HEPATOTOXIC AGENTS: MECHANISM OF ACTION AND DIETARY INTERRELATIONSHIP

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TABLE OF CONTENTS

I. INTRODUCTION

The hepatotoxic action of a number of substances as exemplified by chloroform, carbon tetrachloride or phosphorus has been known for a relatively long period of time. As early as 1882 it was noted by Rosenbaum that liver injury produced by arsenic or phosphorus resulted in a depletion of liver glycogen. The ultimate development of this work was the use of glucose in the treatment of liver injury in both animals and man. Although changes in liver glycogen *per se* are not now believed to influence hepatic damage, such studies in the early 1900s probably served to direct attention to dietary factors. The relation of diet to the toxicity of drugs had been studied by a number of investigators but it was not until 1914 that Opie and Alford investigated the relationship of diet to the effect of hepatotoxic materials. They, and workers following them, demonstrated that the degree of liver damage produced was increased by high-fat diets. With the diets then in use least liver injury was observed when bread and milk was

fed as compared with a high protein diet in the form of meat. Investigation of dietary effects has continued since that time, the technics and diets being more refined in accord with the newer nutritional data. The advent of choline and methionine, as studied by Best and co-workers and others, and the process of transmethylation, soon led to further studies on the relationship of these and other supplements to the action of hepatotoxic agents.

Studies in this field have aimed at obtaining greater knowledge regarding the mechanism by which the liver injury is produced and the relationship of these effects to diet. Most workers have also had in mind the ultimate use of diet, choline, methionine or other substances as therapeutic materials of possible value in the treatment of a chemically-induced liver injury. However, the fact that methionine decreases mortality in exposed animals does not necessarily mean that methionine has acted on the liver. Indeed, the increased survival produced may be unrelated either to the concentration of hepatic lipids or to liver histology, but rather to the prevention of injury in other organs such as the kidney or heart, as will be discussed later. Some such reports in the literature have been misinterpreted and are often quoted as showing a beneficial effect of the supplement on the liver. In other cases the experimental diets used are so abnormal that it remains to be shown that the results have any application at all to injury produced in man.

II. HISTOLOGICAL EFFECTS OF HEPATOTOXIC AGENTS

The administration of a variety of substances will produce fatty changes and necrosis in the liver, the extent of the injury varying with the dose and route of administration. If a single minimal effective dose is given zonal fatty changes and necrosis will occur, followed by complete regeneration and recovery of the liver. A large dose or injection into the portal vein will produce a massive hepatic necrosis resulting, if the animal survives, in post-necrotic scarring of the liver. The zonal necrosis or the massive necrosis are both acute in nature. The chronic administration of many substances, initially resulting in zonal injury, will eventually cause a diffuse hepatic fibrosis. In addition to the production of acute or chronic injury a delayed type, as produced by cinchophen, should be mentioned, although its cause is unknown.

The acute injury may also be divided according to the part of the liver lobule that is injured. Substances such as chloroform, carbon tetrachloride, tannic acid or mushroom toxin cause injury in the central part of the lobule, whereas phosphorus, allyl formate or *Proteus vulgaris* endotoxin produce periportal damage. This zonal difference in injury will be discussed later with regard to necrosis and ischemia.

Chloroform and particularly carbon tetrachloride have been studied in greatest detail and the hepatotoxic effects of these substances will be summarized. Other damaging agents will be reviewed chiefly in relation to dietary factors.

A. Acute liver injury

Chloroform. As early as 1850 Casper (38), Langenbeck (150) and Mourlon (192) noted the delayed toxic effects of $CHCl_3$ in man. However, it was not until 1889

that the first delayed death from the clinical use of $CHCl_3$ anesthesia was reported in which the postmortem examination showed fatty degeneration of the liver (251). In 1866 Nothnagel first observed that $CHCl_3$ produced fatty degeneration in the liver of experimental animals (199). Later the production of necrosis was recognized and the effect of $CHCl_3$ on the liver was extended to other species of animals. It is interesting that the liver of the frog, pigeon and terrapin is markedly resistant to the effects of $CHCl_3$ (191). During this time the action of $CHCl_3$ on the cardiac muscle and kidney was also observed. The earlier literature regarding the effects of $CHCl_3$ in animals and man has been reviewed in a number of papers (22, 137, 260).

A careful study of pathological changes produced by $CHCl_3$ in the dog was made by Whipple and Sperry (264). They noted that the essential change was extensive necrosis and fatty degeneration of the liver and that six to ten hours were required following anesthesia before necrosis could be observed microscopically. Cirrhosis did not follow extensive central necrosis and repair. The repair occurred rapidly, the liver being normal two to three weeks following injury, even though the hepatic artery of the dog was ligated. The necrosis produced in the liver was centrilobular, although, depending on the degree of exposure to $CHCl_3$, the necrosis may also extend to the mid-zone and even to the periphery of the lobule. Similar detailed studies have been performed on rabbits (271).

Carbon tetrachloride. Of the hepatotoxic agents, CCl_4 has been the most extensively studied. Most investigations, including the initial studies made by Hall, have been performed on the dog (29, 39, 50, 93, 111–113, 147, 148, 169, 179, 218, 228, 230, 238). Other studies have been performed on the rat (32, 33, 144), guinea pig (208), rabbit (113, 147), and mouse (79). The chicken is evidently very resistant to the effects of CCl_4 (113) and repeated doses of CCl_4 were without effect on the liver of monkeys (146).

The administration of a single effective dose of CCl_4 rapidly produces fatty changes and a centrilobular necrosis of the liver. The maximal histological change is reached within 24 to 48 hours, the severity of the lesions depending in part on the dose and route of administration of the material.

Careful studies of the effects of CCl_4 were first made by Meyer and Pessoa in the dog (179). They noted that the most severe damage was produced in the liver and kidneys. The hepatic necrosis was similar to that induced by $CHCl_3$; it appeared rapidly and was preceded by fatty degeneration. The severity of the lesion was proportional to the dose, although occasional hypersusceptibility was noticed; it also seemed to vary inversely with the age of the dog. Schultz and Marx (230) administered 0.05 cc. of CCl_4 per kg. to eight dogs and obtained fatty degeneration in four and hepatic necrosis in one. Complete studies on the effects of CCl_4 administered in various doses and by various routes to dogs was reported by Lamson and Gardner and their co-workers (93, 147). They observed that the lesions could be detected in 12 hours and were maximal in 48 hours, and although repair began in three to four days, it still may be incomplete after five weeks.

In the rat, subcutaneous injection of 0.016 cc. to 0.03 cc. of CCl_4 per kg. of

body weight produces minimal lesions. Doses of 0.3 to 1.0 cc. per kg. produce more marked changes, usually not fatal (33). Diffuse congestion is first noted, being followed rapidly by hydropic and fatty degeneration and hepatic necrosis. The degree of degeneration and necrosis is dependent on the dose of CCl_4.

Although changes are noted quite soon, the peak effect of a single dose of CCl_4 is reached in 24 to 48 hours. At this time an exaggeration of the lobular pattern may be grossly observed. Microscopically a zonal necrosis centrilobular in distribution is present, involving every lobule of the liver. The region about the central vein is chiefly affected, although, depending on the dosage of CCl_4, the reaction may extend to the periphery of the lobule (32). At the height of the reaction the animals may appear ill but are not clinically jaundiced and ascites is not present. By the fourth or fifth day the animal appears improved and at the seventh day seems entirely normal. During this period necrotic cells are being removed, and repair by means of mitosis is occurring. Residual liver damage may still be present at this time. However, within a period of two weeks the surviving parenchymal cells have completely regenerated, the necrotic cells have been removed, and the liver appears entirely normal (33). Histochemical studies of the mouse liver following a single dose of CCl_4 have also been made (246).

Massive necrosis. The direct injection of $CHCl_3$, CCl_4 or chlorinated benzene derivatives into the portal circulation produces a very intense or massive liver necrosis which may result in post-necrotic scarring (34, 93, 147, 228, 264). Cameron *et al.* (34) have termed this response, "toxic infarction". Similar results are obtained if the toxin is injected into the spleen. Cameron noted that the liver must absorb these substances quite readily from the circulation since 0.025 cc. of CCl_4 injected into the jugular or ear vein of the rabbit is usually fatal at once, whereas ten times this amount can be introduced into the portal circulation without producing death. Following portal injection, the first signs of necrosis are observed in an hour, and consist of nuclear pyknosis and swelling of the cells, most frequently at the periphery of the lobule. Progressive stages may be seen and by 24 hours the necrotic areas closely resemble infarction. Liver tissue between the lesion appears normal. Progressive regeneration is noticed at seven and 18 days, and by the end of a month smaller infarcts may have completely disappeared. At 104 days all livers were normal except for a slight thickening of the fibrous capsule.

Summary. Depending on dose and route of administration, $CHCl_3$, CCl_4 and other compounds can produce various degrees of damage varying from fatty degeneration and necrosis in the central part of the lobule to massive necrosis throughout the lobule. Such acutely induced liver injury is usually followed by complete repair, except for massive necrosis which may be followed by post-necrotic scarring.

B. Chronic liver injury

Cirrhosis. Cirrhosis of the liver can be produced in various species of animals by the repeated administration of $CHCl_3$ (81, 200). Lamson and Wing (148) gave CCl_4 to dogs for five to six months, and obtained early cirrhosis. They noted that the cirrhosis was similar whether small doses (3 cc.) or large doses (25 cc.) of

CCl_4 were given. In confirmation of these studies a more advanced cirrhosis was produced, typical of Laennec's cirrhosis (29, 169). It was also observed that in certain cases restoration to normal hepatic architecture may take place even after extensive injury (29). Cirrhosis has also been produced in rats (144), guinea pigs and rabbits (254) and pigs (266). Many earlier papers regarding cirrhosis produced by $CHCl_3$, CCl_4 and other chemicals may be found in the review by Moon (188). A method has also been described for following the progression of cirrhosis by taking roentgenograms of the liver after the injection of thorium dioxide (245).

The architectural relationship of the connective tissue trabeculae in cirrhotic livers has been studied and most investigators have described the fibrous tissue as originating in the portal areas. However, Ashburn and associates (10), producing cirrhosis by diet or with CCl_4 and alcohol, showed that fatty deposition, ceroid accumulation, and fibrous trabeculae primarily followed the hepatic veins and their terminal (centrilobular) branches. In cases of marked damage, the trabeculae sometimes coursed by or enclosed a portal area. Thus the connective tissue proliferates in the same areas where damage to the liver takes place. Ferris (81), producing cirrhosis in rabbits by $CHCl_3$, also noted that the earliest fibrosis was central, and only later was periportal connective tissue involved.

The studies of Cameron and Karunaratne (33) on the production of cirrhosis in rats are of interest. If, with moderate doses, a recovery period of ten days was allowed between injections, each dose of CCl_4 produced the typical centrilobular necrosis. When the chemical was administered at shorter intervals, a diffuse hepatic fibrosis occurred. If the injections of CCl_4 were stopped at the first sign of fibrosis resolution occurred. If the administration was continued until the fibrosis became more pronounced, then, even on discontinuing the injections the fibrosis continued to develop and eventually produced death. Small doses, sufficient to produce only minimal hydropic and fatty changes, administered twice a week for five months, failed to cause permanent damage. *Thus the development and progression of the fibrosis depend upon both dosage and continued injury to the liver, but the process may be completely reversed if the damaging agent is stopped early.* During such a reversal period the collagen content of the cirrhotic liver may be shown to decrease, either by microscopic examination or by chemical determination (190).

