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# CARBON TETRACHLORIDE HEPATOTOXICITY

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TABLE OF CONTENTS



## I. INTRODUCTION

In laboratories oriented toward experimental rather than clinical pathology, the study of carbon tetrachloride poisoning has attracted more attention than the study of any other hepatotoxin. For investigators not engaged in this work, and for clinicians who see hundreds of alcoholics for every case of carbon tetrachloride poisoning, the concentration on carbon tetrachloride may appear misguided. However, if experimental cellular pathology is ever to be capable of predicting rationally the reactions of cells to injurious agents, it is the properties of the biological substratum that must be studied. This substratum is so complicated and subtle in its responses to foreign agents, that there is a decided advantage in studying the simplest substances capable of producing devastating effects, short of immediate death. If the detailed unravelling of physiological and biochemical mechanisms involved in the reaction of living cells to a relatively simple chemical compound such as carbon tetrachloride cannot be achieved, then even greater difficulties can be expected for the study of the reactions of cells to

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chemically more complex substances. From this point of view, the work reviewed here is to be judged not in terms of its relation to clinical medicine, but rather as the current expression of one of the oldest and richest traditions in medical research, clearly formulated by Claude Bernard (see ref. 323): "Poisons can be used as agents for the destruction of life, or as a means of curing diseases; but besides these two uses well known to everyone, there is a third which particularly interests the physiologist. For him, a poison becomes an instrument which dissociates and analyzes the most delicate phenomena of the living machine, and, by studying attentively the mechanism of death due to different poisons, he comes to know by an indirect way the physiological mechanisms of life." Within this context, work of the past decade has achieved results of exceptional clarity, especially with regard to the fatty changes. The situation with regard to necrosis is less clear. This review will examine critically the major hypotheses that have guided this work.

## *Historical background*

Chloroform was first used as an anesthetic in 1847. In an entertaining review, Hardin (134) cited descriptions by forgotten heroes of the 19th century of their own reactions to chloroform and carbon tetrachloride, taken orally or by inhalation. Acute yellow atrophy of the liver, the key pathological involvement in delayed chloroform poisoning, was well known by the turn of the century. Gradually, use of chloroform as a general anesthetic waned. It was also realized that as an anesthetic agent carbon tetrachloride was both more toxic and less effective than chloroform. Subsequently, the menace of carbon tetrachloride as an industrial poison impressed itself on the medical generation of the late 19th and early 20th centuries. By 1921 the anthelmintic properties of oral carbon tetrachloride became known (134) and in the ensuing years the toxicity of this compound was once again firmly impressed on a new generation of medical workers. In the first 25 years of the present century, work was directed mainly toward study of dietary conditions which either ameliorated or exacerbated the toxic effects (reviewed in ref. 343). Relatively little work was aimed directly at understanding the mechanisms involved. Nevertheless, the major features of the problem had emerged: Why is the liver particularly affected? What is the initial lesion? Which cell structures are initially attacked, and in what manner are they attacked? What is the basis for the lipid infiltration? What is the basis for the extensive hepatocellular necrosis, and for its predominantly centrilobular and midzonal disposition?

An essentially complete frame of reference for study of hepatic lipid accumulation had been achieved by 1944 (322). It was known that the amount of fat in the liver is the result of complex opposing processes. At least in theory, an increased level of triglycerides in the liver could occur as a result of increased delivery of substrates to the liver by the blood, decreased transfer of triglycerides from liver to plasma, increased intrahepatic biosynthesis, or decreased intrahepatic oxidation. More recently, the discovery that $\alpha,\beta$-diglycerides are precursors in the biosynthesis of triglycerides as well as phospholipids, suggested

that a decreased rate of conversion of $\alpha,\beta$-diglycerides to phospholipids might account for increased triglyceride levels. Successive attempts have been made to decide which of these processes has been altered by carbon tetrachloride poisoning. Not until 1960 was the pathophysiological basis for this kind of fatty liver clearly established, and since that time progress in study of cellular and enzymic mechanisms has been rapid. Recent work has permitted new insights into related questions concerned with the unique susceptibility of the liver of some animals to carbon tetrachloride, the possible nature of the primary lesion, and possible biochemical mechanisms underlying hepatocellular necrosis.

## II. THE PHOSPHOLIPID HYPOTHESIS

Until about 1948, much of the thinking in the field of lipid metabolism was dominated by the phospholipid hypothesis. It was believed that long-chain fatty acids were transported from organ to organ in the aqueous phase of the plasma in ester linkage as phospholipids. Since phospholipids contain hydrophilic centers, they are more water soluble than triglycerides. It seemed inconceivable that triglycerides, substances like butter and lard, could be transported as such through the plasma compartment. Dietary fatty livers were attributed to the absence of some component essential for the synthesis of phospholipids, or to the presence of substances which interfered with their synthesis (237, p. 425). The fatty liver of the pancreatectomized dog maintained on insulin, the fatty liver of diets deficient in choline or methionine, the fatty liver resulting from feeding guanidoacetic acid, as well as other facts of nutritionally induced fatty liver disease could be placed into meaningful perspective in terms of this phospholipid hypothesis.

In fatty livers there was an increase in neutral fat, and a relative decrease in hepatic phospholipids. In the parenchymatous cells of various tissues fatty acids available for oxidation were thought to be those incorporated in phospholipid molecules (237, p. 283). It was natural to imagine that in the damaged liver, long-chain fatty acids might be diverted to triglycerides (believed to be metabolically inert) since their presumed pathway of oxidation *via* phospholipids was blocked. The main facts relevant to choline deficiency fatty liver lent further support to the phospholipid hypothesis. It seemed likely that long-chain fatty acids were normally removed from liver to plasma in ester linkage as phospholipids, largely as lecithin, and, with failure of hepatic phospholipid metabolism due to choline deficiency, the fatty acids "backed up" in the liver as triglycerides (237, p. 434). The fact that choline added to the diet induced loss of accumulated liver triglycerides only strengthened the prevailing view that fatty acids left the liver in ester linkage as lecithin. It was easy to imagine that hepatotoxins such as carbon tetrachloride, which produced massive hepatic necrosis, also interfered with hepatic phospholipid synthesis. Thus the hypothesis could account for toxic fatty liver as well as for the fatty liver of choline deficiency.

The phospholipid hypothesis died a slow death. In 1943, Fishler *et al.* (100) showed that the liver was the major, if not the sole, source of plasma phospholipids. These experiments had an important bearing on the prevailing view that

phospholipids represented the transport form of fatty acids in the plasma. It was known that fatty acids stored as triglycerides in the adipose tissues of the body could be mobilized in response to demands for noncarbohydrate fuel. If the liver was the sole source of plasma phospholipids, then mobilization of adipose tissue fatty acids would have to occur in some form other than as phospholipids. The experiments of Fishler *et al.* (100) did not rule out a role for phospholipids as a transport form of fatty acids away from the liver.

