CURRENT CONCEPTS OF CHRONIC BENZENE TOXICITY

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INTRODUCTION

The maladies that have beset mankind throughout history may in general be classified as parasitic (infections, infestations, etc.), nutritional/endocrine disease (hyper. or hypofunctional endocrine organs, etc.), or traumatic injury (accidents, war, etc.). With the development of an industrial society, new forms of disease arose in which people succumbed to illness induced by exposure to toxic materials in the course of their labor. Occupational diseases have been with us for many centuries.'[1,2] but the incidence of industrial toxicity increased markedly with the advent of the industrial revolution. Although efforts to protect workers against jobrelated illness have been vigorous in recent years, economic necessity compels the continued use of hazardous chemicals, and benzene is an excellent case in point. It has been estimated by the National Institute for Occupational Safety and Health that about 2,000 000 perrons in the national work force have potential exposure to benzene.

In 1922 Alice Hamilton,' the noted student of industrial toxicology, reported on "The Growing Menace of Benzene (Benzol) Poisoning in American Industry." She was familiar with the descriptions of benzene toxicity reported by Santesson[4] rnd Selling,[5,6] and she reviewed the incidence of both acute and chronic toxicity with the intention of alerting the medical profession and industry to the hazards of benzene. During the next several years, steps were taken to restrict benzene utilization, and in 1928 it was possible for her to write a follow-up paper, "The Lessening Menace of Benzol Poisoning in American Industry.'" Despite the optimism generated by the title, she reported that benzene utilization remained heavy in industries concerned with the manufacture of rubber, artificial leather, cans, paints, certain artificial furs. and in dry cleaning. With the advent of high-speed printing presses in the 1930s and the consequent need for fast-drying inks. benzene was again pressed into service because it was an excellent solvent for ink and it evaporated rapidly. Thus, despite attempts to decrease overall utilization of benzene both in this country and abroad, by the middle 1930s there remained. unfortunately, an abundance of clinical material in which to studv benzene toxicity.

The development of the chemical industry during World War II and in the postwar era. especially in the field of plastics, firmly reestablished the necessity for large quantities of benzene

as the starting material for chemical syntheses. Benzene is now consumed by the chemical industry in this country at an annual rate of about 1.4 billion gallons, a figure which is expected to increase when additional facilities for manufacturing benzene become available.[8] Furthermore, additional uses for benzene continue to be found, e.g., currently gasoline companies plan to use benzene (among other chemicals) to replace lead as an antiknock component in motor fuels. Thus, the extensive use of benzene continues despite the appreciation that benzene is among the most serious toxic hazards that confront workers in the chemical industries.

The American Conference of Governmental Industrial Hygienists[9] has recommended that workers not be exposed to atmospheric levels of benzene exceeding 25 ppm, while the National Institute of Occupational Safety and Health suggests that exposure should not exceed 10 ppm as a time-weighted average for a 10-hr day or 40-hr work week with no exposure exceeding 25 ppm.[10] Sherwood[11] has suggested that an alternative approach would be to restrict exposure to a total of 5,000 ppm-min per day. These exposure limits are essentially in agreement since time-weighted averages are approximately one third of the maximum values. Thus, an MAC of 25 ppm ≃ TLV of 10 ppm ≃ 5,000 ppm-min/day. Despite these regulations and conscientious attempts to monitor exposure and to evaluate the effects on workers using modern techniques,[12-15] the hazard will probably remain with us as long as the economy demands that vast quantities of benzene must be produced, transported, and utilized. The consequence to the medical community is that we must continue our efforts to gain a complete understanding of benzene toxicity if we are to learn how to prevent or to deal with the illness resulting from exposure to benzene.

The most serious effect of chronic exposure to benzene is depression of the b[illegible]row.[16] The effect is unique among aromatic hydrocarbons and is not shared by simple alkyl derivatives of benzene.[17] Acute exposure to high concentrations of benzene in the atmosphere has led to loa of consciousness since benzene ;Jong with many other hydrocarbons exerts a depressant effect on the central nervous system that appears to be related to its thermodynamic activity in aqueous solution.[18] Both acute and subacute exposure to benzene has resulted in histochemical changes in kidney,[19-21] liver,[22-24] small intestine,[25] and spinal cord.[26] The heart is also affected,[27] and it has been reported that individuals performing strenuous physical labor are especially susceptible to benzene toxicity. Hamilton[28] describes a situation in which a person entered a tank previously used to store benzene, was overcome by the fumes, and lost consciousness. A co-worker who entered the tank without respiratory protection was himself overcome while attempting to rescue the unconscious person and died. The first victim was subsequently removed from the tank by adequately protected personnel, regained consciousness, and survived. Browning[16] summarized the effects on the heart by indicating that during stressful situations excessive catecholamines are produced by the adrenal and that benzene like some other general anesthetic agents such as cyclopropane and the halogenated anesthetics sensitizes the heart to catecholamines.[29] The result may be ventricular fibrillation followed by death. Thus, it may be surmised that the first worker lost consciousness due to the central nervous system depressant activity of benzene and regained consciousness when removed from the benzene-rich atmosphere whereas his would-be rescuer succumbed to the cardiotoxic activity of benzene while struggling to save his companion.

The incidence of acute toxicity is iou hut in stressful situations may be fatal. The symptoms of acute and chronic benzene toxicity are different, and while benzene itself appears to cause acute toxicity, considerable study has gone into evaluating the role of brnzene metabolites as etiologic factors in chronic toxicity. Therefore, because acute benzene toxicity is of sufficient interest and complexity to generate a separate discussion, this review will concentrate on chronic benzene toxicity.

The opening section will describe chronic benzene toxicity in humans and will be followed by a discussion of reports on the mechanism of benzene toxicity derived largely from animal studiec. The discussion will continue with a review of current thought on whether benzene toxicity may lead tu leukemia and on immunological effects of benzene. The next two sections deal with benzene metabolism in vivo and in vitro because there is a strong possibility that a metabolite of benzene rather than benzene itself is responsible for benzene toxicity. The last section reviews studies

specifically aimed at attempting to relate benzene metabolism to benzene toxicity.

CHRONIC BENZENE TOXICITY IN HUMANS

The earliest reports of benzene toxicity[4-6] describe the development of aplastic anemia as a consequence of chronic exposure to benzene. Despite many years of study, our understanding of the effects of benzene on bone marrow is limited in part because the structure and function of the bone marrow have not been well understood and in part because the concept of aplastic anemia has undergone evolution since the first descriptions of benzene toxicity.[5,30-34]

In normal adults blood cell formation occurs in the marrow of the central bones and the proximal ends of the femur and humerus.[34] The total volume of the marrow cavity equals about 30 to 50 ml/kg body weight of which about half contains active marrow. The cells produced in this organ include not only erythrocytes, leukocytes, and thrombocytes but also some types of lymphocyte.[35] In adults the marrow usually has sufficient reserve capacity to obviate the necessity for extramedullary hematopoiesis.

Early studies of benzene toxicity suggested that benzene caused aplastic anemia resulting in pancytopenia.[1] Subsequently pancytopenia was equated with aplastic anemia. Later investigation of bone marrow smears in benzene toxicity revealed cases in which the marrow appeared to be hyperplastic despite pancytopenia of the circulating blood.[6,37] If the current definition of aplastic anemia, i.e., pancytopenia accompanied by fatty displacement of bone marrow,[33] is applied, then pancytopenia accompanied by hyperplastic bone marrow is not aplastic anemia and may be a sign of the preleukemic state or of true leukemia. The possible role of benzene in the induction of leukemia will be discussed later in this review.

In pancytopenia characterized by a hypoplastic bone marrow sternum,[6,36b,38-41] ribs and vertebrae display marked displacement of red marrow by fat, and fibrotic areas in which fit has been replaced by collagen have been described. In some areas ineffective attempts it intramedullary compensation are exemplified by pockets of nucleated red cells. while other pockets contain phagocytes laden with hemosiderin. Eosinophilic urd neutrophilic granulocytes may be present in numbers equal to or less than those of erythroid cells. Megakaryocytes are largely absent. All of these observations coincide with the finding that throughout the marrow mitotic figures are infrequent. These observations made either in the late stages of benzene toxicity or during post-mortem examination provide a reasonable basis for the pancytopenia of benzene toxicity.