Hepatomas. The repeated oral administration of CCl_4 to the C3H or the A or L strain of mice produces a very high incidence of hepatomas (75, 77). The tumors are well-differentiated and resemble those arising spontaneously or induced by *o*-aminoazotoluene (76). Although there is some correlation between the dose of CCl_4 and the incidence of hepatomas, repeated episodes of liver necrosis and its associated chronic regenerative state are probably not necessary for the induction of the tumors (79).

C. Centrilobular liver damage and ischemia

The reason for the production of necrosis by CCl_4 and other compounds about the central part of the lobule, rather than in the periphery of the liver lobule, has only recently been explained. As the blood carrying CCl_4 reaches the liver

the peripheral cells of the lobule are first exposed and yet survive the higher concentration of CCl_4, whereas the more central cells become necrotic. Survival of the cell seems to depend on proximity to blood supply, which would indicate that the blood, when it reaches the centrally located cells, has been depleted of some vitally necessary substance. Evidence, accumulated over a period of years, indicates that CCl_4 and $CHCl_3$ produce parenchymal swelling which constricts the sinusoids of the liver, thus producing ischemia and anoxia with consequent damage to the central cells of the lobule.

In 1925 Loeffler and Nordmann (164) observed that the inhalation of $CHCl_3$ produced narrowing of the intralobular sinusoids in mice and rats. This change, observed directly by transmitted light, occurred within 15 minutes and persisted for several hours. After ten to 24 hours the sinusoids dilated, but three to ten days were required before blood flow returned completely to normal. Later, by the use of a similar technic, the momentary inhalation of CCl_4 in the anesthetized rat was observed to produce an immediate although transient constriction of the sinusoids (258). When the CCl_4 was inhaled continuously for 30 minutes or injected subcutaneously, severe changes were produced. Eight hours later the hepatic cells were definitely swollen and most of the sinusoids were obliterated. Two to four weeks were required before the intralobular circulation returned to normal. With repeated inhalation, to produce cirrhosis, the vascular changes consist in an early and initial vasoconstriction followed by a prolonged secondary obliteration of the vascular channels, chiefly the venous ones, produced by the swelling and fatty degeneration of the hepatic parenchyma.

Injury induced by CCl_4 also interferes with hepatic distribution of substances such as India ink (8, 94, 125). Normally, India ink injected into the spleen, passes to the liver and fills the portal vessels, intralobular sinusoids and hepatic veins. Two hours after administration of CCl_4 the hepatic cells throughout the lobule are swollen and in a majority of the lobules the India ink fails to reach the central zones, which indicates a marked impairment of circulation. Fine vacuoles are also present in the central zone at this time. At four hours the impairment of the sinusoidal circulation is more pronounced and the fine vacuolization in the central cells has increased. Hydropic degeneration is present by the eighth hour. The sinusoids are still closed but penetration of the India ink occurs in a few irregularly distributed areas. At 12 to 24 hours the central hydropic degeneration and necrosis are severe and most sinusoids are still occluded. It is obvious that the degenerative changes produced by CCl_4 follows the restriction of the intralobular circulation. In contrast, allyl formate and the endotoxin of *Proteus vulgaris*, which produce only periportal necrosis, cause dilatation rather than constriction of the sinusoids (125).

Impairment of blood supply to the liver *per se* will produce lesions resembling those induced by chlorinated hydrocarbons. The changes may be produced by ligation of the portal veins or hepatic artery, or both, in various species of animals (35, 163, 177). The necrosis produced is centrilobular, and occurs after approximately the same time interval noted in rats receiving CCl_4. Anoxia alone can produce degenerative changes in the central liver cells (170). On closer analy-

sis the first effect of anoxia is a fine vacuolization of parenchymal cells which progresses to hydropic degeneration (252), similar to the early effects of carbon tetrachloride. Thus, it seems likely that the central necrosis produced by CCl_4 is a result of ischemia (and possibly anoxia) of the central cells.

It has also been shown by Goldschmidt *et al.* that chloroform anesthesia decreases the oxygen saturation of the portal and hepatic venous blood, indicating hepatic anoxemia (96). They also observed that oxygen protected against $CHCl_3$-induced liver injury, and in similar studies oxygen was found to protect against CCl_4 damage (125). If hyperthyroid animals are exposed to low oxygen tensions liver damage is produced which is similar to that seen after CCl_4 (176), and hyperthyroid animals were shown to be sensitive to the effects of $CHCl_3$ (174, 175).

As noted above, the inhalation of $CHCl_3$ or CCl_4 for brief periods can produce practically immediate constriction of the sinusoids. This constriction may occur by a direct effect on the cellular membranes or through a nervous reflex (164). There is some evidence for nervous control over intralobular circulation (103), and although chlorinated hydrocarbons have not been shown to activate this mechanism, such action may take place following momentary exposure. With more prolonged exposure it might be supposed that continued sinusoidal constriction may produce parenchymal swelling. However, parenchymal swelling induced by presumably inert substances such as fat or polyvinyl alcohols (114) may also produce a central necrosis. Such infiltration with fat will cause narrowing of the sinusoids (164). Thus it would seem likely that the chlorinated hydrocarbons, by producing severe parenchymal swelling, can directly decrease circulation and produce changes in the central part of the lobule.

Summary. 1. Large doses of hepatotoxic agents, introduced into the hepatic portal circulation, directly produce hepatic necrosis.

2. In comparison, smaller doses of such agents cause parenchymal swelling which is associated with decreased blood flow through the hepatic sinusoids. The resultant ischemia produces a centrilobular necrosis. Anoxia *per se* will produce a similar necrosis.

D. Miscellaneous

Chlorinated hydrocarbons and young animals. The susceptibility to various hepatotoxic agents varies with the age of the animal. During the first three weeks of life puppies are less susceptible to $CHCl_3$ anesthesia than adult dogs (263). Graham (100) confirmed the resistance in puppies reported by Whipple and noted that when liver glycogen was decreased by fasting, phlorhizin or normal growth, the animals lost their resistance to $CHCl_3$. However, probably more important than decreased liver glycogen is the fact that the newborn animals are markedly resistant to anoxia (80). The administration of $CHCl_3$ to pregnant dogs shortly before delivery or during labor produces the same degree of liver injury as in normal dogs, but the liver of the fetus is not injured (262).

Following this initial period of resistance puppies then appear to become more susceptible to orally administered $CHCl_3$ (264). A similar result was obtained

with oral CCl_4 in that puppies one to three months of age were found to be more susceptible than adult dogs (93, 147). Both the mortality and the degree of liver injury was increased in such animals. On the other hand, the growing liver cells of young rats six to 50 grams in weight seemed to be markedly resistant to CCl_4 (33). Evidently species differences also exist that may depend on circulatory relationships or different methods of elimination.

Hepatotoxic agents and liver regeneration. Ligation of the hepatic artery in the dog does not delay the liver repair following exposure to $CHCl_3$ (264). Partial hepatectomy in normal rats is followed by a rapid regeneration of liver tissue. Mann and co-workers (169) noted that dogs with CCl_4-induced cirrhosis evidenced very little restoration of the liver after partial hepatectomy. Similar results were obtained in rats, in which species it was found that the removal of $\frac{1}{2}$ to $\frac{2}{3}$ of the liver, when advanced cirrhosis was present, was followed by little or no restoration of the liver. With less advanced cirrhosis some regeneration may be noticed. In the pre-cirrhotic stage partial hepatectomy was followed by complete restoration of the organ.

It has been reported that the liver regenerating after partial hepatectomy is more resistant than usual to CCl_4 (144) and $CHCl_3$ (7). Although Cameron and Karunaratne (33) were unable to obtain such results, others have confirmed the original reports. In dogs recovered from necrosis induced by uranium nitrate the livers showed a greater resistance to uranium and also to $CHCl_3$ (167). More recent studies have also demonstrated a resistance or decreased sensitivity of the liver of mice to subsequent doses of CCl_4, and hepatomas induced by CCl_4 were completely resistant (79).

III. DIETARY FACTORS AND LIVER INJURY

The relationship of diet to the hepatotoxic effects of $CHCl_3$, CCl_4 and other substances has been the subject of many studies during the past 35 years. More recent investigations have shown that dietary imbalance *per se* may produce liver injury and that choline and methionine can prevent such damage. Such findings soon led to the study of the effects of choline and methionine on liver injury induced by various toxic agents. Before considering these relationships the relative roles of choline, methionine, cystine and vitamin E in dietary induced hepatic damage will be briefly summarized.

Fatty infiltration and hepatic fibrosis. Prolonged fatty infiltration in the liver will lead to fibrosis. The increase in fat in the liver may be produced by two technics: a) feeding a diet rich in fat; b) feeding a diet low in fat but also low in protein, so that it is deficient in substances that normally prevent an accumulation of fat. The accumulation of fat in the liver may be prevented by substances termed "lipotropic". In initial studies lecithin was demonstrated to be lipotropic and the active material in lecithin was shown to be choline. However, it was soon shown that lipotropic activity was not limited to choline. Casein and other proteins were also observed to prevent the accumulation of fat in the liver; of the various amino acids then studied only methionine was found to be lipotropic. Du Vigneaud then demonstrated that the methyl groups of methionine may be

used for the synthesis of choline. Thus fatty livers may be prevented by diets high in choline or high in protein. Best and Lucas (20) have reviewed the studies in this field. Under certain conditions, still not clearly defined, other substances such as biotin, inositol, thiamine, riboflavin and pyridoxine may influence fatty livers provided by diets high in fat or cholesterol (173). Caffeine, theobromine and theophylline may be partially demethylated and thus possess lipotropic activity (120).

Massive hepatic necrosis. It was first suggested by Daft and associates (48) and later shown that acute massive necrosis can be produced independently of fatty changes and fibrosis in the liver (127, 130, 131). Animals surviving the acute massive necrosis developed postnecrotic scarring and nodular hyperplasia in the liver. The massive necrosis was produced by diets supplying small amounts of casein or containing yeast as a source of protein. The necrosis was prevented by supplements of cystine (130, 131) or methionine (128). Although methionine can be converted to cystine in the body, and cystine can spare the utilization of methionine, it has been shown that dietary induced necrosis is due to a deficiency of cystine (95). Such necrotic livers show only a slight decrease in methionine content and no change in the amount of cystine (55).

However, not all groups, including the author and his colleagues, were able to produce hepatic necrosis simply by feeding a low protein diet (110, 198, 213). In the meantime Schwarz had shown that a deficiency of vitamin E is also necessary to produce necrosis and that vitamin E would prevent massive necrosis (231, 232), a finding later independently confirmed (1, 2, 104, 129). Thus in order to produce massive hepatic necrosis the diet must be deficient not only in cystine but also in alpha-tocopherol, and adequate supplements of either cystine or alpha-tocopherol will completely prevent this type of liver injury.