Zilversmit *et al.* (351) found that the turnover time for plasma phospholipid phosphorus in the normal dog was 8 hours. Three years later it was shown that when the liver was excluded from the circulation the total content of plasma phospholipids did not change (91). If significant amounts of long-chain fatty acids are carried from liver to peripheral tissues as phospholipids, on exclusion of the liver one would expect the plasma concentration to decline as phospholipids entered peripheral sites. When the liver of the animal was removed from the circulation, the turnover time for plasma phospholipids was greatly extended, up to 160 hours (91). It was clear that the liver was the principal organ concerned with the elaboration as well as with the removal of plasma phospholipids. If plasma phospholipids are both synthesized and degraded mainly in the liver, they could not very well serve as a means for net transport of fatty acids from liver to peripheral sites.

Depancreatized dogs maintained on insulin and fed a lean meat diet have normal levels of liver fat. If insulin is withdrawn, large amounts of fat accumulate in the liver. It is believed that the fatty acids which accumulate in the liver under these conditions are derived from adipose tissue triglycerides. If the fatty acids are transported as phospholipids, the turnover of plasma phospholipid phosphorus should increase on withdrawal of insulin. Actually, removal of insulin lowered the plasma phospholipid phosphorus turnover. It was possible to conclude that plasma phospholipids did not function in the transport of the fatty acids (47). It was subsequently shown that the turnover times for the fatty acid and phosphorus moieties of the phospholipid molecule were the same (342). This work eliminated any doubt that studies of plasma phospholipid phosphorus turnover might not be relevant to plasma phospholipid fatty acid turnover. These experiments and much other work formed the base for one of the major achievements in the whole of lipid physiology, *viz.*, the demonstration that fatty acids in ester linkage as plasma phospholipids could not represent the transport form of fatty acids in the plasma. The phospholipid hypothesis in its original form was abandoned. However, the idea that pathological hepatic lipid accumulation is due to failure of transport of hepatic lipid to the plasma has remained at the center of the modern work. The idea that phospholipids play a role in lipid transport has also persisted into the modern era, since it is now recognized that phospholipids are integral parts of the complex lipoproteins which are the transport form of triglycerides in the blood. In short, early workers concerned with pathological accumulation of liver fat were, in the main, on the right track, but failed to penetrate into the heart of the mechanisms involved because of a misconception as to the role of phospholipids.

## III. THE MITOCHONDRIAL HYPOTHESIS

Work on carbon tetrachloride hepatotoxicity from 1954 to 1960 was dominated by the mitochondrial hypothesis. By the beginning of this period development of homogenization and differential centrifugation, coupled with advance in knowledge of the biochemical functions served by various subcellular components, made possible new approaches in the study of experimental cellular pathology. Christie and Judah (49) and Dianzani (65) pioneered in the application of cell fractionation to the study of toxic and nutritional liver injury. They proposed that toxic liver injury for the hepatotoxins studied, and for choline deficiency fatty liver as well (65), was fundamentally a matter of mitochondrial degeneration. A disorganization of the tricarboxylic acid cycle was observed in liver mitochondria 10 to 15 hours after carbon tetrachloride poisoning, and this disorganization was reproduced by the action of carbon tetrachloride on liver homogenates *in vitro* (49). Dianzani (65) found uncoupling of oxidative phosphorylation and suggested that accumulation of fat could perhaps be the result of failure of fatty acid oxidation contingent on a deficiency in the supply of adenosine triphosphate (ATP) needed for fatty acid activation.

That severe mitochondrial damage occurs in the liver after carbon tetrachloride poisoning has since been abundantly confirmed. Liver mitochondria of rats poisoned with carbon tetrachloride are swollen. This has been demonstrated for mitochondria isolated by differential centrifugation of liver homogenates (70), and also by electron microscopic study of the mitochondria *in situ* (7, 9, 17, 225). The swelling of otherwise normal liver mitochondria also occurs *in vitro*, as a result of the action of carbon tetrachloride (7, 199). Recknagel and Malamed (260) showed that the mitochondrial swelling which occurs *in vitro* in osmotically balanced media containing carbon tetrachloride is due to an increase in mitochondrial membrane permeability. As a result, the mitochondria release into the surrounding medium cytochrome *c* (74), pyridine nucleotides (26, 49, 66, 170, 250), vitamin $B_{12}$ (169), thiamine diphosphate (71, 176), adenosine phosphates (67), ubiquinone (1), citrate (258), and potassium (270, 302, 328). Mitochondria from carbon tetrachloride-induced fatty livers exhibit uncoupling of oxidative phosphorylation, loss of respiratory control, and ATP-ase activation (41, 65, 249, 250, 258, 270, 324) and these effects are produced by carbon tetrachloride acting *in vitro* on mitochondria isolated from livers of normal rats (7, 8, 41, 250, 258). Oxidative functions of the mitochondria dependent on pyridine nucleotides are impaired, both in mitochondria isolated from fatty livers after carbon tetrachloride administration to rats, and by the action of carbon tetrachloride on isolated mitochondria (26, 41, 49, 66, 249, 250, 270). Octanoate oxidation is impaired (72, 250, 258, 270) as well as fatty acid activation, both *in vivo* and *in vitro* (282). Without exception the damage induced by carbon tetrachloride on isolated mitochondria *in vitro* was paralleled by the action of carbon tetrachloride *in vivo*. This evidence swayed some investigators (49, 61, 65, 73, 282) to the view that carbon tetrachloride fatty liver was essentially a "disease of the mitochondria" (269). However, other studies have permitted the conclusion that lipid accumulation in carbon tetrachloride poisoning cannot be understood in

terms of a primary attack on the mitochondrial elements of hepatic parenchymal cells.

In 1956, in the first application of the electron microscope to hepatic histopathology following carbon tetrachloride poisoning, Oberling and Rouiller (225) reported that in the early stages of the lesion, during the first 4 to 6 hours, liver mitochondria exhibited minimal structural alterations, whereas marked changes in the appearance of the ergastoplasm were evident. The evidence from the electron microscopic studies could not reveal functional derangements, but it cast doubt on mitochondrial involvement as the primary lesion. As early as 3 hours after intoxication, total liver fat is significantly increased over control levels (250). In the normal liver, triglycerides account for less than 10% of total lipids. Thus, when total lipids are measured, a large percent change in triglyceride content is obscured by nontriglyceride total lipid, mostly phospholipids, which, after carbon tetrachloride poisoning, increase either very little (286) or not at all (Recknagel, unpublished). Schotz and Recknagel (296) determined triglyceride content of the liver after removal of phospholipids on silicic acid. Liver triglycerides were elevated 34% within 1 hour after carbon tetrachloride poisoning, and 195% within 3 hours. The rapid accumulation of triglycerides in the liver after carbon tetrachloride poisoning has been confirmed (197, 242, 286, 293, 320). It was known as early as 1958 (257, 261) that following intragastric administration, carbon tetrachloride reached its peak concentration in the liver within the first several hours, and declined rapidly thereafter. This observation has also been confirmed (8, 11, 178, 179, 181, 204). On the basis of these studies it was apparent that if the mitochondrial hypothesis was to survive it would have to be demonstrated that mitochondrial damage must occur during the first few hours after carbon tetrachloride administration. Carbon tetrachloride *in vitro* induces a clear-cut mitochondrial ATPase transformation (250). This test system was applied to mitochondria isolated from livers of carbon tetrachloride poisoned rats. Transformation of ATPase properties did not occur until 20 hours after carbon tetrachloride poisoning (250). Loss of respiratory control by liver mitochondria also did not occur until 20 hours after carbon tetrachloride administration (250).