Although various stapes of increasing severity during the progression of benzene toxicity in man have been observed in different individuals. the complete course of the disease cannot be experimentally investigated in man because it eventually reaches a stage which is irreversible. Indeed. most cases of benzene poisoning have not voluntarily presented themselves to physicians until the disease reached life-threatening severity. Outwardly the typical picture of an individual in the late stages of benzene toxicity is one of general debilitation displaying purpura and bleeding from mucuous membranes.[39] Helmer[42] reported that among 60 cases of chronic benzene poisoning in various stages, 72% complained of headache, 88% of tiredness, and 44% of cutaneous hemorrhages. The detection and description of the early stages of benzene toxicity have been a less frequent Occurrence because in the absence of routine screening programs at places of employment individuals may not appreciate that thev are victims of benzene poisoning until the disease has become severe. Most of the reported cases of the earlier stages of benzene toxicity were detected when a large group of workers were screened as part of a study subsequent to reports of severe toxicity due to benzene exposure in a specific industry,[3,7,42-46] whereas other reports were more concerned with later, more serious[41-50] examples of the disease. The reconstruction of the progress of the disease comes from synthesis of the observations made on people at various stages of toxicity supplemented with inferences taken from animal studies. In its initial stages benzene toxicity is manifested as a paradoxical alteration of the blood picture. Polycythemia and anemia, leucocytosis and leucopenia, thrombocytosis and thrombocytopenia have all been reported in the same studies. With continued exposure. however, the trend is toward decreased levels of circulating erythrocytes, leucocytes. and thrombocytes. As the disease intensifies, circulating blood cell levels decrease further as pancytopenia develops.

In man decreases in red cell levels are a frequent indicator of early chronic benzene poisoning. Among 89 individuals studied by Hunter,[44] 159 studied by Goldwater,[45] and 60 studied by Helmer,[42] 37, 48, and 97%, respectively, displayed erythrocyte levels below 4.5 million/mm^3. The anemia is described as macrocytic and hyperchromic. The former is a frequent consequence of bone marrow depression, and the latter appears to be related to the fact that while there is a decrease in total hemoglobin concentration the reduction in red cell numbers is much greater, the net effect being hyperchromicity.[16,39,45,50-52]

Because benzene toxicity has been intensively studied through most of this century, the report of a sign or symptom not previously described is rare. Thus, it was with considerable interest that investigators in this field read the report of Aksoy et al.,[41] who demonstrated that workers in Turkey exposed to benzene and suffering from pancytopenia displayed increased levels of fetal hemoglobin (HbF) in their blood. HbF is a prominent hemoglobin variant normally found in the fetus and at birth constitutes between 50 and 65% of total hemoglobin.[53] HbF decreases in early life to below 1% at the age of 10 and is not usually observed in normal adult blood. It is usually elevated in the thalassemias and is often associated with elevated levels of HbA_2, another hemoglobin variant in these diseases.[54,55] It is important to recall that thalassemia may be manifested from childhood as a serious anemia called thalassemia major (Cooley's Anemia) or may occur asymptomatically in carriers for the disease, in which case it is called thalassemia minor. An intermediate form of the disease is also known.

Shahidi et al.[56] and Aksoy and Secer[57] had previously demonstrated that HbF is frequently observed in patients suffering from aplastic anemia, and Bloom and Diamond[58] showed that among a large group of children in Boston suffering from aplastic anemia with elevated HbF levels, those children in whom the HbF was present in excess of 400 mg% survived while those with less HbF all died. Aksoy et al.[41] reported that among 24 patients with chronic benzene toxicity and pancytopenia, 8 had HbF levels above 400 mg% and the remainder were below that level. All patients with the lower level survived, suggesting to Aksoy et al.[41] that the HbF concentration in blood was not necessarily prognostic for recovery from aplastic anemia induced by benzene.

The significance of the relative values of HbF levels in predicting survival during aplastic anemia may lie in the different populations that were studied. Thalassemia is a disease endemic to Turkey, whereas it is relatively rare in Boston. Although Aksoy et al.[41] showed that HbA_2 levels were normal in 21 out of 24 patients, suggesting that they were not thalassemic, it has been reported that elevated HbA_2 is not always seen in thalassemia.[54] Furthermore, the possible role of benzene in modifying HbA_2 levels has yet to be investigated. Therefore, it would be of interest to determine the various hemoglobin variants in a group of workers similarly exposed to benzene who show no signs of benzene toxicity or have not developed pancytopenia. It may be that thalassemia can either predispose or convey protection against benzene toxicity. Alternatively, benzene may either mask or exacerbate some of the signs of thalassemia. Further studies in this area would be welcomed by students of benzene toxicity.

Although there is some evidence for an increase in red cell hemolysis in benzene toxicity,[50,59] the anemia is most commonly thought to be the result of decreased red cell production. Detection of the earliest stapes of reduced hemopoiesis is not possible using cell counting techniques because of the long life span of the red cell. If the normal mechanisms for removal of aged or damaged cells from the circulation are operational, approximately 1/120 of the erythrocyte population is removed and must be replaced per day. It is likely that early in benzene toxicity erythrocyte production is reduced but not prevented and some portion of the red cells can be replaced despite benzene toxicity. The result is that the time necessary to detect a depression in erythrocyte counts may be quite long. Hunter[44] reported that by the time exposure to benzene caused anemia, depression of leukocytes and/or thrombocytes was apparent. Thus, the appearance of anemia may signify benzene toxicity of long standing.

In contrast, the shorter life span of the leukocyte and the fact that there cells tend to leave the bloodstream and not return suggest that detecting decreased white cell production should be simpler than detecting a change in red cells. The picture is complicated by the fact that the white cells are a complex mixture of cell types, some of which do

not arise in bone marrow. Leukopenia has been described as the earliest[6] and most frequent[4,8] sign of benzene toxicity. In a study of 200 women exposed to benzene in the course of their occupation, neutropenia was reported in 50 cases, a more generalized leukopenia in 44, and anemia *in* 41.[4,6] The finding of leukopenia in the absence of other bone marrow abnormalities, however, is relatively rare.[42,44,45] White cell values have been reported to drop from levels of 5,000 to 10,000 cells/mm^3 to below 1,000 in benzene toxicity.[7,7] Despite the general agreement that benzene toxicity results in leukopenia, effective screening for benzene toxicity should not be restricted to determination of leucocyte levels only.

Often individuals who suffered from benzene toxicity did not approach a physician until they began to hemorrhage. Both Santesson[4] and Selling[5,6] described excessive bleeding in benzene toxicity, but Duke[11] was the first to describe the reduction in thrombocyte levels. Nikulina and Titowa[61] suggested that thrombocytopenia may be among the earliest signs of benzolism, and Goldwater[45] showed that platelet counts were depressed in over 30% of his rotogravure workers exposed to benzene. The observations by Mitnik and Genkin[48] and Brocher[47] that platelet levels may drop from about 250,000 cells/mm^3 to below 10,000 are an indication of the severity of thrombocytopenia in advanced benzene toxicity. In addition to decreased numbers of platelets, Saita and Sbertoli[62] reported that the ability of platelets to aggregate was depressed by benzene. Saita et al.[63] suggested that there may be several defects in clotting but that both decreased levels of platelets and a decrease in thromboplastic factor activity were important. Thus, in benzene toxicity clotting may be disturbed both because of morphological and functional damage.

The time required for these events to occur varies greatly among individuals. Some workers have been known to show signs of benzene toxicity after brief exposure to relatively low concentrations, whereas many display resistance for long periods of time. Factors such as genetic differences, conditions of exposure in the work place, nutrition, and the general state of health probably all play a role. In man, however, chronic benzene exposure leads to a progressive disease in which bone marrow function becomes increasingly depressed, as demonstrated by progressive decreases in circulating blood cell levels, until it ceases to function in the production of normal blood cells.