Liver extract, vitamin B_{12}, folic acid and choline. It has been shown that liver extract is effective in preventing fatty infiltration of the liver induced by a high fat or low protein diet (60, 109). Of particular interest is the fact that liver extract does not contain sufficient choline or methionine to account for its lipotropic activity, and other supplements fail to prevent liver injury although they contain more choline than liver extract (60). Vitamin B_{12} concentrate prepared from liver also exerts lipotropic activity in rats fed a high fat diet (68), although the activity varies in different preparations (63). Crystalline vitamin B_{12} does not prevent hepatic necrosis or fatty infiltration resulting from a high fat diet but it can exert a partial lipotropic effect when a low protein, low fat diet is fed (106, 172). The activity of liver extract and vitamin B_{12} concentrate is not due to a sparing effect of vitamin B_{12} on the small amount of choline present (172). A combination of small amounts of crystalline vitamin B_{12}, choline, folic acid or citrovorum factor exerts only a partial lipotropic effect (145), and this does not explain the lipotropic effect of liver extract or vitamin B_{12} concentrate. Vitamin B_{12} concentrate has also been reported to inhibit the histological changes and the deposition of liver fat induced by CCl_4 (210).

Inanition, food intake, and response to drugs. In general the toxicity of many, but not all, drugs is increased in the fasted animal. Earlier studies on the effect of

restricted food intake or starvation on the toxicity of many materials have been summarized (133). When agents that can produce injury to the parenchyma of the kidney or the liver are administered, it is usually noted that such damage is increased by fasting. It is thus important in reading and comparing papers to note whether the animal has received food *ad libitum* throughout the study or whether food has been withheld for 24 to 48 hours before the administration of the hepatotoxic agent.

Even though food is not withheld the food intake will vary considerably, particularly during a chronic study. Many hepatotoxic agents produce a decrease in food consumption, thus limiting not only methionine or choline intake, but also important factors such as the other amino acids. This may in turn influence weight gain and mortality, independent of the degree of hepatic damage that is present.

During treatment, measurement of food intake is also important. A therapeutic substance may be effective because it produces an increase in food intake. For instance, thyroid feeding will increase the requirements for various B vitamins, and the feeding of yeast supplements will delay the onset of liver injury in hyperthyroid dogs. The yeast supplements are effective because they increase the food consumption of the animals (61, 69). The relationship of yeast and food intake to the effects of CCl_4 was studied by Post and co-workers (211). Using isocalorically fed rats, they found that large supplements of brewer's yeast partly counteracted the growth-inhibiting effect of CCl_4 but were without effect on the liver injury. In contrast to the yeast intake, the food consumption modified the severity of the cirrhosis. Animals fed eight to 11 grams of food per day had more severe liver lesions than those receiving 14 grams a day.

IV. LIVER FUNCTION TESTS

Various tests of liver function may be used to follow the progress of damage induced by hepatotoxic substances or dietary means. Drill and Ivy (62) made serial studies of such tests in dogs receiving CCl_4 twice a week. The determination of bromsulphalein retention was found to be the first and most consistent test to become abnormal, being closely followed by an elevation in alkaline serum phosphatase. The intravenous galactose tolerance test and changes in prothrombin time (one-stage technic) were not as sensitive as the other tests. Determination of serum bilirubin in the dog was of no value. The study emphasizes the quantitative association of the functional tests rather than a qualitative dissociation.

A comparative study of liver function tests was also made by Svirberly and associates (248) in dogs exposed to xylidine. Again the bromsulphalein test was found to be the most reliable one for detecting liver damage. The Rose Bengal dye retention test was nearly as sensitive, but had certain disadvantages. Next in order of value were the alkaline serum phosphatase and prothrombin determinations. The determination of bilirubin clearance and urinary urobilinogen gave still less consistent results. The van den Bergh reaction and determination of icterus index or fibrinogen were of little value.

This order of efficiency of liver function tests in dogs agrees well with the results obtained in human volunteers with induced hepatitis (57, 197). The dog differs from man, with regard to liver function tests, in that normal dog serum usually does not contain bilirubin. Injected bilirubin is also excreted faster in the dog than in man. The cephalin flocculation test cannot be used for experimental studies as normal dog serum produces flocculation. The thymol turbidity test was also noted to be valueless in detecting $CHCl_3$-induced injury in the dog (58).

Changes in dye retention (Rose Bengal) and alkaline serum phosphatase have been studied in protein depleted dogs (159). Irrespective of the diet, all dogs with increased liver lipids or cholesterol showed dye retention and an increased serum phosphatase. However, the degree of functional change was not necessarily proportional to the fattiness or cholesterol content of the liver.

The liver function tests are probably not affected by a decrease of body weight in the dog, provided sufficient lipotropic factors are supplied. Hough *et al.* (134, 135) produced an increase in dye retention and serum phosphatase in dogs fed a low protein diet, which could be prevented by the addition of casein, cooked egg white or choline. The changes in liver function do not seem to be related to a fall in body weight, for the animals receiving no supplements or a daily supplement of choline showed in general a comparable decrease in weight. Miller and co-workers (182) reported similar results, obtaining a decreased bilirubin clearance when the low protein, low choline diet of Hough was fed, but not when the low protein diet was supplemented with choline. In studies on the relationship of a chronic vitamin B complex deficiency and protein intake, Drill and Loomis (66) did not obtain changes in liver function in inanition control dogs receiving adequate yeast, even though body weight decreased.

V. HEPATOTOXIC AGENTS AND DIETARY FACTORS

A. Chloroform

Various substances, other than dietary factors, have at times been reported to protect against the effects of $CHCl_3$. Some of these agents are not specific and the effects of others, such as sulfanilamide, are unexplained. Quinine sulfate was earlier observed to exert a marked protective effect against $CHCl_3$ liver injury in dogs (53). Later Forbes and co-workers described an aqueous extract of hog's liver that prevented hepatic necrosis induced by $CHCl_3$ (88). The extract was purified, as described later under CCl_4, and the active material found to be sodium xanthine which also protected against hepatic damage produced by $CHCl_3$ (196). This action of sodium xanthine was confirmed by Ravdin *et al.* (215). However, they also showed that this effect was not specific for xanthines but could also be obtained with substances such as ricinoleate or colloidal carbon.

Sulfanilamide was also observed to protect rats against CCl_4-induced damage (cf. CCl_4). Forbes and Evans (84) extended their studies to include observations on the action of sulfanilamide during $CHCl_3$ inhalation in rats, and again

noticed protection as judged by the degree of hepatic necrosis. Similar findings were obtained in dogs given sulfanilamide for five days and fasted for three days before $CHCl_3$ anesthesia (250). In sulfanilamide-treated dogs, hypoprothrombinemia and histological changes were less severe than in control dogs. Sulfanilamide given during the anesthesia did not protect against the liver injury induced by $CHCl_3$. The possible relation of this action of sulfanilamide to thyroid activity is discussed under CCl_4.

$CHCl_3$ and diet. $CHCl_3$ was the first hepatotoxic substance to be studied in relation to diet. The early observations were made with mixed diets that would not now be considered satisfactory in all respects, and it was not until 1924 that synthetic diets containing known amounts of casein, carbohydrate and fat were used.

Opie and Alford first studied the relation of diet to the hepatotoxic effects of $CHCl_3$ (201–203). They noted that mortality following $CHCl_3$ was increased by a high fat diet. Meat feedings increased the survival period but best protection was obtained with a high carbohydrate diet of oats and sugar. However, in all groups animals which died early showed a similar degree of hepatic necrosis. These observations were later confirmed in dogs, a carbohydrate diet giving best protection, a high fat diet no protection, and a meat diet being intermediate (52). The feeding of casein to two dogs also offered protection whereas starvation increased the severity of the liver damage. At the same time Graham (100) also reported that the administration of glucose at various intervals decreased the hepatic injury produced by $CHCl_3$. Chloroform also increases nitrogen excretion, and it was found that the elimination of nitrogen and the liver injury following $CHCl_3$ were greater in a fasting than in a sugar-fed animal (51). Liver repair was also more rapid and complete on a sugar or high carbohydrate diet than during starvation. In a further comparison of diets, best repair of the liver following $CHCl_3$ exposure was obtained with a diet of bread and skim milk (54). Repair was slower with a diet of lean meat, whereas when fats were fed the rate of regeneration was not influenced and was similar to that observed in fasting animals. These initial studies all showed that protein, in the form of meat, offered less protection against $CHCl_3$ than a diet high in carbohydrate.

Further studies were reported in 1924 by Moise and Smith (187) who were the first to use diets composed of various proportions of casein, carbohydrate and fat in studying the responses to hepatotoxic agents. With such diets the responses to $CHCl_3$ were slightly different from those obtained with the earlier diets of meat or bread and milk. They fed a standard diet containing 18 per cent casein and 22 per cent fat. Increasing the fat in the diet produced more extensive hepatic necrosis whereas decreasing the fat content and increasing either the amount of carbohydrate or casein lessened the severity of the liver injury produced by $CHCl_3$. Both the high carbohydrate diet and the high protein diet thus offered greater protection than the standard diet. Further comparison between the high protein and the high carbohydrate diet could not be made as the fat content of the diets varied. The results of this study show that a high protein diet in the form of casein gives good protection as compared with the poor protection reported earlier for protein in the form of meat. Moise and

Smith also observed that the rate of repair was greatest with the standard balanced diet. In a further study (239) they noted that the protein gliadin did not offer as much protection as casein, and the substitution of gelatin further increased the degree of liver injury resulting from $CHCl_3$. Goldschmidt and coworkers (97) fed six different diets for periods of two to four weeks and found that the incidence and the severity of liver injury 24 hours after $CHCl_3$ anesthesia, increased progressively with an increase in the concentration of hepatic lipids. It is difficult to compare the effects of high and low protein intakes as the fat content of the diets varied. However, when rats with similar concentrations of hepatic lipids were compared, the total incidence of liver damage following $CHCl_3$ was not significantly different in high protein and low protein diets. The severity of damage on the low protein diet appeared to be greater, but the comparison was not strict as eight of 17 rats were fed only sucrose for the seven days before $CHCl_3$ anesthesia. The $CHCl_3$ anesthesia produced hepatic damage in 10 per cent of the animals fed a stock diet; the only synthetic diet giving a comparable low incidence of injury contained 16 per cent casein and 77.5 per cent sucrose. Diets high in carbohydrate or high in protein decreased the hepatic damage following $CHCl_3$ but more direct comparison cannot be made as the diets varied in fat content. The above reported studies are all acute in nature; no observations on diet in relation to chronic exposure to $CHCl_3$ have been reported.

$CHCl_3$ is very toxic to fasting animals and the injury produced to the liver is equal to or greater than that observed in animals fed a high fat diet (52, 97, 187). If dogs are *markedly* depleted of protein they also become very susceptible to $CHCl_3$ (184). Such animals were fed a diet *nearly devoid* of protein for four to nine weeks with, in most cases, additional bleeding or plasmaphoresis further to deplete protein stores. Such dogs, fasted for 24 hours before anesthesia, died 15 to 65 hours following a brief period of $CHCl_3$ anesthesia. It is of interest that even after such depletion a single meat feeding 24 to 48 hours before anesthesia protected against $CHCl_3$ injury (183, 184).