Loss of bound pyridine nucleotides from aged mitochondria of carbon tetrachloride poisoned rats was a key observation in the original argument of Christie and Judah (49). The observation was confirmed by Recknagel and Anthony (250), who showed that the phenomenon as observed by Christie and Judah was an artifact of the conditions of measurement and that as late as 15 hours after carbon tetrachloride poisoning no deficiency of diphosphopyridine nucleotide could be demonstrated. There can be no doubt that in the period from about 10 to 15 hours after carbon tetrachloride poisoning, and perhaps even earlier, the liver mitochondria have been altered in some way, since aged mitochondria from carbon tetrachloride-poisoned rats rapidly lose pyridine nucleotides when subsequently incubated at 38°C in the hypotonic medium of Christie and Judah (49), whereas aged mitochondria from normal livers do not show such a loss under the same conditions. However, the alteration must be an exceedingly

subtle one, since it clearly does not involve loss of pyridine nucleotides from the mitochondria *in vivo*. The biochemical nature of the alteration has not been further elucidated.

The validity of the mitochondrial hypothesis was further questioned by other work. Calvert and Brody (41) confirmed the activation of $Mg^{++}$-dependent ATPase of liver mitochondria as a late phenomenon in carbon tetrachloride poisoning, evident 20 hours after intoxication. Share and Recknagel (302) showed that the capacity of mitochondria to maintain a positive inside-to-outside potassium gradient is rapidly lost when mitochondria are treated with carbon tetrachloride *in vitro*. However, 4.5 hours after carbon tetrachloride poisoning, when triglyceride accumulation is occurring rapidly in the whole animal, there is no defect in the capacity of potassium-depleted liver mitochondria to reaccumulate potassium ions.

Endogenous citrate of rat liver is localized in the mitochondrial fraction (291). Mitochondrial citrate is almost completely lost if the mitochondria are suspended for brief periods *in vitro* at 0°C in isotonic sucrose containing carbon tetrachloride (258). However, as late as 13 hours after carbon tetrachloride administration to rats, endogenous mitochondrial citrate is not only fully retained throughout homogenization and subsequent isolation procedures, but carbon tetrachloride-poisoned rats responded to intraperitoneal fluoroacetate with a marked elevation of mitochondrial citrate levels, none of which is lost during 3 hours of storage (258). When it is kept in mind that fluoroacetate poisoning, although eventually a matter of aconitase inhibition by fluorocitrate, involves the primary formation of fluoroacetyl coenzyme A, in an ATP-dependent activation reaction, and that eventual citrate accumulation requires the integrity of the citrate condensing enzyme and maintenance of the supply of acetyl coenzyme A and oxaloacetate, it must be recognized that the experiments which tested the capacity of carbon tetrachloride-poisoned rats to respond to a subsequent challenge with fluoroacetate represented a severe trial of the structural and functional integrity of their liver mitochondria. There simply was no evidence of serious mitochondrial involvement during the first 13 hours after carbon tetrachloride poisoning, during which time liver triglyceride content is greatly elevated. These experiments offered no hope for the mitochondrial hypothesis. Subsequently, Reynolds *et al.* (270) found that oxidation of octanoate, glutamate, $\beta$-hydroxybutyrate, and succinate, all were at control levels throughout the first 10 hours after carbon tetrachloride poisoning, but were markedly depressed by 20 hours. Oxidative phosphorylation was maintained at normal levels for 16 hours. With large doses of carbon tetrachloride, Artizzu and Dianzani (8) found a significant decrease of the P/O ratio as early as 2 hours, but not at 15 minutes, 30 minutes or 60 minutes. Respiratory control was also unaffected during the first hour. Total lipid was significantly elevated within the first hour. These experiments have demonstrated that the early accumulation of fat following carbon tetrachloride poisoning is independent of mitochondrial damage. The possible involvement of the mitochondria in the necrogenic action of carbon tetrachloride on the other hand is still an open question.

In summary, from 1954 to 1960, the mitochondrial hypothesis had served as the central organizing idea for the most productive and most provocative work of that period. Its most valuable contribution was methodological. Its proponents, by introducing the methods of biochemical cytology and enzyme assay, had raised the study of experimental hepatotoxicity to a technological level of sophistication unknown to earlier workers. However, by 1959 confidence in the substantive generalizations of the mitochondrial hypothesis had vanished and in that year work in this area moved off into 2 entirely new and completely independent directions. One of these lines of work for the most part relied on cell fractionation. Attention was turned to the study of the effects of carbon tetrachloride on enzyme systems of the microsome fraction. Cogent evidence soon appeared that the primary locus for the action of carbon tetrachloride was in the endoplasmic reticulum of the hepatic parenchymal cells. This work was in the classical tradition which assumed that carbon tetrachloride toxicity was fundamentally a question of a toxic reaction in the liver. A review of the main findings and how they led to a new insight into the pathophysiological basis for hepatic triglyceride accumulation will be given subsequently.

A parallel line of work attempted to understand both the hepatic lipid accumulation and the necrosis of carbon tetrachloride poisoning as a manifestation of massive catecholamine discharge. A catecholamine hypothesis for carbon tetrachloride hepatotoxicity was created. A review of this work follows.

## IV. THE CATECHOLAMINE HYPOTHESIS. PART 1: NECROSIS

This subject has been ably reviewed by Plaa and Larson (240) and the following account rests on their treatment. Brody (30) first proposed in 1959 that both hepatic necrosis and hepatic lipid accumulation of carbon tetrachloride poisoning were due not to a direct effect of the toxic agent on liver cells, but to a massive and persistent discharge of the sympathetic nervous system occasioned by an action of carbon tetrachloride on the central nervous system. Supporting evidence for the hypothesis was presented in a series of papers (30–33, 42–44, 219, 319). It was suggested that the alleged massive discharge of the sympathetic nervous system had 2 consequences of importance to the liver: diminished hepatic blood flow with associated centrilobular hypoxia, which would account for the centrilobular necrosis, and enhanced mobilization of fatty acids from peripheral stores, which would account for the fatty liver. The historical roots of this hypothesis were deep in the past (58, 202, 233). In 1947 Himsworth (147) had conceived of carbon tetrachloride acting directly on the hepatic cells causing swelling and mechanical obstruction of sinusoidal blood flow. This theory dominated the thinking of some of the leading workers in hepatic pathology for many years (*e.g.*, 86, 220). In 1963, strengthened by the experimental and speculative work of Brody and co-workers, this view had not been abandoned (87), although sharp criticism of the vascular theory had been presented by Stoner and Magee in 1957 (323).