ANIMAL STUDIES ON CHRONIC BENZENE TOXICITY

The early reports of Santesson[4] and Selling[6] of benzene toxiaty in humans were accompanied by descriptions of experimentally induced benzene toxicity in animals. The observations of pancytopenia and bone marrow depression in animals, similar to those in man following chronic exposure to benzene, suggested that animal models would be useful in the study of the mechanism of benzene toxicity. In animals the evidence suggests that barring cases of hypersensitivity there is in all likelihood a relationship between dose, time of exposure, and degree of toxicity in various species.[64] Four reports can be chosen to exemplify this relationship. Latta and Davies[65] administered benzene to rats subcutaneously at doses of 2 *to* 4 ml/kg/day, whereas Deichmann et al.[66] exposed rats to benzene in the atmosphere and observed toxic effects in the range of 65 to 831 ppm. Selling[6] gave rabbits benzene subcutaneously at a dose of 1 ml/kg, while Weiskotten et al.[67] exposed rabbits to atmospheric benzene at a level which we have calculated to be 240 ppm.* Although an initial transitory leucocytosis was frequently observed, the eventual result in each case regardless of the species or the route of administration was leucopenia. At lower doses more time was required: but the eventual result was the same. The predominant effect was neutro-

*The limiting concentration of benzene (Q(t)) in the exposure chamber was calculated from a first order differential equation:

$dQ/dt = -pQ(t) + Q$

where P = outflow rate and Q = inflow rate. The general solution of the equation is of the form:

$Q(t) = e^{-\int p dx}\left(\int Q e^{\int p dx}\, dx + c\right)$

using the data supplied in Weiskotten et al.

penia accompanied by an apparent lymphocytosis which gradually disappeared as benzene attacked lymphoid tissue. Latta and Davies[65] reported that lymphatic tissue was more sensitive to benzene than myeloid tissue in rats, while Selling[6] reported that the opposite was true in rabbits. The neutropenia is characterized by a shift to the left in the Arneth count, which suggests that leucocyte maturation is Impaired. The leucopenia can occur rapidly, and cell counts may reach extremely low levels prior to death.

The use of animal models to determine which cell type is most sensitive to benzene in order that monitoring of that cell type in man might be used to monitor benzene toxicity may in retrospect have been relatively unprofitable. In man arguments have been advanced to demonstrate that each of the cell types may be an early indicator of benzene exposure if its level in circulating blood decreases. In animals larger doses of benzene are usually given than those to which man is exposed in an attempt to condense the course of the disease in time. Because of the extremely long half-life of the red cell, it is difficult to observe changes in red cell counts during benzene exposure unless animals are exposed to low doses for long periods of time. Platelets are rather more difficult to measure and are not commonly used as indicators of benzene exposure. Therefore, leukocytes which suffer from neither drawback have usually been reported in animal studies as the first cell type to be depleted. It must be stressed, however, that no conclusive evidence exists to show that benzene preferentially depresses the production of any individual cell line in the bone marrow.

In an attempt to detect early effects of benzene on red cell production, we[68,69] devised a method for studying the effect of benzene on erythropoiesis using the incorporation of ^{59}Fe into hemoglobin of maturing red cells as a measure of red cell synthesis in the mouse. We found that after a single dose of benzene a reduction in the incorporation of ^{59}Fe into red cells occurred at a time when no inhibition of white cell production was apparent, based on cell counting techniques. Using this technique it was possible to show, in agreement with Steinberg[64] and Moeschlin and Speck,[70] that early stages in red cell maturation involving pronormoblasts and normoblasts were more sensitive to benzene toxicity than were stem cells, reticulocytes, or the process of hemoglobin synthesis. Thus, the effects of benzene on erythrocyte production occur quite early after exposure, but their detection requires methods more sensitive than counting the number of circulating cells.

The mechanism of benzene toxicity has been studied in bone marrow of experimental animals. The classical studies of Selling[6] included a description of rabbit bone marrow following chronic intoxication with subcutaneously administered benzene. The first signs of bone marrow damage were evident on the 2nd day of injections with 1 ml/kg of benzene, and by the 9th day aplasia was complete. All of the cell types were damaged and gradually disappeared during the course of treatment. Considerable evidence has been developed to support the thesis that benzene produces its effect by inhibiting mitosis. Fewer mitotic figures are observed in marrow of benzene-intoxicated animals, and abnormal mitotic figures have been described by Parmentier and Dustin[71] and Pollini et al.[72] Chromosome aberrations following benzene treatment or exposure have been reported by Kissling and Speck,[73,74] Forni et al.,[75] and Tough et al.[76] Although cellular damage which results in mitotic arrest is readily observed, the initial attack may well have been inflicted at any of several stages in the cell cycle. Thus, Moeschlin and Speck[71] and Kissling and Speck[73,74] treated rabbits with benzene subcutaneously and reported inhibition of incorporation of tritiated thymidine into DNA and tritiated cytidine into RNA, respectively. They claim that benzene inhibited the synthesis of both types of nucleic acids. Boje et al.[40] exposed rats to atmospheric benzene until signs of benzene toxicity were apparent in peripheral blood and also administered tritiated thymidine to determine the effects of benzene on the incorporation of thymidine into DNA. When they measured tritium uptake into nucleic acids radioautographically 1 hr after thymidine administration (i.e., the method used by Speck and coworkers[70,73,74]), they also found a decrease in the uptake of tritium. Their interpretation, however, was that the results may have been due to the effect of benzene on synthesis, degradation, reutilization, or pool size of thymidine. However, in the event that DNA synthesis was in fact inhibited as a result of exposure to benzene, they questioned whether this technique could distinguish between direct effects on nucleic acid synthesis during S phase and effects of benzene on other portions of the cell cycle, i.e., G_0, G_1, or G_2. They pointed out that alterations in the cell cycle

may account for the difference in uptake by altering factors which regulate the rate of cell proliferation. Thus. although there may well be decreased nucleic acid synthesis in bone marrow of benzene-treated animals. the damage caused by benzene may actually have occurred in any part of the cell cycle but was observed as an effect on nucleic acid production within the experimental design

BENZENE. LEUKEMIA AND CARCINOGENESIS

A cause and effect relationship between benzene and leukemia has been difficult to establish. Despite some negative reports,[35] it now seems fairly clear that chronic exposure to high concentrations of benzene may lead to one of several types of leukemia, the most prevalent of which is acute or subacute myeloblastic leukemia.[1,6,39,78] Chronic granulocytic, lymphatic. aleukemic, and erythroleukemic leukemia and Hodgkin's disease[16,39,78-84] have also been reported to result from chronic benzene toxicity, but there are fewer of these and in some cases they are not well documented. Vigliani and Forni[78] reported that in two studies in France and Italy involving a total of 77 fatalities resulting from chronic benzene poisoning, approximately half died from aplastic anemia and the remainder from leukemia. Leukemia frequently followed aplastic anemia. Saita[39] suggested that the incidence of benzene-induced leukemia may be greater than was previously recognized because many of the earlier diagnoses were made without studying bone marrow aspirates. Therefore, pancytopenia may have erroneously been equated with aplastic anemia when the marrow might have demonstrated that the disorder was in fact aleukemic leukemia, a form of leukemia characterized by leucopenia.

The suggestion[33,34] that hyperplastic bone marrow associated with benzene toxicity may indicate a preleukemic or leukemic state suggests the necessity of reevaluating previous descriptions of bone marrow in benzene toxicity. For example, the description by Mallory *et al.*[36] of hyperactive bone marrow in benzene intoxication closely parallels Saita's[39] description of the bone marrow in acute leukemia. The marrow displayed extreme proliferation of immature cell forms with numerous multipolar mitoses, and large multinucleated cells containing extreme vesiculations were observed. Saita[39] suggested that if the marrow is atrophic. leukemic metaplasia may be observed in the liver or spleen. and Mallory et al.[36] reported that the sinusoids contained large numbers of undifferentiated cells with many mitotic figures. Although they appeared to be primarily erythrogenic, some myeloid elements and occasionally apparently normal megakaryocytes were observed. Later in the disease they observed the progressive invasion of the pulp with many immature cells. In both marrow and spleen the authors noted a general similarity to the picture observed in Hodgkin's disease.

