  $^{14}\text{C}$ -benzene to rats was excreted in the bile.

Benzene biotransformation has also been studied in several other animal species and to a lesser extent in humans (355, 405). The biotransformation seems to result qualitatively in the same products as described for rabbits. The major differences do not lie in the metabolites formed but in the extent of the different conjugation pathways (355).

The specific pathways of benzene biotransformation have also been elucidated by Williams and co-workers. Their suggestion that the hydroxylated phenols all are products of further phenol oxidation (324) was rejected on basis of experiments where phenol (141, 311), catechol (139), and quinol (140) were administered to rabbits. Only catechol gave excretion of hydroxyquinol in the urine, whereas phenol was oxidized mainly to quinol which was not hydroxylated further.

Direct administration of quinol gave only its conjugates in the urine. Catechol was formed in a small amount after administration of phenol, which did not explain the relatively large amount of catechol formed from benzene. Later experiments by Sato *et al.* (362) suggest that an additional step may be involved in benzene biotransformation. Urine of rabbits treated with benzene contained trans-1,2-dihydro-1,2-dihydroxybenzene. This substance, given to rabbits, gave rise to catechol in the urine (200). It may therefore be concluded that catechol is formed from the dihydrodiol directly but perhaps also via oxidation of phenol. The dihydrodiol is probably also the precursor of trans-trans-muconic acid (311). Another step in benzene biotransformation has been postulated to be the initial formation of an epoxide. Incubation of liver homogenates with benzene oxide gives the metabolites found *in vivo* (201). It is thus suggested that benzene is first oxidized to benzene oxide which may subsequently be transformed in several ways;

- 1/ spontaneous rearrangement to phenol
- 2/ reaction with glutathione
- 3/ enzymatic hydration to a dihydrodiol.

A fourth possible reaction may involve the covalent binding of the electrophilic benzene oxide to nucleophilic sites in tissue macromolecules, recently postulated to occur in rat liver DNA (259).

The liver appears to be the most active organ in benzene biotransformation (355). Results from studies of benzene biotransformation *in vitro* (156, 408) strongly support the hypothesis that the NADPH dependent mixed function oxidase system in liver microsomes is responsible for the conversion of benzene to phenolic metabolites. Phenols are known to be

mitotic poisons and after the demonstration by Williams and co-workers that benzene is metabolized to various phenols, these were supposed to cause benzene hematotoxicity (108). Nomiya (293) administered the phenols known to be products of benzene biotransformation to rats and found leukopenia after catechol only. In contrast, Mitchell (277) administering phenol, catechol and quinol in high doses to rats found no hematopoietic toxicity. Yet, rats receiving benzene developed aplastic anemia. A link between the biotransformation of benzene and benzene bone marrow toxicity has been sought in experiments where benzene biotransformation was stimulated or inhibited. The results so far are conflicting. Treatment with phenobarbital, a stimulator of liver microsomal benzene biotransformation (408), protected against benzene hematotoxicity in rats (192, 277). Various treatments such as administration of 3-amino-1,2,4-triazole (292), piperonyl butoxide (277) or partial hepatectomy (277), all shown to inhibit or decrease benzene biotransformation, also protected against benzene induced cytopenias in animals. However, as pointed out by Snyder *et al.* (407), these treatments all influence benzene biotransformation in the liver, whereas biotransformation in the critical target organ, the bone marrow, may not be changed. The bone marrow has been shown to possess mixed function oxidase activity (407). Andrews *et al.* (20) found a high concentration of benzene metabolites in the bone marrow shown in a later investigation by Snyder *et al.* (406) to be irreversibly bound to a small extent. Benzene metabolites administered directly to mice did not concentrate in the bone marrow. Using toluene, a competitive inhibitor of benzene bio-

transformation, Andrews *et al.* (20) also found a reduction of the concentration of benzene metabolites in the bone marrow as well as a reduced hematotoxicity, as evidenced by the  $^{59}\text{Fe}$ -incorporation technique, when benzene and toluene were given simultaneously to mice. The bone marrow level of benzene was not changed by co-administration of toluene. They concluded that benzene is metabolized *in situ* in the bone marrow and that an active toxic metabolite rather than benzene itself is responsible for benzene hematotoxicity. The structure of this metabolite is not known, but benzene oxide, formed initially during benzene biotransformation, is considered as a most probable candidate.

## TOLUENE

Toluene (methylbenzene),  $\text{C}_6\text{H}_5\text{CH}_3$ , is a clear, colorless and flammable liquid. Due to the addition of a methyl group to the aromatic nucleus, toluene is more fat soluble and less volatile than benzene (Appendices 1 and 2) but exerts a high enough vapor pressure at room temperature to make inhalation the most probable mode of contact (51, 146). Toluene is extensively used as a solvent for fats, resins, paints, varnishes, plastics and similar materials. Toluene is also a constituent of motor and aviation fuels and an important starting material in the chemical industry. The U.S. production of toluene in 1977 was about 3.5 million tons (432).