What was new in the speculations of Brody and co-workers was emphasis on the brain as the primary locus of action of carbon tetrachloride. The hypothesis

was based on indirect evidence. Hepatic blood flow was not monitored during the 24-hour postintoxication period, and tissue hypoxia was not demonstrated. Distressing uncertainties attend any efforts to determine hepatic cellular oxygen tensions *in situ* (23). Plasma fatty acid levels also were not monitored. The experimental evidence marshalled in support of the hypothesis rested on results obtained with high cord section, adrenalectomy, reserpine, and adrenergic blocking agents. The most dramatic results were obtained when rats previously subjected to transection of the spinal cord at the vertebral level of C-6 were studied 4 to 24 hours after surgery. In these animals, the usual liver injury did not occur 20 hours after an oral dose of carbon tetrachloride. There was no centrilobular necrosis, and no lipid accumulation. The liver mitochondria exhibited no activation of latent $Mg^{++}$-dependent ATPase activity. Oxygen consumption and associated esterification of inorganic phosphate were maintained at near normal levels.

Adrenalectomized rats given carbon tetrachloride exhibited little or no hepatic lipid accumulation. Protection against hepatic necrosis was minimal, and although mitochondrial P/O ratios tended to be maintained, both oxygen uptake and inorganic phosphate esterification were both depressed about 60%. Protection against activation of latent mitochondrial $Mg^{++}$-dependent ATPase was minimal and variable.

Treatment with reserpine for 5 days prior to carbon tetrachloride administration also gave some protection against the usual mitochondrial degeneration seen 20 hours after intoxication. Subcutaneous administration of phenoxybenzamine, ergotamine, or tolazoline either shortly before or coincident with carbon tetrachloride administration tended to maintain mitochondrial P/O ratios at normal levels, although here also both oxygen uptake and inorganic phosphate esterification were depressed 50 to 60%. Necrosis was much reduced, but the adrenergic blocking agents were not very effective in preventing hepatic lipid accumulation. Pretreatment of rats with large doses of $\beta$-TM10 [2-(2,6-dimethylphenoxy)propyl trimethylammonium chloride] maintained mitochondrial oxidative phosphorylation. Necrosis was minimal, but lipid accumulation was marked. Guanethidine and bretylium were less effective (31).

These results show that certain indices (mitochondrial function, fatty infiltration, necrosis) indicative of the usual response to carbon tetrachloride could be prevented or ameliorated by suitable prior manipulations of the experimental animals. The authors proposed that carbon tetrachloride initiated a sequence of events, beginning with central excitation and ending with massive and persistent sympathetic discharge. Any interruption of anatomical or chemical pathways leading eventually to discharge and effective action of catecholamines would protect against the toxic agent. An alternative hypothesis is embodied in the notion of permissiveness. Permissiveness in the physiological sense implies that even though a particular physiological system may not be directly involved in the reaction of the whole animal to an imposed environmental change, nevertheless the functional integrity of such a permissive system must be maintained if the imposed environmental change is to elicit some specific effect. Physiological

systems with a permissive role, although indispensable, are nevertheless only supportive. Brody and co-workers did not consider the possibility that in their experimental animals, carbon tetrachloride did in fact concentrate in the liver, as had been reported in 1958 (257, 261) for intact rats, and that the liver was in fact the primary site of the response to the toxic agent, as had been held for so many years, but that their particular experimental animals, cord-sectioned, adrenalectomized, or dosed with adrenergic blocking agents were simply not capable of responding to carbon tetrachloride with the usual hepatic involvement as would an otherwise intact animal. Put still another way, Brody and co-workers did not ascertain whether their procedures yielded experimental conditions with far more restricted boundary conditions than are implied in work with intact animals. There is little doubt that the experimental results obtained with cord-sectioned rats can be understood only in terms of the latter possibility, and not in terms of the catecholamine hypothesis chosen by Brody (30) and Calvert and Brody (42).

The remarkable protection afforded by spinal cord transection was confirmed by Larson and Plaa (179) and by Larson *et al.* (183), but the degree of protection depended on the dose in a curious way. Intact rats exhibited increasing microscopic damage in liver cells as the oral dose of carbon tetrachloride was raised from low to high levels. Prior cord section at C-7 gave maximal protection against a large dose of carbon tetrachloride, in confirmation of Brody (30) and Calvert and Brody (42), but at the lowest doses the effect was more severe in cord-sectioned rats than in the corresponding intact controls. With their suspicions aroused, Plaa and Larson (240) began a systematic and penetrating analysis of the problem. They first eliminated the possibility that reduced absorption might be at the root of the protective effect (179, 181, 183). The correct line of work was found when it was noted that control over temperature regulation was lost after high-cord section. The fact that cord-sectioned animals cannot maintain normal body temperature has been known for a long time (see 303). Rats rapidly become poikilothermic after transection at C-7; within 1 to 2 hours rectal temperatures drop to about 28°C (180, 181). Larson and Plaa (180) then carried out the critical experiment. When cord-sectioned rats were given carbon tetrachloride as usual, but were maintained at 36°C in an incubator, severe centrilobular necrosis developed (192). Hypothermia induced by immersion of intact rats in cold water resulted in a protective effect comparable to that of cordotomy (181), and it was shown that the latter effect was not due to failure of carbon tetrachloride to reach the liver. Furthermore, cord-sectioned rats at room temperature did develop hepatic lesions if carbon tetrachloride was administered in 3 successive doses over a 36-hour period (180), and even after a single dose of the toxic agent, cord-sectioned rats exhibited extensive hepatic lesions within 55 to 65 hours (181). It was also shown (179, 183) that the degree of protection afforded by cordotomy was lessened as the level of transection was lowered (transection at L-4 failed to protect), and that low-cord section did not result in hypothermia.

The fact that normothermic, cord-sectioned rats are not protected rules out a

key role for the central nervous system as the primary locus for the action of carbon tetrachloride. Conversely, the fact that hypothermic, intact rats are protected supports the view that the protection is due to the hypothermia. Why do hypothermic rats exhibit such a remarkable resistance to the toxic effects of carbon tetrachloride? Larson and Plaa (181) noted that whole-body oxygen consumption of C-7 transected rats maintained at room temperature dropped to 50% of normal within 1 hour, and to 30% by 5 hours. At 28°C, which is body temperature for rats within 1 to 2 hours after high-cord section (181), oxygen consumption of the isolated perfused liver was only 20 to 30% of control values at 37.7°C (24). It would thus appear that in hypothermic rats hepatic oxygen uptake must be considerably reduced, and that this reduction in metabolism in some way results in the protection.

A number of other studies have not supported the idea that "histotoxic anoxia" is responsible for centrilobular necrosis, or that the catecholamines could be playing a significant, direct role in the response to the toxic agent. Brauer *et al.* (25) showed in 1961 that if the isolated, perfused rat liver was exposed to anesthetic levels of chloroform, within 30 minutes there was massive glucose "dumping," arrest of bile secretion, sodium space expansion, and histological changes similar to those seen *in vivo*. These changes occurred in preparations perfused with fully oxygenated blood, and in the face of perceptible vasodilation. Brauer (23) rejected the notion that the primary liver lesion resulting from exposure to chloroform or carbon tetrachloride is dependent on any vascular changes. Heimberg *et al.* (143) have reported that addition of small amounts (5 to 10 $\mu$l) of carbon tetrachloride to the portal vein catheter of an isolated, perfused rat liver results in inhibition of the release of triglycerides into the perfusion medium. Mediation of the inhibition by catecholamines in such a system is not likely. The inhibition of triglyceride release caused by carbon tetrachloride could be demonstrated when perfusion flow rates were unaffected, and addition of adrenergic blocking agents or reserpine did not diminish the inhibitory action of carbon tetrachloride.