The Occurrence of acute leukemia, usually in the later stages of benzene toxicity subsequent to marrow aplasia, is characterized by high fevers, hemorrhages, serious rapidly developing anemia, infections, and ulcerations about the nose and mouth. Although red cells and platelets are usually severely depressed, white cell levels may vary considerably from below 5,000 to above 50,000/mm^3. As in the case of nonbenzene-induced acute leukemias, the disease is usually fatal.

The study of chromosomal abnormalities and their relationship to leukemia is currently undergoing intensive study. Kissling and Speck[73,74] observed chromosomal aberrations in bone marrow samples from rabbits, and they have been reported in both bone marrow and peripheral white cells of humans exposed to benzene.[76,78,85,86] Newell[87] suggests that abnormal marrow chromosomes indicate the presence of neoplastic cells and thus serve both as a means of identification of preleukemics or leukemics and may indeed be helpful in prognosis. He cautions, however, that people who have displayed abnormal clones in the marrow have not always developed neoplastic disease, while others who did not display chromosomal abnormalities were leukemic. Unfortunately for purposes of diagnosis, chromosomal abnormalities in the acute leukemias are relatively rare. Nevertheless, Vigliani and Forni[78] recommend cytogenic studies as a useful diagnostic tool in helping to identify benzene toxicity, which may or may not eventually develop into leukemia, provided that exposure to X-rays or other drugs likely to alter chromosomes is ruled out.

In view of the apparent role of benzene in inducing leukemia, an analysis of benzene as a carcinogen is appropriate. Empirical observations

have demonstrated that aromatic hydrocarbons can be classified according to whether or not they are carcinogenic. Using this classification the electronic structure of these compounds has been studied to determine quantitative parameters useful for predicting carcinogenicity. The work of Coulson[88] and Pullman and Pullman,[89] recently confirmed by Herndon,[90] suggests that carcinogenicity of aromatic hydrocarbons can be predicted from calculations of resonance energies of bonds in the K and L region on the basis of the localization theory of chemical reactions. When expressed in terms of chemical reactivity, the theory predicts that compounds in which the K region is chemically reactive but the L region is not (or is lacking) are carcinogenic. Sims and co-workers'[91-96] have reported that the activity of the K region resides largely in the ability to form epoxides which can be degraded by several pathways or can react with nucleic acids. Although benzene is thought to be converted to an epoxide.[97] the theoretical calculations predict that it is nonarcinogenic.

The prediction of carcinogenicity based on calculations of resonance energy is subject to error. Despite accurate predictions for such highly carcinogenic compounds as 3,4-benzo(a) pyrene and others and the accurate prediction of noncarcinogenicity of 20 known noncarcinogens, it was shown that I6 noncarcinogens were predicted to have arcinqenic activity while a number of other compounds known to be carcinogenic were not predicted by the theory.[97] The errors may be related lo secondary structural features of the compounds, the route by which they are metabolized, inaccuracy in measuring carcinogenicity, or inability to determine the specific type of cancer in animal models. The latter may be true in the case of benzene since it has not been possible to reproducibly demonstrate the formation of kukemia in animals after giving benzene.[16,98] Furthermore, benzene has not been shown to produce skin cancer or other types of tumors produced by polycyclic aromatic hydrocarbons which were used to develop the theory. The evidence described by Vigliani and Fomi[97] that links benzene to the production of leukemia suggests either that the theory does not predict for induction of leukemia or that benzene is an example of a case where the theory ern.

The study of the K region and K region epoxides, however, may prow to be useful in predicting how benzene (or its epoxide) may react with nucleic acids either tc depress bone marrow activity or to produce leukemia. In genera! two types of reactions of carcinogens with nucleic acids have been demonstrated.[99,100] Noncovalent interactions include intercalation or external binding. whereas covalent linkages to DNA have been suggested to occur at guanine moieties.[96,101] While the former may be difficult to detect because of the possibility of removing noncovalently linked benzene from DNA during isolation and removal of unreacted benzene. the prcsena of covalently linked benzene should present fewer difficulties. Preliminary attempts to defect covalently bound benzene in this laboratory have shown that small quantities of benzene may be bound to liver microsomes. but binding specifically to DNA has not yet been studied.

Detailed studies of induction of leukemia by benzene haw been hampered by the lack of a suitable animal model. It has not been possible to reproduce the Jingle report of leukemia in benzene-trattd mice.[97] Several types of studies might, however, contribute useful information. For example, morphological studies of benzene-treated animals in which the bone marrow is examined to evaluate the Incidence and significance of hyper- versus hypoactive bone marrow in benzene toxicity might provide a model for studying the preleukemic condition in the absence of true leukemia. It would also be of value to measure the binding of radiolabeled benzene to proteins and nucleic acids in bone manow of benzene-intoxicated animals. Thw, biochemical lesions leading to leukemia in animals may be studied in the absence of the full-blown disease.

IMMUNOLOGICAL ASPECTS OF BENZENE TOXICITY

Early in this century it was recognized that benzene had an adverse effect on immunological mechanisms. It was demonstrated that susceptibility to tuberculosis'[102] and pneumonia'[103,104] was increased m benzene-treated rabbits. The reports of decreased production of red cell lysins, agglutinins for killed typhoid bacilli and opsonin,[105] and the absence of antibacterial anti. bodies'[106,107] in benzene-intoxicated rabbits were all indications of depression of the production of various components of the immune mechanism. Developments in the fkld of immunology have led

to studies of the effects of benzene in humans on several immunological components which have been identified in recent years. Smolik and co-workers[108,109] studied a large number of workers exposed to but not seriously intoxicated by benzene. They found that serum complement levels, IgG, and IgA were decreased bur that IgM levels did nut drop and were in fact slightly higher. Taken together these observations may explain why benzene-intoxicated individuals readily succumb to infection and the terminal event in severe benzene toxicity is often an acute overwhelming infection.

These authors also evaluated levels of leukocyte agglutinins and found them elevated in selected individuals exposed to benzene.[110] They extended this observation to suggest that in some persons the picture of benzene toxicity may *in* part be accounted for as an allergic blood dyscrasia.

Alterations of immunological function may also play a role in the development of acute leukemia resulting from benzene intoxication described above. Current concepts of immunology suggest that a mechanism referred to as "immune surveillance"[111,112] is constantly at work to weed out cells which result from mistakes in cellular genetics or genetic changes caused by carcinogenic agents. The mechanism, while not completely understood, appears to involve a recognition of surface components of abnormal cells followed by immunological destruction of the cell or clone of cells. Since some forms of benzene intoxication result in hyperplasia of bone marrow with the Occurrence of many bizarre cellular species, it may be presumed that some of these may be neoplastic. Damage to immunological mechanisms in benzene toxicity may then impair the immune surveillance response, with the resulting development of leukemia.

BENZENE METABOLISM IN VIVO

Although metabolic modifications of foreign chemicals usually result in the formation of more polar, less biologically active metabolites, it is not uncommon for the products to possess enhanced biological activity.[113] Thus, prontosil, the first sulfa drug, is metabolically inactive until converted to sulfanilamide, the active antibacterial agent.[114] There are also many adverse effects resulting from metabolic activation. Carcinogenesis by 2-acetylaminofluorene, dimethyl nitrosamine, *N*-methyl-4-aminoazobenzene, and aflatoxins. hepatotoxicity by carbon tetrachloride. and carcinogenesis or mutagenesis by nitrofurans are but a few examples of the activation of foreign chemicals to more toxic compounds.[115-118] Before undcrtakinp a discussion of the relationship between benzene metabolism and its toxicity. benzene metabolism both in v.vo and in vitro will be reviewed.