The degree of susceptibility to $CHCl_3$ depends a great deal on the severity of the protein depletion. In a further study dogs were fed a moderately low protein diet (eight per cent casein) and a normal (20 per cent casein) diet, both adequate in all other respects, for prolonged periods (58). $CHCl_3$ was then administered to each group and the changes in bromsulfalein retention, serum bilirubin, alkaline phosphatase and thymol turbidity studied. After nine weeks on the diets, $CHCl_3$ was administered orally and no difference in response between the two groups was observed. Dogs fed the eight per cent casein diet for 16 weeks and then anesthetized with $CHCl_3$ for one hour showed a slightly greater retention of bromsulfalein and a slower return of this function to normal, when compared with the 20 per cent casein group. In a further test the low protein diet was fed for 35 weeks, and such dogs showed only a slight degree of sensitivity to oral $CHCl_3$. In all cases the only difference between the groups was obtained with the bromsulfalein test; the results of other tests, although they became abnormal, did not differ significantly between the two groups.

Choline and methionine. Supplements of choline did not prevent hepatic necro-

sis following $CHCl_3$ anesthesia in rats receiving a low protein, high fat diet, or in rats fasted for 24 hours (97). The known effect of $CHCl_3$ to cause an increased excretion of nitrogen and sulfur (47, 137) and the fact that casein and beef muscle contain methionine and cystine soon led to a trial of these substances for the prevention of liver injury. This problem was first investigated by Miller, Ross and Whipple (183) who showed that methionine, and to a lesser extent cystine, given 24 to five hours before $CHCl_3$ anesthesia, offered almost complete protection to protein-depleted dogs. The dogs were fed a diet with nearly zero protein content for four to 10 weeks and were fasted 24 hours before the anesthesia. Supplements of other non-sulfur containing amino acids did not protect against the $CHCl_3$ poisoning. They also observed, in similar protein-depleted animals, that methionine protected against $CHCl_3$ toxicity when administered three to four hours after anesthesia, but not when given four to six hours later (185). In view of later studies with CCl_4 it is not now known whether the liver, kidney or other organ was the site of action of the methionine. In other studies rats fed a diet moderately deficient in protein (eight per cent casein) and a normal (20 per cent casein) diet were exposed to chronic inhalation of $CHCl_3$ for a 70-day period. The $CHCl_3$ caused a decrease in the weight gain of each group, not corrected by methionine. Similarly the hepatic lesions produced by $CHCl_3$ were not significantly changed by methionine in rats on either diet (59). These results are similar to the lack of effect of methionine on the liver of rats during chronic CCl_4 exposure. It is obvious that the response to $CHCl_3$ and the effect of methionine will vary tremendously with the degree of protein restriction and perhaps also with the chronicity of the study.

It has also been reported that methionine and sodium thioglycollate protect against liver injury induced by $CHCl_3$ anesthesia in dogs receiving a normal stock diet (31). Liver injury was estimated by the bromsulfalein test and the data based on $CHCl_3$ exposures two months apart. Twenty-four hours after the initial $CHCl_3$ exposure the bromsulfalein retention for each group varied between 22 to 39 per cent. A similar average spread and variation of 17 per cent were present after the second $CHCl_3$ exposure. Further, the control group and the methionine-treated group differed from each other by about 10 per cent retention of dye, the values still lying within the spread of 22 to 39 per cent retention. With this variability it is difficult to conclude that there is a positive effect of methionine, particularly because each curve is only an average, without the range or the standard error being given. The bromsulfalein test, although sensitive in detecting liver injury, varies considerably with regard to individual animals and on different days, as may be seen in related data on dogs (65, 67).

Clinical studies. Although no clinical studies of methionine or choline therapy during $CHCl_3$ exposure have been reported it should be pointed out that the best effects of methionine have been obtained in animals fed a diet nearly devoid of protein for many weeks. Such conditions are not likely to be comparable to those seen in man, a fact overlooked by many who have misinterpreted the experimental studies with $CHCl_3$ and applied the conclusions to other instances of liver injury under different dietary conditions.

Summary. Both a high fat diet and fasting increases the hepatotoxic effect of $CHCl_3$. Diets low in fat and rich in carbohydrate and protein give the best resistance to $CHCl_3$ and allow active regeneration of hepatic cells. Moderately protein-deficient diets only slightly affect the response to $CHCl_3$ whereas diets nearly free of protein markedly increase susceptibility. Methionine has been the chief supplement studied, and the best effect of this material has been obtained in animals fed a diet nearly devoid of protein for a prolonged period.

B. Carbon tetrachloride

Various substances exist which display a protective effect against CCl_4-induced injury. The effects, although reproducible, often are not specific, and do not seem to be related to known actions of dietary factors. In 1936 Forbes and coworkers reported the preparation of an aqueous extract of hog's liver that protected against hepatic necrosis induced by CCl_4 in rats (88). The material also prevented cirrhosis following repeated exposures to CCl_4 (83). A crystalline material was then prepared from the extract which was identified as sodium xanthine (87, 196). Either the purified crystals or sodium xanthine offered protection against the hepatic necrosis induced by CCl_4 (14, 82, 136, 196). The protective effect of xanthine does not seem to be related to the concentration of liver lipids (85). However, similar effects were obtained with guanine, hypoxanthine, uric acid, nucleic acid, nucleoside guanosine and pyrimidine uracil (196). In view of related studies with $CHCl_3$, in which protective effects were obtained not only with sodium xanthine but also with caffeine, sodium ricinoleate and colloidal carbon (215), the effect of sodium xanthine must certainly be regarded as non-specific, although unexplained. In acute studies with CCl_4, protection against liver injury was reported with sodium thioglycollate, glutathione, sodium thiomalate, sodium glycollate and sodium malate (30). Theophylline is also reported to decrease mortality following the intraperitoneal injection of CCl_4 in rats receiving a 10 per cent casein diet (136).

Leach and Forbes noted that sulfanilamide protected the liver of rats from CCl_4 (154). In a subsequent study sulfanilamide also retarded the development of hepatic cirrhosis produced by CCl_4 (86). This effect has also been confirmed in studies with $CHCl_3$. Leach and Forbes suggested that the activity of sulfanilamide might be related to its known ability to inhibit thyroid function. It is of interest that subsequent studies demonstrated a preventive effect of thiouracils or thyroidectomy on dietary induced liver injury (115, 107). However, the lowering of thyroid activity by thyroidectomy or thiouracil, in experiments with pair-fed controls, failed to prevent the liver injury induced by CCl_4 (9), which suggests that the mechanism of the protective effect of sulfonamide in CCl_4 injury is different from that in dietary induced damage.

CCl_4 and diet. All investigations have shown that diets high in fat increase the acute toxicity of CCl_4 (50, 85, 93, 147, 218). Similar results were obtained with the chronic inhalation of CCl_4 in rats (108). In one study a high fat diet did not increase mortality in dogs receiving CCl_4, but the diet was fed for only four days (46).

Best protection against liver injury induced by CCl_4 seems to be offered by diets high in carbohydrate. Initially Davis (50) reported less hepatic damage from CCl_4 with a carbohydrate diet. The administration of glucose before the inhalation of CCl_4 was noted to protect the liver of cats but not of dogs (39). In rats, glucose or sucrose was without effect when given before CCl_4 inhalation, but when administered at intervals after exposure prevented the usual increase in liver fat (56). Similar results were obtained in dogs. Treatment for 24 hours did not influence the degree of hepatic necrosis but if continued for two to four days less liver injury was evident. Inanition will also increase susceptibility to CCl_4. Rats with fatty livers from a low protein, high fat diet, starved for 24 hours, were only slightly more susceptible to CCl_4 than normal animals starved for the same period of time (85). Bollman, in various studies, always obtained best protection with a high carbohydrate diet (26–29). His dogs fed a high protein diet (meat) showed greater histological damage after CCl_4 than those fed a high carbohydrate diet. Similarly, in studies on rats a high carbohydrate diet gave the least evidence of histological damage and the longest period of survival. In a discussion of one paper (28) Soskin states that his unpublished results are in agreement with those of Bollman.

The death of exposed animals may be due to factors unrelated to liver injury. Cutler (46) noted that dogs fed a diet of lean meat were much more susceptible to CCl_4, as judged by mortality, than animals receiving a mixture of lean meat and dextrin or bread and milk. However, although the mortality differed, he was unable to demonstrate any consistent difference in the amount of liver injury produced by CCl_4 with either diet. It is known that intoxication with CCl_4 and other substances produces an increase in blood guanidine which in turn produces hypoglycemia. It has been shown that the increased toxicity of CCl_4 with a high meat diet is correlated with an increase in blood guanidine followed by a severe and often fatal hypoglycemia (45). In a series of studies (186), it has been repeatedly shown that calcium administration, by counteracting guanidine, will markedly increase the survival of meat-fed animals without affecting the incidence or the severity of the liver injury. Thus the susceptibility of animals fed high protein diets in the form of meat is not necessarily related to the lipotropic activity of the diet.

More recent studies also fail to show that a low casein diet increases the liver injury produced by CCl_4. With a constant fat intake, a high carbohydrate, low protein diet increased mortality during chronic CCl_4 exposure but did not produce any significant difference in hepatic histology when compared with a high protein, low carbohydrate diet (108). However, when rats receiving low casein and normal casein diets were pair-fed no difference in survival time was found after CCl_4 administration (116). In studies of liver injury in dogs, as measured by the bromsulphalein test and histologically, hepatic damage was greater in animals fed the normal protein diet than in those fed a low protein, high carbohydrate diet for three and one-half or 16 weeks (67). The fat content of both diets was constant and there was no significant difference in food intake between the two groups. In other studies rats were placed on a protein-free diet, 18 per

cent casein diet or 54 per cent casein diet for three days and then injected with CCl_4 (36). Liver injury was least on the protein-free diet. Those fed the 18 per cent casein diet showed the greatest degree of hydropic degeneration and those on the 54 per cent casein diet the greatest deposition of neutral lipids.

Choline, methionine and vitamin E. The effect of methionine has been studied under conditions of high, normal and low protein intakes. Drill and Loomis were unable to obtain any added protection against hepatic injury induced by the oral administration of CCl_4 when methionine was added to a high or normal protein diet (64, 65). The results were similar in dogs receiving CCl_4 for short periods of time or up to 64 days. In order to eliminate the factor of absorption Shaffer *et al.* injected CCl_4 intraportally but also failed to find an effect of methionine on liver function tests, hepatic histology or survival of the dogs (235). They were also unable to find any effect of subacute inhalation of CCl_4 on the nitrogen-sulfur ratio of the livers of rats fed a normal diet (236). Supplements of methionine, choline or cystine were also found to be without effect on the hepatic lesions induced in rats fed a normal diet (30, 234).

Choline supplements do not prevent an increase in hepatic lipids of rats receiving a low protein diet and injected with CCl_4. On the other hand, the addition of choline increased the rate of removal of excess fat from the liver of the poisoned animals (13). With a low protein diet supplements of methionine during chronic CCl_4 inhalation increased survival but did not influence the liver injury produced (108). The results were similar with either a normal or high amount of fat in the diet. A sex difference in response was also noted, male rats being more susceptible than female rats. The methionine supplements increased survival time not by affecting the liver but by preventing the renal necrosis induced by CCl_4. Methionine was also without effect on the liver injury produced by CCl_4 in dogs receiving a low casein diet, as judged by functional and pathological changes in the liver (65).