Stern and Brody (319) reported that catecholamine excretion is enhanced after exposure to carbon tetrachloride and Brody (31) considered this as evidence in favor of his hypothesis (see also 284). Larson and Plaa (181) administered large doses of norepinephrine, epinephrine, or mixtures of these to rats, intraperitoneally or by direct infusion into the hepatic portal vein or into the aorta above the coeliac axis. Continuous infusion was carried out in some cases as long as 20 hours. The rats were unanesthetized and normothermic. No hepatic lesions characteristic of carbon tetrachloride poisoning resulted even in animals which received by direct intrahepatic infusion during 20 hours amounts of norepinephrine 2.5 times the *total* amount of catecholamines and their metabolites which they calculated could have been excreted by the whole animal in a comparable length of time on the basis of the data of Stern and Brody (319).

Further evidence against the catecholamine hypothesis has come from application of methods developed by Levi-Montalcini (190) for producing immunological sympathectomy in rats. Larson *et al.* (182) prepared immunologically sym-

pathectomized rats with antisympathetic nerve growth factor. Even after adrenal demedullation, these animals developed hepatic lipid infiltration and necrosis after administration of carbon tetrachloride. The animals were normothermic.

This work permits the firm conclusion that the catecholamines could not play a critical role in the effects of carbon tetrachloride that culminate in hepatocellular necrosis.

## V. THE CATECHOLAMINE HYPOTHESIS. PART 2: HEPATIC LIPID INFILTRATION

The catecholamine hypothesis, in addition to postulating massive and persistent discharge of the sympathetic nervous system as a precondition for carbon tetrachloride-induced hepatic necrosis, also postulated that hepatic lipid accumulation depended on an oversupply of fatty acids released from adipose tissue *via* catecholamine discharge (30, 42). The core of this hypothesis is clearly the assumption that the hepatotoxic agent, *via* hormonal and neurohumoral discharge, elicits a rise in plasma fatty acid levels sufficiently high and sufficiently sustained to result in pathological accumulation of fat in the liver.

There is no doubt that experimental conditions can be found in which hepatic triglyceride accumulation can be induced as a result of increasing the supply of plasma fatty acids. Nestel and Steinberg (223) produced triglyceride accumulation in the isolated rat liver by greatly increasing the concentration of fatty acids in the perfusion fluid. However, the pertinence of such an experiment to the problem of pathological fatty liver is highly questionable. In their perfusion system, oxidative removal of added fatty acids would be highly unlikely. For every mole of fatty acids oxidized, a very large number of moles of fatty acids could be converted to triglycerides. This no doubt accounts in part for the well known capacity of the liver to convert fatty acids rapidly to triglycerides, which their experiments demonstrated. The problem is not to make triglycerides; the problem is to get rid of the triglycerides. As discussed elsewhere in this review, movement of triglycerides from the liver, as very low-density lipoprotein, is a major pathway over which the flux of fatty acids is very large. In the intact animal, low-density lipoprotein triglycerides are rapidly removed at various peripheral sites freeing the carrier apoprotein, and presumably other nontriglyceride components, for re-entry into the hepatic triglyceride secreting mechanism. This system accomplishes the efficient removal of liver triglycerides in the intact animal. In the perfused liver experiments of Nestel and Steinberg (223) quantitatively significant exit of triglycerides from the liver was a virtual impossibility. The perfusion medium contained no apoprotein carrier and even if any had been present it soon would have been loaded with triglycerides, which could be deposited nowhere else but back into the liver. Looked at another way, the experiment of Nestel and Steinberg (223) is an excellent demonstration in support of the view that exit of triglycerides from liver to plasma does indeed depend on availability of carrier apoprotein in the plasma. But their experiment, which permitted deposition of hepatic triglycerides but did not provide for their removal, cannot be regarded as evidence in support of the view that fatty liver depends on oversupply of fatty acids from the periphery.

Fatty liver can be produced in the whole animal by raising the plasma fatty acid level. On subcutaneous injection of an extract of lyophilized hog pituitary glands, plasma fatty acid levels rose almost 10-fold within 2 hours and remained elevated for 18 hours, and there was a gradual, progressive rise in liver fat content (287). In a dog anesthetized with thiopental, on intravenous infusion of norepinephrine, plasma fatty acid levels rose promptly to 10 times control levels and remained elevated as long as norepinephrine infusion was maintained; liver triglyceride content rose progressively during the period when plasma fatty acid levels were elevated (97). It should be particularly noted that in these cases hepatic triglyceride accumulation occurred as a result of a marked and sustained rise in plasma fatty acid levels. However, to understand the mechanism of action of hepatotoxic agents, the pertinent question is whether during the time when a hepatotoxin is producing an increase in liver triglycerides in the intact animal has there in fact been an *accelerated* release of free fatty acids from adipose tissue, and, if so, whether the over-supply is of sufficient magnitude and duration to account for the hepatic lipid accumulation. From this point of view the evidence is not convincing.

A moderate elevation of plasma fatty acid levels for a short period of time does not lead to increased liver fat. For example, Brodie and Maickel (28) noted that a single dose of catecholamines is not sufficient to produce an increase in liver triglyceride levels. In our laboratory we gave rats 2 successive subcutaneous doses of norepinephrine at 2-hour intervals. Each dose of norepinephrine was sufficient to maintain plasma fatty acid levels elevated for 2 hours. These rats were sacrificed after 4 hours. Liver triglyceride levels were not elevated.

After intragastric or intraduodenal administration of carbon tetrachloride, hepatic triglyceride rises linearly for 20 to 24 hours (286; Recknagel, unpublished; see also 320). If this lipid accumulation were the result of elevated plasma fatty acids, the elevation ought to begin promptly after administration of carbon tetrachloride and persist for at least 20 hours. Yet investigators have found that plasma fatty acids do not rise under these conditions (197, 242, 251, 294) or that their elevation is either too little or too late to account for the accumulation of triglycerides in the liver (205, 265, 320).

Control of plasma fatty acid flux appears to be exercised in the adipose tissue (6, 99, 209). Turnover time in the plasma compartment is not dependent on plasma concentration, and increases and decreases in the steady state concentration of fatty acids are indices of corresponding increases or decreases in flux. Stern *et al.* (320) recognized the possibility that control of fatty acid flux might be disturbed in carbon tetrachloride poisoning and suggested the possibility that a constant steady state level of plasma fatty acids might not reflect a change in turnover rate. However, Schotz *et al.* (294) and Recknagel and Ghoshal (251) found that flux of fatty acids through the plasma compartment 4 hours after carbon tetrachloride poisoning was unaltered in comparison with control animals (see table 1).