Porteous and Williams'[119,120] administered benzene to rabbits orally and using chemical methods determined that the principal metabolites in urine were ethereal sulfate and glucuronide conjugates of phenol, catechol, and quinol. They also detected muconic acid in the urine. More accurate and quantitative determinations of the metabolites were performed when radioactive labeled benzene became available. Parke and Williams'[121,122] administered ^{14}C-benzene (0.34 to 0.5 ml/kg, orally) to rabbits and recovered 84 to 89% of the dose as radioactivity in the expired air, urine, feces. and body tissues. In the expired air 43% was recovered as unchanged benzene and 1.5% as $^{14}CO_2$. The urine contained 34.5%, of which phenol accounted for 23.5%, and the remainder consisted of hydroquinone (4.8%), catechol (2.2%), hydroxyhydroquinone (0.3%), frons-transmuconic acid (1.3%). and L-phenylmercapturic acid *(0.5%).* The phenolic metabolites were found to be conjugates which were liberated by hydrolysis in strong acid. No free phenols were found in the urine. The feces and body tissues contained about 5 to 10% of the dose. In other studies Williams and co-workers[123] found that 1% of a dose of benzene was excreted in the bile.

Snyder'[124] studied benzene metabolism as a function of dose in the mouse. When mice were given ^{3}H-benzene at 880 mg/kg subcutaneously in oil. 70% of the dose was recovered in the expired air within 8 hr. The rate of benzene metabolism was evaluated over a 24- hr period by collecting urine and quantitating the labeled metabolites of benzene from mice given 440 to 8.800 mg/kg of ^{3}H-benzene. Using a double reciprocal plot it was estimated that a 25-g mouse can metabolize at most approximately 1 mmol of benzene per day. The major metabolite of benzene was phenol, but trace quantities of catechol were also identified. The phenolic compounds were found primarily as conjugates in the urine, but a small percentage (e.g., 5%) of the metabolites was consistently found to be unconjugated phenol. Glucuronide

accounted for about 50 to 65% of the dose and ethereal sulfate about 26 to 38%.

Benzene metabolism in the rat was studied by Cornish and Ryan,[124] Gerarde and Ahlstrom,[126] and Van Rhees.[127] Cornish and Ryan[125] gave benzene to either fed or fasted rats (88 mg/kg intraperitoneally) and measured free phenols, glucuronide conjugates, and total conjugated phenols in the urine. In fed controls glucuronides accounted for 17% of the dose, nonglucuronide conjugates 70%, and the remainder was unconjugated phenols. In fasted rats the percentage of glucuronide rose to 48% and the nonglucuronide conjugates fell to 44%, with the remainder free phenols. Gerarde and Ahlstrom[126] used the formation of ethereal sulfate as a measure of benzene metabolism. They determined the sulfate ratio (inorganic sulfate/organic sulfate) in control and treated rats, and a decrease in the ratio was used as a measure of benzene metabolism. They showed that with doses up to 889 mg/kg, benzene metabolism was complete within 24 hr, but longer time periods were required for higher doses. Van Rhees[127] gave rats either 2 or 4 mg of benzene intraperitoneally and measured urinary metabolites after 8 and 24 hr by hydrolyzing the conjugates, steam distilling the phenol, and measuring it colorimetrically. At these low doses benzene metabolism was largely complete at 8 hr.

Benzene metabolism in dogs has not been studied in recent years, but inferences from the older literature can be made. Callow and Hele[128] were interested in sulfur metabolism and attempted to distinguish between ethereal sulfate formation and the production of mercapturic acids. Dogs were given benzene at doses of 180 or 230 mg/kg. As a result, inorganic sulfur in the urine decreased with a corresponding increase in organic sulfur. The so-called "extra" urinary sulfur after giving benzene was reported as either ethereal sulfate or "neutral sulfur" (mercapturic acid). Thirty to forty percent of the dose was excreted bound to sulfur, of which 50 to 75% was ethereal sulfate and the remainder was mercapturic acid. The authors indicated that the ratio of free to total phenol in the urine suggested that 60% of the phenol was excreted unchanged, thereby accounting for the remainder of the phenol not bound to sulfur. Although glucuronic acids were known at that time, there was no indication of the finding of these compounds in the urine. More recently, Oehme[129] in extensive studies on the metabolism of phenol in the dog, has reported that within 24 hr 55 to 80% of an injected dose of ^{14}C-phenol was recovered in the urine at doses ranging from 10 to 100 mg/kg. Glucuronides were prominent metabolites at each dose level. For example, at doses of 50 to 100 mg/kg which would be closest to the doses of benzene reported by Callow and Hele,[128] 60 to 65% of the urinary metabolites were recovered as glucuronides and 15 to 25% were recovered as ethereal sulfate. No report of mercapturic acid was made.

Williams and co-workers[122,130,131] also studied the metabolism of the major metabolites of benzene in vivo in the rabbit. When they administered ^{14}C-phenol orally they recovered approximately 90% of the dose in the urine almost equally divided between phenylsulfate and phenylglucuronide. Approximately 10% was recovered as quinol and less than 1% as catechol. In contrast to benzene, phenol did not give rise to muconic acid, suggesting to the authors that catechol was not a substrate for ring opening. Parke and Williams[122] suggested that 1,2-dihydrobenzene-1,2-diol might be a more immediate metabolite of benzene that could give rise to muconic acid. The formation of the dihydrodiol has since been confirmed by Jerina et al.[134]

Oehme[129] administered ^{14}C-phenol to dogs, cats, pigs, and goats intravenously and measured the urinary metabolites. The dog excreted approximately equal quantities of free phenol, phenylsulfate, and phenylglucuronide. In contrast, the cat excreted 80% as phenyl sulfate with the remainder equally distributed between free phenol and the glucuronide. The pig converted 60% to the glucuronide, and 30% was recovered as free phenol, with very little as the sulfate. Phenylsulfate represented 75% of the radioactivity in the goat urine, 25% was glucuronide, and about 1% was free phenol. When the dose of phenol was increased in these studies, the percentage of total metabolites in each species recovered as phenylsulfate decreased and the glucuronide portion increased, suggesting that sulfate availability may be limited and that glucuronidation may provide a reserve mechanism following depletion of sulfate.

In a more extensive study performed in Williams' laboratory, the metabolic fate of phenol was studied in 19 species including man.[133] ^{14}C-Phenol was given orally at doses of 25 mg/kg except for man, where the dose was 0.01 mg/kg, and the rhesus monkey, where 50 mg/kg was

given The major route of metabolism in all species was conjugation, and all species with the exception of the cat and the pig put out both ethereal sulfate and glucuronide conjugates. The pig excreted the total dose as phenylglucuronide and the cat as phenylsulfate (87%) and quinol sulfate (13%). Among the other species. some conjupated phenol primarily via the ethereal sulfate route (man, hedgehog, chicken. jerboa). others favored glucuronide formation (squirrel monkey, capuchin, fruit bat. guinea pig). while in the remainder neither predominated (rhesus monkey, ferret. dog, rabbit, mouse, gerbil, hamster, lemming). Quinol was a significant metabolite in many of the species and exceeded 20% of the dose in the ferret, dog. mouse. lemming, squirrel monkey, capuchin, and hamster. Although the results of Williams and co-workers and those of Oehme are largely in agreement, some slight discrepancies are apparent which may be related to differences in specific strain or breed of animals, route of administration of the phenol. and dose.

Williams and co-workers'[130,132] also investigated the fate of the two minor metabolites of benzene found in greatest quantity – catechol and quinol. Rabbits were given about 200 mg/kg orally of each compound and the urine was found to contain ethereal sulfate and glucuronide conjugates of each. There was no evidence of the addition of another hydroxyl group to quinol, but trace amounts of hydroxyquinol were recovered after giving catechol. In each case the polyhydroxylated benzene derivatives were excreted as the monoconjugates.

The rate of benzene metabolism can be determined by the dose of benzene and by compounds which either stimulate or inhibit benzene metabolism. Thus, both Gerarde and Ahlstrom[126] and Snyder'[24] demonstrated a dose dependency for the rate of benzene metabolism in the rat and the mouse, respectively. Pretreatment with phenobarbital has been shown to increase benzene metabolism in the rat by 40%[135] and in the mouse by 70%.[136] Cornish and Ryan[125] reported that SKF525A inhibited benzene metabolism in the rat. Toluene inhibits benzene metabolism in both the rat[127,135] and the mouse. Because benzene is metabolized via the hepatic microsomal mixed function oxidase,[136] it is not surprising that compounds which stimulate the activity of that enzyme system might increase the rate of benzene metabolism, while those which react with cytochrome P450 might inhibit benzene metabolism.