The influence of diet and various supplements on cirrhosis induced by CCl_4 has been studied by Sellers, Lucas and Best (233). When such animals were placed on a low casein diet the hepatic lesions did not improve but, as expected, progressed. However, after treatment for two to three months with the low casein diet plus adequate supplements of choline or methionine the liver appeared grossly normal and it was difficult to find fibrous tissue on microscopical examination. A high protein diet containing naturally occurring methionine produced similar results. Supplements of inositol were without effect. It is obvious that the cirrhosis produced by CCl_4 was still in a reversible stage, as discussed previously (cf. II, B), and a more advanced cirrhosis probably would remain unaffected. The authors conclude, "While the experimental evidence indicates that the presence of the lipotropic agents is essential for the repair of the damaged liver under the conditions of our experiments, it does not suggest that the addition of choline or its precursor, methionine, to diets already containing adequate amounts of these factors will enhance the therapeutic effect. . . ."

It has also been recently reported that the addition of methionine or vita-

min E to a low (10%) casein diet will decrease the mortality from acute toxic doses of CCl_4 (136). Protection was not obtained when the diet contained 22 per cent casein. Studies were not made to determine whether the effect of vitamin E or methionine was on the liver or other organs. However, these findings are of interest, particularly with regard to vitamin E, for as summarized in Part III a deficiency of tocopherol or cystine can produce hepatic necrosis.

Clinical studies. The use of methionine received impetus from the report of Beattie and associates (18) who described its administration, along with casein digest, in a patient who ingested an estimated 30 to 40 cc. of CCl_4. They concluded that methionine exerted a beneficial effect. However, survival from as much as 118 to 148 cc. of CCl_4 has been reported before the advent of methionine (156), so that it cannot be definitely stated that methionine was of value in the above case. Methionine has also been administered to patients following industrial exposure to CCl_4 (71, 72), but a critical reading of these papers does not reveal any evidence of beneficial effect of methionine in the treatment of liver injury.

Summary. Diets high in fat increase the toxicity of CCl_4. A diet high in carbohydrate and protein offers the best protection against the effects of CCl_4. Moderately protein-deficient diets do not increase the hepatotoxic action of CCl_4 although mortality may be greater due to renal injury. Choline does not prevent the increase of liver fat produced by CCl_4, but increases its rate of removal from the liver.

Supplements of methionine have not been shown to decrease the liver injury produced by CCl_4 in animals fed a normal diet or a moderately low protein diet, although renal injury occurring on the low protein diet may be prevented. Thus there is no experimental support for the use of methionine to combat liver injury induced by CCl_4 in patients and certainly, despite claims to the contrary, there is no good clinical evidence for such an effect. This does not deny a possible effect of methionine on other organs, or its possible value in liver lesions in the face of very severe protein depletion, which has not as yet been studied. When early cirrhosis is induced in rats by CCl_4, repair of the liver occurs when a normal diet is fed, but there are no indications for supplemental methionine.

C. Ethylene dichloride (1,2-dichloroethane)

Ethylene dichloride is one of the more toxic chlorinated hydrocarbons. Single inhalation exposure has been reported to produce fatty degeneration in the heart, liver and kidney (153, 195). More complete studies have recently been reported by Heppel and co-workers (118, 119). They noted that pulmonary congestion is a constant finding after inhalation, and that many animals develop adrenal cortical necrosis. Renal and liver damage are also produced but the liver injury is very mild compared to that resulting from acute exposure to carbon tetrachloride or chloroform (119).

Normal diet and protective agents. Various chemical agents were found to protect mice, fed a normal diet, against death from dichloroethane (119). Protection was obtained by the oral administration of *p*-aminobenzoic acid, methionine, sulfanilamide and aniline. In one additional test, methionine was administered

after exposure to a high dose of ethylene dichloride, but failed to decrease the ensuing mortality. These compounds, with the exception of methionine, are without known effects on the liver and the liver injury was so slight the decrease in mortality cannot be due to the prevention of liver injury. Since the compounds were administered 30 to 60 hours before the inhalation of ethylene dichloride they probably did not act by depressing the thyroid gland. It is interesting to note that the compounds, other than methionine, all contain an amino group in a benzene ring, but the mechanism of their protective action is at present unknown.

In a further study in rats fed normal diets containing 15 to 25 per cent casein, good protection against death from the inhalation of ethylene dichloride was obtained by the addition of *l*-cystine, *l*-cysteine, methionine, thiourea or thiouracil to the diet (121). Partial protection was obtained with dithiodipropionic acid or 2-thiobarbituric acid. These compounds all can furnish sulfhydryl groups, either present in the compound, or by reduction to thiol compounds, or by existing in a tautomeric equilibrium with a thiol form. Cysteine was not effective when its SH group was blocked as in S-benzyl-*l*-cysteine. Also inactive were cysteic acid, $Na_2S_2O_3$, Na_2SO_4, Na_2S, taurine, tyrosine, thiolactic acid and thiomalic acid, although NaCNS gave a partial effect. Although *p*-aminobenzoic acid offered protection when given by stomach tube it was without effect when added to the diet. Others have also noted a beneficial effect of thiouracil (270). It is doubtful, however, if all the above effective compounds act through the thyroid gland. In current observations activity seems to be correlated with SH groups although earlier studies demonstrated an effect of other unrelated compounds. It is also unknown whether the decreased mortality in animals fed a normal diet is due to an effect of the compounds on the liver or on other organs.

Methionine and choline. Heppel and co-workers also studied the relationship of dietary protein, fat, methionine and choline to ethylene dichloride exposure (118). A low casein or low casein-high fat diet increased mortality following single or multiple inhalations of ethylene dichloride. A supplement of choline in pair-fed rats fed a low casein, high fat diet decreased mortality after one exposure, but did not significantly change mortality following two exposures. When a lower concentration of ethylene dichloride was used, a combination of choline and methionine decreased mortality following multiple inhalations to the level obtained in control rats. Although the diet and supplements can markedly alter mortality, correlations between liver fat and mortality are less definite. Increased liver fat is not associated with increased mortality, for in single exposure studies rats may die with an average liver fat of 7.5 gm. % and survive with 12.6 gm. %. This lack of correlation is seen even more clearly following repeated exposures to ethylene dichloride, as follows:

	Av. liver fat
1. Normal diet, died	6.7 gm. %
2. Normal diet, survived	4.5 "
3. Low casein diet, died	14.8 "
4. Low casein diet, plus methionine and choline, died	7.0 "
5. Low casein diet, plus methionine and choline, survived	10.7 "

It is evident that the effect of choline and methionine in decreasing mortality is probably not related to lipotropic or other effects on the liver. As pointed out above, in animals on normal diets, ethylene dichloride produces only minimal hepatic lesions. This slight hepatotoxic effect is not enhanced by low protein or low protein-high fat diets for Heppel and associates (118) state: "In fact, the histological appearance of liver of rats dying after dichloroethane showed nothing to distinguish them from livers of rats fed similar diets but not exposed." Although it is only concluded that methionine and choline reduce mortality when added as supplements to low choline diets, statements in the literature often erroneously imply that the protective effects of methionine and choline are lipotropic in nature. The adrenal cortex may be important and should be further investigated. Ethylene dichloride produced necrosis and hemorrhage of the adrenal cortex in a large number of animals fed low casein, high fat diets but only in a few of the animals receiving the dietary supplements.

In a further study the addition of methionine, *l*-cystine or choline plus cystine to a low protein diet decreased mortality from ethylene dichloride, whereas choline, arginine, valine or alanine were without effect (121). Choline decreased liver lipids but did not affect mortality whereas cystine protected, but did not decrease the concentration of hepatic lipids. The data would seem to show that the fat content of the liver is less important than the availability of sulfhydryl groups in decreasing mortality from ethylene dichloride.

In a recent histological study, the mortality and the incidence of fatty changes following a single injection of ethylene dichloride were reduced by methionine, 1,2-dimercaptopropanol (BAL) or cysteine (124). The most consistent and pronounced reduction in the incidence of fatty changes were obtained in the heart, to a lesser extent in the liver, and least of all in the kidney.

Summary. A variety of agents will decrease the mortality of animals fed a normal diet and exposed to ethylene dichloride. The mechanism of these effects is unknown. When animals are fed low protein diets the change in liver lipids is not related to mortality. Similarly the protective effects of methionine or cystine and the lack of effect of choline, when added to low protein diets, are unrelated to changes in hepatic lipid. Such protective effects of methionine or cystine may be on the kidney or other organs of the exposed animals.

D. Propylene dichloride (1,2-dichloropropane)

Propylene dichloride is one of the more toxic halogenated hydrocarbons producing injury to various organs after oral administration (268) and particularly following inhalation (123). The inhalation studies are the most extensive; necrosis occurs in the liver, kidney and adrenal cortex.

Mortality in young rats following multiple inhalations of dichloropropane was markedly increased by a low casein diet or a low casein, high fat diet (117). The addition of choline to the low protein diet did not affect mortality whereas the addition of methionine or choline plus *l*-cystine markedly increased resistance to the effects of dichloropropane. Pathological changes were found in many organs and it is not known whether the protecting substances acted on

the liver, kidney, heart or adrenal cortex to decrease mortality, although evidence does seem to point more toward a renal effect.

E. Ethyl alcohol

Many studies have been performed to determine whether alcohol exerts a direct toxic effect on the liver. In earlier studies Opie and Alford (202) were unable to influence the mortality or produce significant hepatic lesions when single doses of alcohol were administered to rats receiving various diets. In the meantime it was shown that alcohol would increase the hepatotoxic effects of carbon tetrachloride (147) and chloroform (223). Since then a number of studies have been performed with alcohol, only a few of which will be reviewed here. Lowry and co-workers (166) reported that alcohol increased the severity of cirrhosis produced by deficient diets in pair-fed rats. In 1947 Ashworth reported that ethyl alcohol could directly produce hepatic lesions in rats (11). However, the total caloric intake of his two groups was different, for the added calories provided by the ethyl alcohol were not controlled. Further, the alcohol was administered in amounts that often produced coma, leading to a marked restriction of food intake and weight loss. The problem was re-investigated by Best *et al.* (19) who concluded that alcohol does not exert a specific toxic effect on the liver. They used pair-fed alcohol and non-alcohol rats who received isocaloric amounts of glucose in place of alcohol. The diet used contained adequate vitamin supplements, allowed a good food intake and growth of the animal, but was slightly deficient in choline and methionine. Under these conditions fatty infiltration and an early fibrosis of the liver was produced in rats receiving ethyl alcohol or isocaloric equivalents of glucose. These hepatic changes could be prevented by supplements of choline, methionine or casein. It was concluded that when the diet was marginal with respect to lipotropic factors calories in the form of alcohol or glucose would induce a relative choline deficiency producing liver injury. Further studies were reported by Klatskin and co-workers in 1951 (140). They re-investigated the problem as to whether alcohol increases choline requirement by augmenting the calorie content of the diet, and secondly whether alcohol will produce fatty infiltration of the liver when the diet is adequate with respect to protein, lipotropic factors and vitamins. In general the results are in agreement with those of Best. With the diet they used neither the alcohol nor isocaloric glucose-fed controls developed significant fatty infiltration or cirrhosis of the liver. Chemical analyses for liver fat showed only a slight increase, but still within the normal range, in the groups receiving ethyl alcohol. However, this increase in liver fat was noticed only in animals sacrificed at 117 days. No significant change in liver fat was obtained in animals sacrificed at 180 days.