Brodie and co-workers (27, 29) and Horning *et al.* (155), on the basis of gas-chromatographic analysis of liver triglycerides following carbon tetrachloride

poisoning, concluded that the fatty acids of the deposited triglycerides had originated in the adipose tissue. However, a demonstration that newly deposited liver fatty acids have come from adipose tissue is not equivalent to a demonstration that the rate of movement of fatty acids from adipose tissue has increased. If the normal flux is of sufficient magnitude to supply fatty acids to the liver at a rate equal to or greater than the rate at which fatty acids are trapped as triglycerides in the liver, then no oversupply need be postulated. Evidence presented below indicates that the normal flux of fatty acids is more than adequate to supply the fatty acids trapped in the carbon tetrachloride-poisoned liver.

Further evidence against the view that carbon tetrachloride intoxication leads to a massive and persistent discharge of the sympathetics was offered by Schotz and Recknagel (295). They showed that administration of epinephrine to rats resulted in an increased rate of release of fatty acids from subsequently excised adipose tissue *in vitro*, *i.e.*, the excitatory effect of catecholamines *in vivo* survived extirpation of the target organ. However, no increase in rate of release of fatty acids could be detected from adipose tissue extirpated from rats ½, 4 or 9 hours after intragastric administration of carbon tetrachloride. Since the dose of carbon tetrachloride was sufficient for production of marked triglyceride accumulation in the liver, the latter can certainly occur either without a supporting adrenal discharge, or with an adrenal discharge so weak that it cannot survive extirpation of adipose tissue. In this connection, Rubinstein (284) reported that plasma levels of epinephrine were elevated 2 hours after intraduodenal administration of carbon tetrachloride to rats, but by 4 hours plasma epinephrine levels were normal. This observation again shows that although there may be some excitation of the sympathetic nervous system after administration of carbon tetrachloride to rats, such excitation is minimal and transitory.

Part of the evidence cited by Brody (30) and Calvert and Brody (42) in support of the catecholamine hypothesis consisted in the observation that adrenalectomy, although only minimally protective against hepatic necrosis, did protect rats against carbon tetrachloride induced lipid accumulation. The experimental situation here is an exact parallel of that discussed above in connection with the effects of high-cord section on carbon tetrachloride induced hepatic necrosis. Two different interpretations are possible: (1) The hepatotoxin initiates a sequence of events beginning with activation of either or both the sympathetic and the pituitary-adrenal systems leading to an outpouring of lipid mobilizing humoral and neurohumoral substances, which in turn accelerate the flux of fatty acids through the plasma fatty acid pool. The oversupply of fatty acids from the periphery is then deposited in abnormal amounts in the liver. (2) Hypophysectomy, adrenalectomy, dosing with sympatholytic drugs, or adrenal demedullation plus reserpine yields an experimental situation with far more restricted boundary conditions than are implied in work with intact animals. The rats may be so deficient in their capacity to mobilize depot lipid that hepatic triglyceride accumulation would not be possible, or would be greatly attenuated, even though a primary hepatic lesion had in fact been sustained. It has been known for many years (247) that adrenalectomy markedly diminishes hepatic

lipid accumulation ordinarily seen with a variety of agents or conditions, including phosphorus poisoning, partial hepatectomy, diabetes mellitus, exposure to cold, fasting, and administration of colchicine, ethionine, or anterior pituitary extract. It is most unlikely that all of these agents or conditions produce fatty liver in intact animals *via* catecholamine discharge and oversupply of fatty acids from adipose tissue. A far more plausible unifying assumption is that the ameliorating effect of adrenalectomy on hepatic lipid accumulation ordinarily produced by such a wide variety of drugs, poisons, endocrinological disturbances, dietary conditions and stresses is due to a failure to mobilize adipose tissue fatty acids. If carbon tetrachloride fails to elicit fatty liver in hypophysectomized or adrenalectomized rats, then the most plausible assumption is that the toxic agent has exerted its effect on the liver more or less as usual, as witnessed by the fact that adrenalectomy is only minimally effective against carbon tetrachloride hepatonecrosis, but fatty infiltration does not develop because of restriction in the supply of necessary raw materials from which to construct hepatic triglycerides. The latter possibility is in fact given strong support by recent work of Poggi and Paoletti (243), who showed that body temperature of adrenalectomized rats given carbon tetrachloride drops to about 30°C within 4 hours and that liver triglycerides do not increase in hypothermic, adrenalectomized rats within the first 4 hours after carbon tetrachloride feeding. They concluded that the "... presence of adrenal hormones is only important because it abolishes the fall in body temperature induced by $CCl_4$ administration in adrenalectomized animals and it is nonessential when body temperature is kept normal with physical means. . . ." However, the above conclusion is not supported by their data. For example, in otherwise normal rats given carbon tetrachloride, the data of Poggi and Paoletti (243) reveal that the increase in liver triglycerides was almost 4 times the increase in liver triglycerides recorded for normothermic, adrenalectomized rats given the same dose of carbon tetrachloride. Even though normothermic, the adrenalectomized rats are not responding to the hepatotoxin as are their eucorticoid controls. Again, the data show that after adrenalectomy, post-Triton triglyceridemia (see below) was markedly reduced in rats not given carbon tetrachloride. Since the livers of such animals are presumably intact, the result indicates that adrenalectomy has reduced the flow of free fatty acids from adipose tissue. Further, Poggi and Paoletti (243) gave Triton intravenously to adrenalectomized rats maintained on corticosterone. These animals presumably had intact livers and were normothermic. Yet post-Triton triglyceridemia in these animals was only ¼ that which these authors reported for intact animals. These observations are not consistent with the view that the role of the adrenal gland in carbon tetrachloride poisoning is confined to maintenance of body temperature. On the contrary, they indicate rather clearly that maintenance of the normal flow of fatty acids from adipose tissue depends on the presence of the adrenals. It may be recalled that in otherwise normal animals given carbon tetrachloride, increase in liver triglycerides begins within the first hour and continues for many hours. It seems highly unlikely that the combined effects of carbon tetrachloride and adrenalectomy on reduction of body temperature could

seriously affect the destructive action of carbon tetrachloride on the hepatic triglyceride secreting mechanism, an action which takes place very rapidly after intoxication.

Adrenalectomized rats given carbon tetrachloride fail to develop fatty livers because in such animals, in addition to any hypothermic effects as reported by Poggi and Paoletti (243), the flux of fatty acids from adipose tissue to liver is too low to sustain hepatic triglyceride synthesis at rates sufficient to achieve abnormal accumulation. A similar situation appears to obtain in hypophysectomized rats given carbon tetrachloride. In unpublished work in our laboratory we showed that carbon tetrachloride administration to hypophysectomized rats resulted in no demonstrable increase in liver fat over the first 11 hours. We showed also that post-Triton triglyceridemia in hypophysectomized rats was reduced to about half that of normal Tritonized controls; this finding indicates that mobilization of fatty acids following hypophysectomy is reduced.