BENZENE METABOLISM IN VITRO

Early studies of benzene metabolism in vitro relied on the colorimetric determination of phenol production as a measure of benzene hydroxylation. Sakamoto et al.[137] attempted to identify the intracellular locus of benzene hydroxylation using differential centrifugation of rabbit liver homogenates, but because they used g forces too low to isolate microsomes they were forced to conclude that the enzyme resided in their 19,000 g supernatant fraction. They were the first, however, to describe the requirement for reduced pyridine nucleotides in benzene hydroxylation and reported that phenol was the principal metabolite. They also showed that the enzyme was inhibited by heavy metals and was sensitive to dialysis. Although these key observations were reported relatively early in the study of benzene metabolism in vitro. they were not appreciated because they were published in Japanese in a rather obscure and inaccessible journal. Using a similar procedure, Posner et al.[138] demonstrated that benzene was converted to phenol in microsomes isolated from rabbit or dog liver.

Because of the lack of sensitivity and the possibility of interference by other phenolic compounds, the colorimetric method of phenol determination was not considered adequate for detailed studies of benzene metabolism. Thus, Snyder et al.[139] instituted the use of radioactive labeled benzene to measure benzene metabolism in subcellular fractions of liver from rabbits, rats. and mice. Both hydroxylation of benzene to phenol and subsequent conjugation to form either phenylsulfate or phenylglucuronide were measured in elther whole homogenates or 9,000-g supernates, whereas only phenol formation occurred in isolated microsomes. In 9,000-g supernates about 50% of the phenol was conjugated but upon addition of ATP and sulfate over 90% of the phenol was converted to phenylsulfate. In contrast UDPG glucuronic acid or its precursors ATP, UTP, NAD, and glucose were less effective in promoting the formation of phenylglucuronide.

The rate of benzene metabolism in these studies appeared to be related to the degree of conjuga-

tion because wtwn ATP and sulfate were added more benzene was metabolized.[140,141] It was suggested that perhaps there was either inhibition of benzene hydroxylation by competition of phenol with benzene for a site on benzene hydroxylase or phenol exerted a negative feedback on the reaction. Under these circumstances conjugation would remove phenol and thereby stimulate the reaction To test this idea sodium fluoride was added to these preparations. Sodium fluoride inhibits ATPase and would therefore amplify the effectiveness of small amounts of ATP. It also has the effect of inhibiting ethereal sulfate formation.[142] The result was that fluoride prevented the conversion of a large percentage of phenol to phenylsulfate but the increase in benzene metabolism was not altered. The stimulation of metabolism appeared to be a function of the effects of ATP and fluoride on benzene hydroxylase rather than on conjugation. and, indeed, when added to isolated microsomes both produced an increase in the rate of benzene hydroxylation. The mechanism has yet to be determined. but the effect is not observed in the course of the metabolism of chlorpromazine, zoxazolamine, neoprontosil, *p*-nitrobenzoic acid, aniline, or *N*-methylaniline.[14] It was of interest that the oxidation of aldrin to dieldrin was stimulated by ATP and F in vitro.

Liver microsomes contain a relatively nonspecific enzyme system called the mixed function oxidase which is primarily responsible for the metabolism of a great many xenobiotic compounds.[143,144] The system requires molecular oxygen and NADPH for activity. Mixed function oxidase activity revolves about a series of reactions of a heme-containing protein called cytochrome P450, which is the active site at which both substrate[145-147] and oxygen react.[148,149] The substrate reacts with oxidized (i.e., Fe^{+++}) cytochrome P450, and the resulting enzyme-substrate complex is reduced to the Fe^{++} form by reducing equivalents originating from NADPH and transmitted via a flavoprotein enzyme called NADPH-cytochrome P450-reductase.[150,151] Alternatively, some of the electrons may come from a second source of reducing equivalents involving NADH, another flavoprotein called NADH-cytochrome b_5-reductase, and another hemeprotein called cytochrome b_5.[152,156] The latter is itself incapable of reacting with oxygen, binding substrates, or catalyzing hydroxylation, but it may rid in the reduction of cytochrome P450. The sequence uf events continues with the reaction between reduced enzyme substrate complex and oxygen; the substrate undergoes hydroxylation and the cytochrome is reoxidized. The demonstration of benzene metabolism in liver microsomes strongly suggested that the mixed function oxidase was benzene hydroxylase.[157]

Several pieces of evidence taken together indicate that benzene is hydroxylated by cytochrome P450 and the mixed function oxidase. Benzene hydroxylation occurs in liver microsomes of rabbits, rats. and mice and requires oxygen and NADPH.[136,138,139] Benzene reacts with cytochrome P450 to yield a Type I spectral change indicative of the formation of an enzyme-substrate complex and with cytochrome P448 to yield a Type RI spectral change in much the same manner as many other substrata for the mixed function oxidase.[136,158] Aniline, metyrapone, aminopyrine, and SKF525A, all of which inhibit the metabolism of other compounds by cytochrome P450 or interact with cytochrome P450, inhibit benune metabolism.[136] Cytochrome *c*, which inhibits mixed function oxidase reactions apparently by diverting electrons from cytochrome P450,[159] also inhibits benzene hydroxylation.[136] Benzene hydroxylase, like other microsomal hydroxylases, is inducible, and its induction will be described below. Finally, benzene metabolism is inhibited by carbon monoxide, and the inhibition is best reversed by light at a wavelength of 450 nm.[136] From these data it seems fair to conclude that benzene hydroxylase is a form of the mixed function oxidase.

The mechanism of benzene hydroxylation by the mixed function oxidase has not yet been determined largely because of the difficulties encountered when trying to isolate and purify the membrane-bound components of the microsomal enzyme system. Studies by Jerina and Daly[7] and their associates have concentrated on the chemical aspects of the problem and have suggested that the reactions probably occur via the formation of an arene oxide intermediate. Upon the addition of benzene oxide to a microsomal preparation, the products of benzene metabolism were formed.[34] Benzene oxide production has not been demonstrated in microsomes probably because of its extreme lability, but naphthalene oxide was recovered when naphthalene, an aromatic hydrocarbon closely related to benzene, was incubated with liver microsomes.[160] On theoretical grounds,

Hamilton[161] has proposed that it would be expected that mixed function oxidation reactions proceed through arene oxides. Furthermore, arene oxides of a number of aromatic hydrocarbons haw been reported.[93-97,162,163] Therefore, benzene oxide appears to be a likely intermediate in benzene metabolism.

The products of arene oxide degradation result from either enzymatic or nonenzymatic reactions.[ ] Nonenzymatically benzene oxide can undergo isomerization to form phenol by either of two mechanisms, one of which is favored at low pH while the other is pH independent. Since both in vivo and in vitro phenol is the major metabolite of benzrne and biological systems function in the range of neutrality, the latter appears to be the preferred mechanism. Enzymatically benzene oxide may be hydrated to 8 dihydrodiol by the action of epoxide hydrase[134,164,165] followed by subsequent reduction to catechol. The significance of the latter pathwry has yet to be determined since very little catechol is formed in the course of benzene metabolism. Another enzymatic reaction is the transfer of glutathione to arene oxides by the enzyme arene oxide-glutathione transferase. Perhaps the mort significant pathway for the degradation of benzene oxide b the reaction with cellular nucleophiles such as nucleic acids or proteins which could result in cellular damage indicative of benzene toxicity. Thus, *the* metabolic fate of benzene can be visualized as the formation of an arene oxide followed by the rearrangement to the less chemically reactive phenol, the interaction of the oxide with cellular nucleophiles, or the enzymatic conversion to either the dihydrodiol or a premercapturic add.