It seems most likely that alcohol in doses that can be sufficiently tolerated to allow a gain in weight may produce fatty livers when the diet contains a minimal amount of choline. This effect is probably chiefly due to the calories supplied by the alcohol during a fixed intake of lipotropic factors, although it is possible that under some conditions alcohol may increase the requirement for choline by another means, perhaps by blocking in some manner its action within the liver.

Clinical studies. From the various data in the literature it can at least be said that cirrhosis of the liver is more commonly found in alcoholics than in non-drinkers. Clinically the effect of alcohol is believed to be only indirect inasmuch as it supplies calories but not lipotropic factors, decreases appetite and tends to lead to an imbalanced diet. This view is supported by the more recent experimental evidence. The treatment of the liver injury by dietary means received much impetus from the work of Patek and co-workers (206). They treated such patients with a diet high in calories and protein plus supplements of crystalline B vitamins and yeast. Such a diet will supply an adequate amount of lipotropic factors but there is no good evidence in the literature for an added effect of supplements of choline, methionine or inositol. In other studies reporting an effect of such supplements, control experiments are lacking, the need for which is particularly emphasized in a recent paper by Klatskin and Yesner (141) who obtained marked histological improvement in the liver by removal of alcohol, bed rest and an average hospital diet.

F. Benzene

The effects of chronic benzene exposure vary markedly with the diet and this relationship has been carefully studied by Li, Freeman and their associates (158, 160, 161). Dogs fed an adequate stock diet or a low fat, high protein diet were quite resistant to chronic benzene poisoning, surviving the 52-week period of study with a gain in weight. A high fat, high protein diet increased susceptibility to benzene slightly. Survival period was greatly decreased and there was loss of weight in animals on diets low in protein, independent of a low or high fat content. There was no relationship between the length of survival and the liver lipids or the level of serum phosphatase. Hepatic dye clearance (Rose Bengal test) tended to change early but was not associated with changes in liver lipids. The production of leucopenia, thrombocytopenia and anemia was most marked in animals fed the low protein diets. The percentage of conjugated sulfates in the urine increased in all animals during exposure, but most markedly in those fed the low protein diets (161).

Similar results were obtained during the chronic exposure of rats to benzene. Although benzene produced a leucopenia in all groups the change was greatest in those animals fed low protein diets (158). Total liver lipids were increased in control rats fed low protein diets, being higher than in the corresponding benzene-exposed groups. Supplements of methionine increased growth and tended to reduce the incidence of leucopenia in protein deficient rats receiving benzene (160). However, as judged by the growth curve and the number of leucocytes, the benzene exposure was still toxic. The low protein diet increased liver lipids and methionine was equally effective during benzene exposure or control exposure to air in maintaining a normal liver fat content. The expected lipotropic effect of methionine is not interfered with by benzene exposure.

Summary. A low protein diet increases the susceptibility to benzene, which may be partially counteracted by methionine. Neither the increased susceptibility to benzene nor the effect of methionine seems to be related to total liver lipids.

G. Brombenzene, methyl chloride

Brombenzene. Both brombenzene and iodobenzene are excreted in the urine as the respective halogen-phenylmercapturic acid. The incorporation of brombenzene into a low protein (six per cent) casein diet will retard growth. Supplements of cystine or methionine, which cause an increased excretion of organic sulfur, will promote growth of rats receiving brombenzene, whereas taurine or sodium sulfate is ineffective (265).

Methyl chloride. The survival of rats inhaling methyl chloride is decreased by diets low in protein. Supplements of cystine and methionine increase survival time even when added to a diet containing 20 per cent casein (244).

H. Trinitrotoluene

Although exposure to trinitrotoluene (TNT) has been reported to produce liver damage in man, it has been difficult to produce hepatic lesions with this substance in laboratory animals. In earlier studies single doses of TNT produced fatty degeneration in only two, with necrosis in one, of ten rabbits (189), although some died too early to develop hepatic lesions. The chronic administration of TNT to three cats and two rabbits failed to produce any significant change in the liver (269). Similar difficulties in producing lesions were experienced by other investigators (205, 255).

Dietary factors. In a more recent study, Himsworth and Glynn (126) were able to produce fatty changes and liver necrosis during the chronic administration of TNT to rats fed a low protein, high fat diet. The lesions were centrilobular and occasionally the necrosis was acute and accompanied by intravascular clotting in the central vein. The liver injury produced is of a delayed type for the animals dying during the first ten days were free of hepatic lesions. They concluded that the injury depends on a high fat intake inasmuch as the lesions were absent in rats receiving a high protein diet or a low protein, high carbohydrate diet. Although the two diets, low protein-high carbohydrate and low protein-high fat, contain a similar amount of protein, the protein content per 100 calories of diet will be lower in the high fat diet. Thus the injury produced by TNT could be more related to the lower protein intake per day, which would be obtained with the high fat diet.

In studies on rats, using a higher oral intake of TNT, Smith and co-workers (243) were able to produce hepatic injury in 20 per cent of the animals receiving a normal protein diet. These changes were produced with a low percentage of fat in the diet. The incidence of liver injury was not decreased by increasing the casein content of the diet to 37 per cent. It is difficult to judge from this report whether TNT added to a five per cent casein diet increased the severity of the hepatic lesions that would be expected in rats given a low (five per cent) casein diet alone for 60 days. Supplements of cystine or ascorbic acid were without effect on the liver damage. However, the addition of one per cent methionine to the diet prevented fatty livers in animals fed the low casein diet, whereas rats fed 20 per cent or 37 per cent casein diets still showed fatty changes. Methionine would seem to be of greater value than the high protein diet.

Commercial TNT contains three isomers and Barger and Tutin (12) have shown that the β and γ isomers, but not the α isomer, can combine with certain amino acids. A high protein diet might therefore offer protection by allowing the TNT to combine with protein and thus to become nontoxic. However, as pointed out by Himsworth and Glynn (126), pure α TNT produces the same changes as commercial TNT so that this possibility seems unlikely.

Clinical studies. Exposure of man to TNT may produce toxic effects among which are hepatic necrosis and fibrosis (49, 149, 249). The injury is of a delayed type, as seen in rats, and histologically is similar to that produced experimentally by Himsworth and Glynn. Eddy has reported that methionine produced remarkable improvement in patients with hepatitis due to TNT (71, 74). Although the number of cases treated is not given, some are outlined in detail. Without any controls for comparison it is difficult to say that methionine had any effect, particularly since the patients were also treated with bed rest, a high protein diet, vitamin B complex and a multiple vitamin product. (Two other patients with epidemic hepatitis were treated with methionine, which is now known to be without beneficial effect, with striking improvement in 48 hours.) From a critical reading of these publications it must be concluded that no beneficial effect of methionine on hepatitis resulting from TNT exposure has been demonstrated.

I. Pyridine

Pyridine is reported to have produced serious renal and hepatic damage following prolonged administration in two of five subjects (209). Experimentally pyridine will produce renal and hepatic injury; if the liver damage is continued for a sufficient length of time, it results in cirrhosis (16).

Diet and methionine. The addition of pyridine to a diet low in protein (10 per cent casein) rapidly affects growth and produces death with liver and renal injury (15). Increasing the amount of casein to 20 per cent or the addition of small amounts of methionine increased growth but did not affect survival. Larger amounts of methionine, even when added to a 25 per cent casein diet, increased survival time.

In further studies survival was increased by supplements of methionine, cystine, cystine plus choline, but not choline alone, when added to diets containing from 10 per cent to 25 per cent casein (17). This protective effect is not due to an increase in food intake. It is stated that the increased survival was "apparently due to prevention or reduction of liver and kidney injury," although no data on this point are given. Mention is made that choline, although not affecting mortality, did modify the hepatic lesions in the pyridine-treated animals receiving the 10 or 18 per cent casein diets. Other studies confirmed the increased survival time in pyridine-exposed animals when methionine or cystine was added to a 25 per cent casein diet, whereas choline was again ineffective (43). It is difficult to interpret the histological data presented in this paper, particularly because the authors rely more on increase in liver water as reflected in liver weights than on histological study. Liver fats, for the same duration of

exposure to pyridine, were slightly decreased by methionine and choline, but as the determination was on pooled livers the significance of the difference cannot be determined.

Although the above supplements may increase survival to toxic doses of pyridine, it cannot be stated definitely at the present time that this effect is actually related to prevention of liver or renal injury or of damage to other organs.

J. Azo compounds

Azo compounds and diet. It has been shown that various azo compounds may produce liver cancer in rats. Dimethylaminoazobenzene, first reported by Kinosita (cf. 247), is highly active in this regard. In initial studies animals were fed diets with rice as the basal food (rice-carrot diet), so as to correspond to the diet of the rice-eating countries of the Orient, where the incidence of hepatic cancer is high. With this diet dimethylaminoazobenzene will produce hepatic tumors in 80 to 100 per cent of the animals (cf. 247). The pathological changes induced have been studied in detail (78). The development of the cancer is inhibited by supplements of rice bran extract, liver or yeast (139, 247). The addition of 18 per cent casein to the rice-carrot diet has been reported to be either without effect (247) or to decrease the incidence of the tumors (105, 139).

Using a more balanced diet, Miller *et al.* (181) obtained a protective effect with casein, liver, eggs and yeast protein. They noted that best protection against the hepatic tumors was obtained when the diet was rich in both protein and the vitamin B complex, particularly riboflavin. In one report tumors were not produced when the rats were fed synthetic diets containing six or 18 per cent casein (105). However, with a higher intake of dimethylaminoazobenzene, tumors were produced but the incidence was not significantly different in diets containing five or 37 per cent casein (241). Yeast and egg protein were protective. White and Edwards (267) also obtained tumors in rats fed a six per cent casein diet.

The riboflavin intake and content of the liver are also important, and supplements of casein and riboflavin to the rice-carrot diet markedly decreases the tumor incidence (139). Biotin is also reported to exert a procarcinogenic effect (70, 152). Variation in the amount of such substances in the diet may account for some of the above reported differences, particularly with casein supplements.

Lipotropic substances. Miller *et al.* (181) were unable to prevent the tumors by supplements of xanthine, cystine, inositol or choline. Gyorgy *et al.* (105) reported that supplements of cystine with choline, but not choline or cystine alone, prevented the occurrence of the tumor in rats fed the rice-carrot diet. However, others were unable to obtain such a protective effect with cystine, cystine plus choline, or methionine (267).

K. Phosphorus

Phosphorus produces chiefly fatty changes and necrosis in the liver but may cause parenchymatous changes in the kidney, muscle and other organs. Cirrhosis of the liver can be produced by the chronic administration of phosphorus (168).