The central idea in this analysis is that the pituitary-adrenal axis and sympathetic system play mainly, or exclusively, a supportive role permitting a normal supply of fatty acids. In animals subjected to the action of hepatotoxins, the fatty acids are retained in the liver because of a primary hepatic dysfunction. The importance of this analysis of the problem is the following: The fact that animals rendered nonfunctional with respect to pituitary-adrenal and sympathetic activity do not develop fatty livers in response to hepatotoxic agents cannot arbitrarily be used to support the peripheral oversupply hypothesis, since a cogent, alternate explanation of the experimental findings, with supporting data, is available. Support for the oversupply hypothesis must rest on an unequivocal demonstration that the hepatotoxin has in fact produced an accelerated flux of fatty acids of sufficient magnitude and duration to account for the hepatic lipid infiltration.

In summary, the available data do not support the view that after carbon tetrachloride poisoning an accelerated movement of fatty acids from peripheral adipose tissue occurs. If the hypophyseal-adrenocortical axis, or the sympathetic nervous system are activated, the activation is minimal and transient, and certainly not of an intensity sufficient on the one hand to maintain plasma fatty acid concentrations at the very high levels needed to produce hepatic infiltration by oversupply alone, and on the other hand of sufficient duration to produce the observed continuous hepatic infiltration over periods as long as 20 to 24 hours following a single dose of the toxic agent.

## VI. HEPATIC TRIGLYCERIDE SECRETION

### *A. The pathophysiology of carbon tetrachloride fatty liver*

When the mitochondrial hypothesis was abandoned, work on the fatty liver problem developed in 2 different directions. One of these was the catecholamine hypothesis just discussed. Another began with study of enzymes of the microsome fraction. In 1946, Rosin and Doljanski (281) had reported that as early as 1 hour after carbon tetrachloride poisoning the centrilobular parenchy-

mal cells were free of pyroninophilic granules, a finding that suggested loss of cytoplasmic ribonucleic acid or dispersal of ribonucleoprotein particles (see 93). In 1958 Leduc and Wilson (188) reported that in mice, loss or dispersal of centrilobular basophilia occurred within 15 to 30 minutes after carbon tetrachloride poisoning. The latter authors and especially Gupta (130) may be consulted for earlier theories as to the causes and significance of this phenomenon. The key point is that early loss of cytoplasmic basophilia indicated structural damage to the endoplasmic reticulum.

In 1956 Oberling and Rouiller (225) reported their electron microscopic study that showed early pathological involvement of the endoplasmic reticulum before evident mitochondrial damage. Neubert and Maibauer (224) reported that the microsomal system catalyzing detoxication of aminopyrine was destroyed in livers of carbon tetrachloride-poisoned rats at a time when mitochondrial oxidative phosphorylation was intact. Recknagel and Lombardi (258) noted a ⅓ reduction of hepatic glucose-6-phosphatase activity 2 hours after carbon tetrachloride poisoning, accompanied by a significant increase in microsomal DPNH-cytochrome reductase activity.

These studies indicated that the endoplasmic reticulum of the hepatic parenchymal cells was affected within the first few hours after administration of carbon tetrachloride. The knowledge (296) that liver triglycerides began to increase immediately after carbon tetrachloride administration suggested that some process, intimately concerned with rapid turnover of a hepatic triglyceride pool of some sort, was most probably localized in the endoplasmic reticulum of the hepatic parenchyma. What the nature of such a process could be was not immediately apparent. Dr. M. C. Schotz recognized that before a solution to the problem of hepatic triglyceride accumulation could be reached, it would be necessary to bring to bear a large body of work relevant to whole-body lipid transport (for reviews see 84, 106, 231, 233, 276, 316, 317).

Earlier efforts designed to obtain information on rates of renewal of liver lipids were highly misleading. Stettin and Boxer (321) followed uptake of deuterium into the fatty acids of total liver lipids in rats in which body water had been enriched with the isotope. They obtained a turnover time of 2.7 days. In a similar experiment, Pihl *et al.* (239) found a turnover time of 1 day for liver fatty acids. These results were misleading for 2 reasons. First, fatty acids of the various liver lipids were regarded as a single pool. It has since been recognized that there are many separate fatty acid pools within the liver, each with its distinctive renewal rate. To avoid misleading conclusions the total liver fatty acid pool must be divided not only into meaningful lipid subclasses (free fatty acids, glycerides, various phospholipids, steroid esters, *etc.*), but into subclasses based on subcellular anatomy. We now know that the rates of renewal of some of these pools of fatty acids are much faster than the estimates in days given by the earlier workers. A second aspect of the earlier work which led to grossly misleading notions of liver fatty acid turnover times was the choice of model. The experiments were of the inverse exponential or build-up type (see 334, 350). In such an experiment the specific activity of the pool being monitored reaches an

upper limit equal to the specific activity in the precursor pool. The inappropriateness of this model for study of liver fatty acid turnover was first pointed out by Harper *et al.* (135). The major defect of the isotope build-up experiment as a model for studying liver lipid turnover is that the fatty acids of certain liver fatty ester pools are in rapid equilibrium with fatty acid[2] and fatty ester pools in the plasma and adipose tissues. The rate of mixing in the hepatic-extra hepatic pool is very fast in comparison with the rate of entry of label into newly synthesized liver fatty acids from deuterium in the body water or $C^{14}$-acetate in the diet. Hence as label is incorporated into newly synthesized fatty acids in the liver it spreads rapidly into a relatively small pool made up of liver fatty acids, some liver triglycerides, part of the plasma triglyceride pool, adipose tissue fatty acids, part of the adipose tissue triglyceride pool, and plasma fatty acids. This is the so-called "superficial layer" of Olivecrona (228). The label then spreads further and more slowly into extremely complex and very poorly defined pools of liver and tissue glycerides, phospholipids, cholesterol esters, *etc.*, *i.e.*, into the "deep layer" of Olivecrona (228). With an improved experimental design Harper *et al.* (135) obtained an estimate of neutral lipid fatty acid flux more than 10-fold greater than the corresponding flux estimated by use of the isotope build-up model.

From 1956 to 1960, a number of key discoveries confirmed and extended the views of Harper *et al.* (135) and set the stage for a new approach to the study of both toxic and nutritional fatty liver. Before 1956 the plasma long-chain fatty acids were thought of mainly, if not exclusively, as products of the action of heparin clearing factor on plasma lipids (129, 273). In 1956, Dole (82) and Gordon and Cherkes (126) showed independently that the plasma level of fatty acids in human subjects increased during fasting, rose dramatically after epinephrine administration, and fell after oral glucose. Dole (82) found no change in plasma fatty acid levels during alimentary lipemia, and, in patients with chronically elevated blood lipids, the plasma fatty acid levels were not abnormally high. He reported a sharp drop after insulin administration. These observations indicated that the plasma fatty acid fraction was responding to the nutritional needs of the subject, rising during fasting and falling when plasma glucose was available. It was suggested (126) that unesterified fatty acids represent the blood lipid fraction immediately available to cells in general as a supply of lipid to meet energy demands, and that their release into the blood is regulated by some mechanism sensitive to the need for fat oxidation.