The hepatic microsomal *mixed* function oxidase system can be stimulated by enzyme induction by at least two general classes of compounds: (I) polycyclic aromatic hydrocarbons and (2) phenobarbital plus a number of other compounds which act similarly.[166] The former appear to induce the formation of a type of heme protein called cytochrome P448, whereu phenobarbital induces tht formation of cytochmme P450.[167,178] Benzene hydroxylase activity in vitro increases after treating animals with phenobarbital or 3-methylcholanthrene, a polycyclic aromatic hydrocarbon.[139,179,180] Administration of benzene itself or DMSO also increases the rate of benzene metabolism.[139,179,181] When benzene or DMSO is administered subcutaneously or intraperitoneally, benzene metabolism in liver preparations increases but there is no increase in cytochrome P450 levels.[24,182] Recently Norpoth et al.[183] reported that exposure of rats to benzene vapor at about 450 ppm for 10 days resulted in an increase in cytochrome P450 levels of about 65%, and Drew et al.[179] reported that exposing rats to levels of benzene in the atmosphere above 4,000 ppm for 4 hr/day fur 3 days resulted in an increase in microsomal benzene metabolism. Nevertheless, the reports of increases in benune metabolism by benzene or DMSO in the absence of increased levels of cytochrome P450 suggest that other factors may play a role in determining the rate of benzene metabolism.

When benzene is added to liver microsomes, it is possible to observe a Type 1 spectral change typical of substrates for the mixed function oxidase.[36] When the technique of Schenkman et al.[146] for titrating microsomes to obtain the apparent spectral dissociation constant (K_s) was applied, it was found that in liver microsomes from benzene-treated mice there was no change in the K_s but the maximum *change* in optical density (ΔOD_m) was increased. By the same token kinetic studies showed that metabolism in liver microsomes of benzene-treated mice was characterized by no change in the K_M for benzme but the V_{max} increased by about the same percentage (a1663% of controls) as the ΔOD_m.

In addition to increasing the rate of benzene metabolism, parenteral administration of benzene also increased the rate of zoxazolamine hydroxylation and the reduction of *p*-nitrobenzoic acid in rats.[177] Stimulation of the metabolism of both benzene[179] and aminopyrine[183] has been reported following exposure of rats to benzene in the atmosphere. Therefore, benzene appears to function as a microsomal stimulant which can increase the rate of drug metabolism without necessarily increasing cytochrome P450 and as a result poses questions regarding the nature of the induction which may be dealt with in the context of our understanding of microsomal enzyme induction.

In the course of developing current concepts of hepatic microsomal enzyme induction, it became clear that many drugs, among which phenobarbital and the polycyclic aromatic hydrocarbons are most prominent, were capable of initiating protein synthesis in liver. Increased microsomal protein,[167] increased incorporation of amino acids

into hepatic microsomal protein.[185-187] increased activity and synthesis of nucleic acids and enzymes that mediate their activity,[187-194] prevention of induction by inhibitors of protein synthesis.'[67, 186,195] proliferation of smooth endoplasmic reticulum (SER),[196-199] and increased synthesis of cytochromes P450 and P448[158,200,201] have been determined during enzyme induction. Induction by benzene is characterized by an increase in metabolism and in incorporation of amino acids into microsomal protein within 24 hr of a single dose, but no proliferation of SER is observed at that time.'[40,184] After 1 or 2 weeks of benzene treatment, SER appears to be proliferated and the metabolisms of benzene, zoxazolamine, and neoprontosil remain slightly stimulated. whereas those of hexobarbital and *p*-nitrobenzoic acid are depressed.[184] Benzene induction is characterized by requiring a longer time and producing less proliferation than phenobarbital. In contrast, it is more similar to polycyclic aromatic hydrocarbons, which also induce rapidly and do not cause proliferation of SER at the time of induction.'[97] There may be a greater similarity to dieldrin, which after several weeks causes the formation of hypertrophic, hypoactive smooth endoplasmic reticulum.[202] In all, these observations suggest that there does not seem to be a relationship between the degree of proliferation of SER and the rate of benzene metabolism.

Since hepatic microsomal enzyme induction involves protein synthesis, the data suggest that at least two different types of protein may be synthesized to account for increases in the rate of benzene metabolism. The finding by Gonasun et al.[136] that the increase in benzene metabolism after induction with benzene was not accompanied by elevated levels of cytochrome P450 is consistent with induced synthesis of a minor component of the microsomal cytochrome P450 population which is responsible for benzene hydroxylation but which represents a relatively small fraction of the total microsomal carbon monoxide binding heme proteins. Thus, a sizeable percentage increase in that component might go undetected when measuring cytochrome P450 but become apparent only when measuring binding spectra. Recently, Norpoth et al.[183] demonstrated that after exposing rats to benzene vapor for 10 days (5 hr/day), cytochrome P450 levels in liver increased 65%. Therefore, benzene can increase cytochrome P450 levels but not rapidly enough to observe the effect within 24 hr, the time at which benzene can increase the rate of its metabolism.

An alternative possibility was suggested by Remmer[203] several years ago but has not yet been evaluated. He showed that phenobarbital stimulated the metabolism of hexobarbital and aminopyrine and DDT stimulated the metabolism of hexobarbital, but in neither case was there an increase in cytochrome P450 levels commensurate with the increase in the rate of metabolism. He also observed that male rats metabolized hexobarbital faster than female rats but that the difference could not be explained on the basis of cytochrome P450 levels. In each case the rate of metabolism was more closely associated with the optical density changes in the binding spectra than with cytochrome P450 content of the microsomes. Increases in binding spectra following enzyme induction have also been reported by other workers.'[77,204,205] Remmer[203] suggested that regardless of the effect of inducing agents on cytochrome P450 concentrations, the relationship between the rate of metabolism and the binding spectrum may be caused by the induced synthesis of a so-called "binding protein" which facilitates the binding of the substrate to the cytochrome. If the rate-limiting step in the hydroxylation of benzene is the rate at which the enzyme substrate complex can be reduced,[206] then a protein which increases binding may hasten the rate of reduction and thereby stimulate benzene metabolism. Both of these possibilities deserve further study.

RELATIONSHIP BETWEEN BENZENE METABOLISM AND TOXICITY

Relatively few studies have been aimed at correlating benzene metabolism with benzene toxicity. Following the definitive studies of Parke and Williams,[121,122] in which the metabolites of benzene were identified as phenol and polyhydroxylated phenols, Dustin[207] suggested that the metabolites might be responsible for benzene toxicity through either of two mechanisms. In one, quinone-yielding metabolites such as catechol, quinol. or pyrogallol could react directly with chromosomes and interfere with mitosis. An alternative proposal was the following sequence of events: (1) depletion of sulfate, derived from sulfur amino acids. for purposes of conjugation, leading to (2) subsequent depletion of glutathione

In bone marrow, resulting in (3) disturbances in redox reactions in bone marrow, leading to (4) bone marrow depression. No evidence has yet been developed to support either of these suggestions, bur they represent some of the first thought indicating that benzene metabolism may play a role in benzene toxicity.

Nomiyama et al.[208-211] reported that young rats displayed a high rate of benzene metabolism in vitro and were more susceptible to benzrne given subcutaneously than old rats in which the rate of benzene metabolism was slower. They demonstrated that 3-amino-1,2,4-triazole, which inhibited benzcne metabolism in rat liver homogenates, protected against benzene toxicity. They therefore concluded that a metabolic product of benzene was responsible for benzene toxicity rnd proceeded to test several benzene metabolites for their potential as hemotoxic agents. Among phenul, catechol, quinol, hydroxyquinol. *trans*-transmuconic acid, D,L-phenylmercapturic acid, and potassium phenylsulfate, only catechol appeared to depress bone marrow function by causing anemia rnd leucopenia.