Phosphorus and dietary factors. Opie and Alford (202) noted least mortality

with a high carbohydrate diet and the intensity of necrosis was greatest when a meat diet was fed. Rettig also observed protection with a high carbohydrate diet (216). The fatty infiltration of the liver was less with such a diet and the usual increase in nitrogenous elimination following the administration of phosphorus to fasting animals was decreased or prevented by carbohydrate. A protective effect of sugar against phosphorus poisoning is also reported (237), and Laszt and Verzar (151) state that iodoacetic acid will prevent the fatty infiltration resulting from phosphorus.

The effect of choline on such liver injury was first studied by Best, MacLean and Ridout (21). They noted in rats fed a low protein diet that choline increased the rate of disappearance of fat from the liver during the recovery phase of phosphorus poisoning but did not inhibit the deposition of fat which occurred after the administration of the phosphorus. The choline influenced only the rate of removal of fat and was without effect on the other degenerative changes produced in the liver by phosphorus.

Radioactive phosphorus and diet. Severe protein depletion, induced by feeding a diet containing 1.9 per cent protein with an associated loss of 25 per cent of body weight, increased the susceptibility to total body irradiation (138). Other studies have been reported on the subacute toxicity of radioactive phosphorus with diets varying in their casein and fat content (42). In rats fed a 10 per cent casein diet the LD_{50} of radioactive phosphorus was not influenced by fatty infiltration of the liver, induced by a deficiency of choline with or without partial poisoning with CCl_4 or by supplementing the diet with choline to prevent the fatty infiltration. Thus, although severe protein depletion may increase radiosensitivity, moderate depletion is without effect. In fact, survival following P^{32} was shortest when the diet contained 25 per cent casein. Supplements of folic acid and vitamin B_{12} had no significant effect on the survival of mice injected with P^{32}. However, when succinylsulfathiazole was added to the diet to decrease bacterial synthesis of vitamins in the intestine the animals became more susceptible to P^{32}. The administration of vitamin B_{12} and folic acid to such animals returned the survival rate nearly to normal (41).

L. Arsenic

Hepatic injury can be produced by various compounds containing arsenic, such as arsenates, arsenious oxide, arsphenamine, neoarsphenamine and dichlorophenarsine (89, 90, 113, 142, 143, 257). Large single doses of organic arsenicals can produce widespread necrosis particularly about the central and middle portion of the lobule. After repeated administration of these materials a focal necrosis is generally produced particularly in peripheral portion of the lobule. Periportal fibrosis of various degrees can also be obtained.

Arsenates and diet. The chronic administration of arsenates to rabbits will produce cirrhosis. Modification of the diet by the addition of carrots or a carbohydrate diet decreased the incidence of cirrhosis (257). In a further study with rabbits it was observed that supplements of yeast, added to a normal diet, increased survival and decreased the incidence of liver injury (256). The mecha-

nism of this effect is unknown, although it is possible that the action of the yeast is chiefly to increase food intake.

Organic arsenicals and diet. Dietary studies tend to show that a high fat intake increases the susceptibility to liver injury, although with some of the diets used the results are often difficult to interpret. In initial studies, a diet of bread, oats and milk increased survival to single doses of arsphenamine as compared with a high protein diet of lean beef heart (133). However, hepatic necrosis was not common, the chief lesion produced being necrosis of kidney epithelium. Conclusions regarding the effect of high fat diet on liver injury produced by arsphenamine are not in agreement (44, 226).

More recent studies have been performed by Hawkins and associates. They noted that arsphenamine produced severe liver injury as judged by icteric index and histologic changes when a high fat diet was fed (178). Either a protein (meat) or a carbohydrate diet was beneficial, the protein diet giving slightly better protection against hepatic damage. It is of interest that the carbohydrate diet was effective even though it contained a very low amount of protein (maximum 3.5 per cent protein). Further, the high fat diet was grossly unbalanced, being devoid of protein or carbohydrate. It was also demonstrated that methionine protected against oxophenarsine-induced liver injury in protein depleted dogs (98). All dogs were fasted for one week and then placed on a diet nearly devoid of protein for various periods. Some were also subjected to plasmaphoresis. Under such dietary conditions it is difficult to transfer the beneficial effect of methionine in experimental animals to conditions that may exist in man. Such diets will themselves produce liver injury as shown by Fouts (91), with less dietary restriction.

Clinical studies. In 1916 Westrope reported that a milk diet 24 hours before the administration of arsphenamine decreased the incidence of vomiting and headache (261). Later Peters and co-workers made a detailed study of post-arsphenamine jaundice (207). The rate of recovery of patients was observed by serial estimation of serum bilirubin and it was concluded that a slight but statistically significant increase in the rate of return to normal was obtained in patients receiving cystine or methionine. Control patients required an average of 25.7 days for the serum bilirubin to fall below four mg. %, whereas methionine-treated cases showed a similar change in an average of 19.7 days. However, in patients treated with casein the serum bilirubin did not decrease to four mg. % until 32.9 days had elapsed. Although the casein may not have supplied sufficient methionine to effect recovery, it is doubtful that it would delay recovery. If we then assume the casein to be ineffective the patients may be considered as another control group, which would then show a difference between controls (25.7 and 32.9 days) of 7.2 days. With such a variation among two control groups the difference produced by methionine would not be significant. Part of the variation is probably due to the fact that the date of onset of jaundice was elicited by questioning, and was thought in most cases to be accurate within two days.

It is probable that the jaundice studied in the above report was due to hepati-

tis virus rather than arsphenamine. The time of onset of jaundice following the start of arsphenamine therapy, the symptoms, and the clinical course of the patients are typical of homologous serum jaundice. It is now known that the virus may be easily transmitted by syringes, needles or lancets that have been in contact with infected human blood (23, 37, 224). Minute quantities of blood are infective. Methionine is without effect in patients with infectious hepatitis or homologous serum hepatitis.

M. Selenium

Grain grown in soil containing selenium, or selenium added to the diet, will produce a typical disease picture in many farm animals, causing, among other changes, damage to the liver, kidney, spleen and adrenal cortex. Details concerning selenium poisoning may be found in the review of Moxon and Rhian (194). Animals fed such materials remain normal for a few weeks, following which time some will die and show at autopsy acute massive necrosis of the liver. Others survive for longer periods and develop fibrosis of the liver, evidently the result of post-necrotic scarring. In acute toxicity studies selenium produces death by effects on the central nervous system. The subacute or chronic administration of selenium produces necrosis, cirrhosis and hemorrhage of varying degrees in rats and dogs (92, 162, 217). Depending on the dosage selenium may cause slight anorexia to complete refusal of food.

Dietary factors. Diets high, normal and low in protein have been studied and it was concluded that selenium poisoning is least severe on the diet with a high protein content. The high protein diets increased growth rates and decreased mortality (157, 193, 240, 242). Different proteins vary in protective effect (99, 242). Smith (240) and Stohlman (242) express the toxicity of selenium in relation to protein, concluding that a protein-selenium ratio (per cent protein in diet to micrograms selenium per 100 grams of diet) of 1:30 or less is non-toxic while a diet with the same selenium content but with a protein-selenium ratio of 1:100 is very toxic. The ratio of protein to selenium may account in part for differences in species response to selenium, but the problem has not been sufficiently studied. The feeding of diets with a low or high content of fat did not affect mortality from selenium (193).

Seleniferous wheat, combined with a low protein, high carbohydrate diet, resulted in a marked nodular cirrhosis but produced only slight damage when a high protein, low carbohydrate diet was fed (162, 240). However, control data on the liver lesions were not given for rats fed normal wheat combined with the low protein, high carbohydrate diet. Similar results were obtained when sodium selenite was added to synthetic diets containing various amount of protein (242), but control data on the liver injury produced by the low protein diet were lacking. In other studies cirrhosis was not obtained when seleniferous wheat was fed with a low protein, high fat diet (193). Microscopically such animals showed fatty degeneration in the liver which was not unlike that obtained, as stated in a footnote, with a similar diet and normal wheat (240). Therefore, although low protein diets increase mortality and decrease growth when selenium is fed, evi-

dence is still not conclusive that the liver injury produced is any greater than what might be expected by feeding a low protein diet alone for a prolonged period.

Cystine, methionine, choline. Cystine has consistently been without effect in counteracting the growth depressing effects or lethal action of selenium in animals fed a low protein diet (157, 193, 227, 242) or a normal diet (234). The addition of methionine to a six per cent casein diet gave some protection, as evidenced by increased growth rate and slightly longer survival, but not always to the extent obtained with a high protein diet (157). Some effect of methionine was also stated to be present in pair-fed animals. However, gain in weight and longer survival do not necessarily indicate a protective effect of the diet or dietary factors on the hepatotoxic action of selenium. Pathological changes in the liver have been noted in rats which were rapidly gaining weight and appeared otherwise normal (99). Supplements of methionine and cystine added to a low protein diet did not prevent the cirrhosis caused by selenium (242). Interesting results have been obtained by Sellers *et al.* (234) in animals fed a normal diet. They found that methionine would protect against the hepatotoxic effect of selenium, but only if alpha-tocopherol was also supplied. Supplements of choline were without effect.

It has been postulated that selenium is in part detoxified and eliminated as the volatile dimethyl selenide (132) and it is possible that methionine may serve as a source of methyl groups in this detoxification. However, in further studies it was shown that 16 to 52 per cent of injected selenium is excreted in eight hours as a volatile compound which is still unknown, the excretion of which was not influenced by choline or methionine (229).

It is known that selenium can inhibit certain cellular respiratory enzymes, but no correlation of these effects to diet has been established (cf. 240). The possibility remains that selenium produces hepatic necrosis because it replaces sulfur in sulfur-containing amino acids. In growing plants selenium can replace sulfur, for the metabolism of sulfur and selenium is similar in cereal plants, although not identical in all respects (204). The deposition of selenium in cereal proteins follows that of sulfur in most cases, but there are a few wide variations in the S/Se ratios. Selenate, however, does not seem to be formed even though a seleniferous plant is high in sulfate.

VI. POSSIBLE MECHANISMS OF DIETARY EFFECTS

Various theories have been suggested to explain the mechanism by which hepatotoxic agents produce injury, and to account for the manner by which certain diets influence the hepatic damage. Graham postulated in 1915 that the toxic effects of chloroform were due to hydrochloric acid liberated during its decomposition (101, 102). However, further investigation failed to confirm some of his results or his suggestions and the theory was soon discarded (53, 253). Other explanations have received more attention and warrant discussion in greater detail.

A. Carbohydrate and liver glycogen

Many hepatotoxic agents have been noted to decrease liver glycogen. This effect was observed as early as 1882 by Rosenbaum during experimental arsenical and phosphorus poisoning (219), and has since been reported by various investigators. However, in such studies food intake was not controlled and the fall in liver glycogen may have been due wholly or in part to a decreased food consumption following $CHCl_3$ anesthesia or to the administration of other toxic materials. Although the experiments were not properly controlled, such reports of low liver glycogen were used as a basis for the administration of glucose in attempts to decrease hepatotoxic reactions, particularly in man.

The studies of Rosenfeld also suggested the use of glucose to prevent liver injury in man during $CHCl_3$ anesthesia. He noted various conditions, including $CHCl_3$ poisoning, in which fatty changes in the liver were associated with a decrease in glycogen content. He believed that fat could not be metabolized unless carbohydrate was simultaneously oxidized, and therefore as liver glycogen decreased lipid would accumulate in the liver (220–222). It was concluded that a toxic substance such as $CHCl_3$ increased the need of the liver for carbohydrate which, when supplied, would increase the detoxifying power of the liver and prevent fatty infiltration. These data also led to the use of dextrose in patients with hepatic disease. Since then various investigators have reported that depletion of liver glycogen increases the toxicity of $CHCl_3$, CCl_4 and phosphorus (25, 50, 100, 225). During this time glucose and sucrose were observed to decrease the liver injury resulting from $CHCl_3$ (51, 52, 100) and CCl_4 (39, 56). However, it was noted that glucose and insulin did not increase liver glycogen during phosphorus and $CHCl_3$ poisoning (5, 6).

The viewpoint developed by Rosenfeld went unchallenged until it was more closely studied by Ravdin and associates. It had been assumed that the decrease in liver glycogen was due to an increased rate of metabolism in the liver, but careful studies in dogs did not confirm this belief (214). They demonstrated that the administration of glucose resulted in less deposition of liver glycogen in dogs following $CHCl_3$ exposure than in the normal dog. Further, the protective effect of oxygen on the liver during $CHCl_3$ anesthesia was not associated with any effect on the rate or degree of discharge of hepatic glycogen. They believed that a decrease in liver glycogen is best explained by an inability of the damaged cells to retain glycogen, rather than by any increased metabolic activity of the liver cells. They also noted that fat began to accumulate in the liver, following $CHCl_3$ anesthesia, at a time when considerable hepatic glycogen was still present. Therefore, the early accumulation of fat does not seem to be solely related to a deficiency of carbohydrate, as claimed by Rosenfeld.

A further study of the relation of liver glycogen to lipid content, made by Goldschmidt, Vars and Ravdin (97), failed to confirm the hypothesis that a high content of glycogen protects the liver from $CHCl_3$ injury. An increased susceptibility to $CHCl_3$ was, however, correlated with an increase in liver fat. When rats with equal concentrations of hepatic lipids were compared the incidence of liver damage was not significantly different between groups with high

and low liver glycogen. If the content of liver fat is not considered, it may be erroneously concluded that the increase in liver injury was related to a decrease in liver glycogen. Thus, the early reports correlating a decrease in liver glycogen with increased susceptibility to hepatotoxins must be regarded as based on fortuitous results, it being more likely that the increase in toxicity was related to changes in hepatic lipids.

It is well known that the starved animal is more susceptible to liver injury (52, 239). Even when rats with the same content of hepatic lipids are compared, the animals not fed for 24 hours suffer greater liver damage than fed animals (97). From the above discussion of liver glycogen it does not seem that a deficiency of this substance is responsible for increased damage to liver when food is withheld. It is more likely that the sensitivity of the unfed animal is due to a depletion of body stores of protein, which diminish quite rapidly, particularly in the liver, of the starved rat (3, 4). $CHCl_3$ anesthesia will increase the excretion of nitrogenous products and this may be partially counteracted by the administration of glucose (47, 51), thus allowing a more rapid regeneration of liver cells. In fact, Opie and Alford (202, 203) initially suggested that the necrosis produced by $CHCl_3$, phosphorus or other agents may be a histological expression of protein breakdown, and that carbohydrate may limit necrosis by virtue of its protein sparing action.

B. Fat storage of toxic agents

An increase in hepatic lipids, produced by dietary means, renders animals more susceptible to hepatic damage. This has been shown for a number of compounds, as discussed individually above, and is perhaps best demonstrated in the studies of Goldschmidt *et al.* (97) with $CHCl_3$.

Such an effect of fatty livers, in increasing toxicity, was first noticed during the early clinical use of $CHCl_3$. This led Wells to suggest in 1908 that such susceptibility to $CHCl_3$ may be based on the known solubility of the chemical in fats (260). He believed that a fatty liver would absorb more $CHCl_3$ from the blood than a normal liver and thus enable the $CHCl_3$ to be in contact with hepatic cells for a longer period of time and to exert a more toxic effect. Opie and Alford (202, 203) accepted this explanation and pointed out that the induced necrosis was in the region where fat was generally deposited. The studies of Goldschmidt and associates led to the same conclusion and they believed that the fat within the cells of the liver acted as a reservoir for $CHCl_3$, maintaining a toxic concentration for a prolonged period of time.

Although such a hypothesis is plausible, at least for the fat-soluble materials, there are no data in the literature relative to the concentration of $CHCl_3$ in the liver at different levels of fat concentration. More recently McCollister *et al.* (171) studied the distribution of radioactive CCl_4 in monkeys and noted a very high concentration of radioactivity in fat. A distribution ratio was calculated and, with the ratio for blood equal to 1.00, that found for fat was 7.94, for liver 3.03, and for bone marrow 3.00. All other tissues studied had a distribution ratio of less than one. These data show the affinity of CCl_4 for fat and it would be of

interest to have similar data for the concentration of CCl_4 and related materials in the liver at different levels of hepatic lipids.

The solubility of $CHCl_3$ and CCl_4 in fat probably explains their increased toxicity in animals fed a high fat diet. Comparative studies are not available for other compounds, but one would suspect that solubility in fat, like the Meyer-Overton theory in anesthesia, would not hold for all compounds. In such cases where solubility in fat is a factor, the damaging agent would then be able to exert a greater toxic effect, as might be evidenced by more severe central ischemia, or by a greater effect on other cell constituents such as SH groups.

C. SH groups and dietary effects

The function of certain enzymes is known to be associated with the presence of SH groups, and it is possible that certain chlorinated hydrocarbons may exert their toxic effects by affecting such enzyme systems. The SH groups can readily be inactivated chemically, thus preventing the SH radical from functioning in an oxidation-reduction system (180, 212). It has also been reported that $CHCl_3$ administered orally will decrease the glutathione content of the liver (24). It is thus possible that chlorinated hydrocarbons may produce liver injury, particularly necrosis, by virtue of their ability to inactivate sulfhydryl groups.

As previously pointed out, hepatic necrosis can also be produced by a deficiency of cystine or tocopherol and it would be logical to assume that the underlying mechanism of the damage caused by these deficiencies and by toxic agents is similar. Preliminary data correlating such seemingly unrelated substances are available. Cysteine has been shown to increase the glutathione content of the liver (165). A deficiency of cystine will markedly decrease the glutathione content of the liver, which can, however, be increased by the administration of glutathione (155). Little is known about the effect of vitamin E deficiency on the SH radical although it is believed that the primary effect of alpha-tocopherol is to act as a strong reducing agent needed to preserve the reduced form of certain intracellular enzymes (122). Methionine will increase more than three-fold the antioxidant activity of alpha-tocopherol (40). Although further studies are needed, it is evident that hepatic necrosis may have a common basis related to a reduction of sulfhydryl groups caused by various hepatotoxic agents, vitamin E deficiency or cystine deficiency.

VII. DISCUSSION AND SUMMARY

A number of hepatotoxic agents have been discussed that can produce, depending on dosage and duration of administration, fatty changes, necrosis and fibrosis of the liver. Various studies, since 1925, have shown that the compounds that produce injury to the center of the liver lobule do so because a centrilobular ischemia is induced. Anoxia seems to be the most important factor in the induced ischemia, and probably acts to produce necrosis by upsetting oxidation-reduction potentials within the liver cell. The cirrhosis caused by the prolonged administration of certain compounds has also been carefully studied, with chief

emphasis being placed on the reversibility of the process during early stages and on the architectural relationship of the new connective tissue.

Dietary changes will also affect the toxicity of such agents. Diets high in fat will increase the toxicity of most of the chemicals discussed. Liver injury may also be increased under such conditions and this effect is probably due to the greater solubility of many of these substances in fat, which then allows a more prolonged toxic action of the compound.

Whereas diets high in fat increase toxicity to many drugs, best protection is obtained with diets high in carbohydrate and protein and low in fat. *However, it should not be assumed that an increase or decrease in toxicity of the hepatotoxic agents with various diets is due to an increase or decrease in the severity of liver injury.* The mortality may be affected because of changes induced in other organs such as the kidney or the heart; the adrenal gland, although little studied, seems to be affected during ethylene dichloride exposure.

Diets low in protein will produce liver injury and can also affect the toxicity of the compounds discussed, but again one should not assume that increased toxicity is synonymous with increased hepatic damage. Diets with a zero, or nearly zero content of protein can markedly increase susceptibility to substances such as $CHCl_3$. Such diets, in addition to producing liver injury *per se*, can also affect other organs. It is also known that prolonged protein depletion can impair antibody production, decrease the leucocytic response of bone marrow and decrease the activity of various enzymes. Diets only moderately deficient in protein (containing about eight per cent casein), even when fed for long periods of time, either have no influence on the hepatotoxic effect of the chemical or only slightly increase the degree of liver injury. In some cases the increased injury is found in the kidney or the heart.

Depending on the hepatotoxic agent being used, its dose and route of administration, and the dietary conditions of the study, mortality may be influenced by choline, methionine, cystine, other compounds supplying SH groups, or totally unrelated agents. Changes in mortality obtained by such supplements may be independent of effects on the liver. One would expect supplements of choline to act chiefly with regard to the prevention or alleviation of fatty changes, and methionine and cystine to affect the incidence of necrosis. The effects of methionine and choline will depend on several factors. Methionine functions not only as a lipotropic agent by virtue of its labile methyl group, but also in maintenance of growth and as a source of sulfur for the formation of cystine and other sulfur-containing metabolites. These last two effects are apparently more essential to the body and sufficient methionine must be present for them before a lipotropic effect is obtained. Thus differences in response may occur depending on whether a growing rat or an adult dog is being studied. Choline can only replace the lipotropic effect of methionine. In a similar manner methionine and cystine are interchangeable maximally when the deficiency is one of organic sulfur, provided the minimal requirements for methionine are supplied and an adequate amount of choline is present. Other supplements, such as alpha-tocopherol, require further study.

The more unbalanced the diet, particularly with regard to protein deficiency, the more likely it is that various supplements will decrease the toxic effects of hepatotoxic agents, either by affecting the liver, or the kidney or other organs. In applying such results to conditions that may be present in man the experimental diet should be carefully considered. It is, for instance, unlikely that man would subsist on a protein-free diet for a period of weeks, although his diet may be moderately deficient in protein for a number of years. The various supplements may be of value in the treatment of a patient with moderate protein deficiency who has been exposed to a hepatotoxic agent, although at the present time there are no adequate clinical data on this point. With certain compounds survival has been increased by some supplements even though a normal diet was being fed to the experimental animal, and it is possible that the same effect may be obtained in man.

High carbohydrate diets or glucose injections also seem to offer protection in certain cases. The protective effect of carbohydrate is not related to maintenance of liver glycogen, but rather to a decrease in the degree of protein breakdown as measured by nitrogen excretion.

The mechanism by which the various compounds and deficient diets produce liver damage is unknown. Preliminary data tend to point to an effect on SH groups and oxidation-reduction potentials.

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