At about the same time Ikeda[212] exposed rats to benzene in the atmosphere at 1,000 ppm for 7 hr/day, 5 days/week and showed that the order of increasing sensitivity to benzene based on depression of white cell counts was adult males > young males > adult females > young females. He then evaluated the levels of several enzymes involved in benzene metabolism. Aryl-4-hydroxylase was assumed to be the *enzyme* that hydroxylates benzene, and its activity was determined using aniline as the substrate. Ethereal sulfate and glucuronide-forming enzymes were evaluated using *p*-nitrophenol as the substrate. He concluded that the best correlation between benzene toxicity and metabolism could lk made between the rate of ethereal sulfate formation and toxicity. Thus, the relationship among the various groups of rats with respect to decreasing ethereal sulfate formation was identical to the relationship stated above for increasing susceptibility to benzene. No correlation between metabolism and toxicity for the other enzymes was found. Ikeda suggested that Nomiyama's results may have differed from his own because Nomiyama used whole homogenates rather than the cell fractions in which greatest enzyme activity could be observed and because Nomiyama failed to fortify his homogenates with pyridine nucleotides. It b apparent, however, that Ikeda, in attempting to study enzymes involved in benzene metabolism in vitro. used neither benzene nor its metabolites as substrates. Although the mixed function oxidase is the enzyme that hydroxylales both benzene and aniline, there is no reason to assume the rates of these reactions will be equal nor can it be assumed that the rates will necessarily vary among the groups of rats that he studied in the same way for both substrates. The relationship of these studies to events in humans is even more tenuous when it is considered that many instances of sex differences in drug metabolism have been reported in rats which do not occur in other species.'" Although it was previously suspected that women were more susceptible to benzene toxicity than men, that idea has not stood the test of time rnd further experience.'[14]

Ikeda[135,215] then went on to study benzene metrbolism in vivo and reported that rats treated with phenobarbital metabolized more benzene and were more resistant to benzene-induced leucopenia. These results were later confirmed by Drew et al.[179] Ikeda'[15] therefore suggested that metabolism of benzene was a detoxifying procedure and that inhibition of benzene metabolism should result in increased toxicity. It must be argued that in fact these studies could not distinguish between toxicity by benzene or a metabolic product because the increase in metabolism produced by phenobarbital might either have detoxified benzene or hastened the removal of a toxic intermediate, a mechanism similar to that originally suggested by Ikeda.'[12]

In this laboratory we have studied the relationship between benzene metabolism and toxicity by administering labeled benzene to mice and (1) using the labeled metabolites in the urine as a measure of the rate of benzene metabolism and (2) using the reduction of the incorporation of ^{59}Fe given the next day into erythrocytes of the same mice as a measure of hematopoietic toxicity produced by that dose of benzene.'[24] When the mice were given toluene, a competitive inhibitor of benzene metabolism, the rate of benzrne metabolism was reduced and ^{59}Fe uptake into erythrocytes increased, indicating that benzene toxicity was alleviated. In similar studies in which phenobarbital was given to mice to increase the activity of the mixed function oxidase, mice with higher rates of benzene metrbolism demonstrated a greater decrease in ^{59}Fe uptake than mice in which less benzene was metabolized. These studies

would rend to support the view expressed by Nomiyama that a metabolic step was important in causing benzene toxicity.

A common thread seems to run through the studies renewed above. In each case the effect of benzene on a parameter of bone marrow activity was conelated with the rate of benzene metabolism either in vivo or in vitro using liver preparations. Some recent studies suggest that these approaches might be modified to yield further information. Acetaminophen[216-219] induced liver necrosis appears to be directly related not only to the rate of acetaminophen metabolism but also to the extent of acetaminophen binding to hepatic protein. Stimulation of acetaminophen metabolism by phenobarbital increases both necrosis and binding; inhibition of acetaminophen metabolism by piperonyl butoxide or cobaltous chloride protected against necrosis while decreasing binding. Bromobenzene[2] also produced hepatic necrosis, but inhibition of bromobenzene metabolism by SKF525A coincided with reduction of liver damage. The effect of enzyme induction on bromobenzene-induced liver hepatotoxicity, however. depended on the metabolic pathway induced. Thus, phenobarbital stimulated the formation of a hepatotoxic metabolite, while 3-methyl cholanthrene increased the activity of a detoxifying pathway. The rate of benzene metabolism may affect bone marrow depression by a mechanism similar to that by which acetaminophen or bromobenzene produces liver damage. Furthermore, there may well be both detoxifying pathways as well as pathways which lead to bone marrow depression which could help to explain differential sensitivity of humans to benzene as a result of enzyme induction. In any event, the role of covalent binding to subcellular constituents in benzene toxicity should be evaluated.

Although there is considerable suspicion that benzene metabolism plays a role in benzene toxicity, there is good reason why the evidence is not yet definitive. The study of benzene metabolism whether in vivo or in vitro has In actuality been a study of events that occur m the liver. The liver is responsible for most of the metabolism of benzene, and the metabolites of benzene in the urine are essentially the result of hydroxylation and conjugation in liver. However, benzene toxicity is manifested in the bone marrow rather than the liver. It must either be argued that a toxic metabolite travels from the liver to the bone marrow or that the metabolite is indeed formed in the bone marrow. If benzene oxide is the active metabolite, its chemical reactivity is too great for it to survive transport through the circulation. Phenolic metabolites might well be transported free or as conjugates which may be hydrolyzable in the bone marrow. However, there is some evidence that conjugates of benzene are neither degraded nor are they toxic.[31-211] It appears, therefore, that although the liver may serve as a good model for the study of the enzyme systems which metabolize benzene, the most profitable tissue for studying the role of benzene metabolism in toxicity is the bone marrow. The techniques are difficult, and bone marrow is not available in large quantities. It may be necessary to use the methods of tissue culture. Among the problems to study are the metabolism of benzene in bone marrow and the effects of the metabolites of benzene formed either in the liver or the marrow on bone marrow activity. In any event, a point has been reached in the study of benzene toxicity where we must recognize that there can be no more direct approach to studying the disease process than to investigate it in the organ where it occurs.

SUMMARY AND CONCLUSIONS

Although the morphological aspects of damage to hematopoietic organs caused by chronic benzene exposure have been understood for some time, the mechanism by which benzene acts is not known. The evidence suggests that benzene inhibits the maturation of early blood cell precursors; i.e., the Arneth count is shifted to the left in the leucocyte series and maturation of pronormoblasts and normoblasts is inhibited in the erythrocyte wries. In advanced stages the result can be pancytopenia due to bone marrow aplasia. DNA synthesis is reduced in bone marrow of benzene-treated animals either because of inhibition of enzymes involved in DNA synthesis or because a lesion revealed as reduced incorporation of tritiated thymidine in DNA occurs at some point in the all cycle. How benzene mediates this inhibition is not yet clear.

Although epidemiological studies among workers in industries where benzene exposure is a hazard have failed to demonstrate a correlation between the Incidence of kukemia and benzene exposure, many individual cases of leukemia have been linked to benzene. The suggestion that

hyperplastic bone marrow sometimes seen in benzene toxicity is indicative of the preleukemic state and the concept that aleukemic leukemia may he more frequent in benzene toxicity than was previously recognized are ideas that must be more fully explored. The evaluation of chromosome abnormalities in benzene-intoxicated individuals might be useful in determining leukemia, but it must be recalled that acute leukemia, which is frequently related to benzene exposure, yields chromosome abnormalities relatively rarely. Furthermore, abnormal chromosomes are not necessarily prognostic for leukemia.

Benzene has been known to repress immunological mechanisms for many years. Recently it was demonstrated that specific immunoglobulins such as IgA and IgG as well as serum complement are depressed while IgM is elevated in benzene intoxication. Two observations in benzene-intoxicated individuals may result from immunological damage. Benzene-intoxicated individuals often suffer from serious infections which they are unable to combat and thus may be terminal. Furthermore, in some instances of benzene toxicity the "immune surveillance" mechanism which appears to be partly responsible for preventing the growth and development of neoplastic tissues may not function and the result may be benzene-induced leukemia.

Benzene is hydroxylated by the microsomal mixed function oxidase to phenol and other hydroxylated benzene derivatives probably via the intermediate formation of an epoxide. The epoxide may either rearrange to yield phenol. conjugate with glutathione to yield a premercapturic acid, be hydrated to epoxide hydrase and reduced to catechol, or it may react with various cellular constituents and thereby produce its toxic effects. Despite some contrary evidence. there are considerable data to support the concept that a metabolic step yielding a toxic intermediate is necessary to produce benzene toxicity. Thus, inhibition of benzene metabolism protects against benzene toxicity. Since the bulk of these studies has been performed using whole animals or liver preparations, it is essential that further studies revolve around the metabolism and toxic effects of benzene in bone marrow.

ACKNOWLEDGMENTS

The authors wish to thank their colleagues Drs. E. W. Lee and C. M. Witmer for discussions during the preparation of this review. This work was in part supported by a grant from the USPHS, National Institutes of Environmental Health Sciences (ES00322).

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