That stored fatty acids of adipose tissue were released to plasma as nonesterified fatty acids soon became clear. Gordon *et al.* (128) reported large negative A-V differences in man for fatty acid content of plasma draining areas rich in adipose tissue. Wadström (339) reported that in adipose tissue of the rabbit,

[2] Following a suggestion made by Dole and Rizack (85), when referring to the plasma, we use the simple term "fatty acids," or "long-chain fatty acids" as a synonym for "nonesterified" or "unesterified" fatty acids, although these terms, as well as "free fatty acids" will sometimes be used if deemed desirable for the sake of clarity.

after administration of epinephrine, there was a marked rise in diglycerides and monoglycerides, an indication that lipolysis had occurred. Gordon and Cherkes (127) reported stimulation of release of fatty acids from rat adipose tissue *in vitro* by factors which elevate plasma fatty acid concentrations *in vivo*. Havel and Fredrickson (139) reported a replacement time of the order of 3 minutes for the plasma fatty acid fraction in dogs (see also 19, 107). By 1957 (83) it was realized that the flux through the plasma fatty acid pool, estimated as calories, exceeded the flux through the plasma glucose pool. In 1957, Laurell (184) reported a replacement time for the plasma fatty acid pool in fasting man of the order of 4 to 5 minutes, and estimated that the turnover was large enough to satisfy the bulk of the caloric requirement in the fasting state. The extremely rapid rate of turnover of the plasma fatty acid pool proved eventually to be decisive in understanding the rapid rise of liver triglycerides in certain instances of toxic fatty liver although this was not appreciated at the time these observations were made. As noted above, it had been suspected for many years (see *e.g.*, 237, p. 434; 322) that failure of lipid transport from liver to plasma played a role in pathological fatty liver of various etiologies. This suspicion was reinforced by a number of studies. Olson *et al.* (230, 232) maintained rats for 2 weeks on alipotropic diets and noted a reduction in all classes of serum lipids. Significantly, the low-density lipoprotein fraction, relatively rich in triglycerides, virtually disappeared. Feinberg *et al.* (98) noted in dogs that after 25 to 27 days of ethionine administration, total serum fatty acid, phospholipid, and cholesterol levels were almost zero. Both high- and low-density serum lipoproteins fell to very low levels. On withdrawal of ethionine, serum lipids returned to normal (see also 341). Fredrickson and Gordon (106, p. 618) suggested that these instances of hepatic lipid accumulation might be due to deficient transport of fatty acids because of vehicular failure, and pointed out that the solution to the fatty liver problem would have to wait until such fundamental information as the site of lipoprotein formation itself was definitely established. The missing pieces to the puzzle were soon supplied. Dole (83) had noted widespread distribution of radioactivity in various rat tissues following intravenous administration of palmitate-1-$C^{14}$. Liver lipids were the most radioactive. Bragdon and Gordon (22) observed that 10 minutes after intravenous administration of palmitate-1-$C^{14}$ to rats, from 14 to 16% of injected radioactivity was present in liver lipids. Laurell (186) noted an extremely short replacement time for the plasma fatty acid pool in rats, of the order of 1 to 2 minutes and, more significantly, he showed that injected palmitic acid-1-$C^{14}$ was recycled as plasma triglyceride. Comparison of the specific activities of plasma triglyceride and liver triglyceride suggested that the entire plasma triglyceride pool was derived from the liver. Laurell (186) also studied the rate of turnover of the plasma triglyceride pool in rats and reported values of the order of 6 to 8 minutes. These studies suggested that the flow of fatty acids from the plasma fatty acid pool, to liver triglycerides, to plasma triglycerides was a major pathway, accounting for a substantial fraction of the entire plasma fatty acid flux. After administration of palmitic acid-1-$C^{14}$ to rats triglycerides of the microsome fraction have the highest specific activity,

much higher than the large triglyceride fraction which floats on top of centrifuged liver homogenates; and the specific activity of the microsomal triglyceride fraction falls rapidly from 15 minutes to 30 minutes (315), the fall indicating that radioactivity in this pool is being diluted rapidly by unlabelled fatty acids. These data indicated an extremely rapid turnover of the triglyceride pool of the liver microsome fraction, much faster than the rate of turnover of the bulk of the liver triglyceride fraction. The picture which emerged from this work indicated that a large share of the plasma fatty acid flux entered the liver, where a correspondingly large fraction was rapidly converted to triglycerides in a small, rapidly turning over triglyceride pool, localized in the endoplasmic reticulum, from which the newly formed triglycerides entered either into the plasma lipoprotein triglyceride pool, or into an intrahepatic storage pool of triglycerides. This insight into hepatic handling of plasma long-chain fatty acids suggested the possibility that entrance of plasma long-chain fatty acids into the liver, and biosynthesis of these fatty acids to triglycerides was occurring more or less normally after carbon tetrachloride poisoning, but exit of triglycerides from liver to plasma was blocked. Two pieces to the puzzle were still missing. One of these was evidence that the flux of fatty acids from plasma to liver triglycerides to plasma triglycerides was great enough to account for the rapid rise in liver triglycerides in the carbon tetrachloride-poisoned rat (296), and most important of all, a definitive experimental design that could test the hypothesis was needed. Help came from an unexpected source. Kellner and co-workers (171, 172) had first noted in 1950 that intravenous administration of certain non-ionic surface-active agents produced a sustained hyperlipemia. During the next 10 years, this phenomenon had been much studied in connection with the problem of atherosclerosis. The hyperlipemic effect first observed by Kellner (171) was soon confirmed by Cornforth *et al.* (54) and by Friedman and Byers (110). The non-ionic detergent most used in these studies was Triton WR-1339 (hereinafter referred to as Triton), available from Winthrop Laboratories, 1450 Broadway, New York, N.Y., and described by them as an oxyethylated tertiary octylphenol, formaldehyde polymer. The hyperlipemia produced by this agent is striking. After intravenous administration of 100 mg of Triton into rats, Friedman and Byers (110) noted an increase in plasma neutral fat from 26 mg% to 769 mg%, during 24 hours. They found no histological abnormalities in liver, adrenals, heart and aorta, lungs, or intestines 24, 48, and 72 hours after intravenous Triton, and concluded that the hyperlipemic effect was due to alterations induced by Triton in plasma lipoproteins. Brown *et al.* (34) suggested that Triton combined with the lipoprotein substrate to yield products which are not attacked by lipoprotein lipase. Schotz *et al.* (297) showed more conclusively that Triton inhibited the action of lipoprotein lipase by a physical alteration of the substrate, and this phenomenon has since been studied extensively by Scanu and co-workers (288–290). In a beautiful study, published in 1956, Hirsch and Kellner (148) showed that the expected large rise in serum cholesterol, following intravenous administration of Triton to rabbits, was completely eliminated by prior hepatectomy. In 1957 Friedman and Byers (111) showed that Triton greatly retarded the escape of triglycerides from the plasma compartment of rats, and

in 1959 the effect was confirmed in the rabbit (38a). This work set the stage for an experiment which had an immediate and profound effect on the course of study of toxic and nutritional fatty liver. Friedman and Byers (111) were fully aware of the importance of the plasma fatty acid fraction in supplying various tissues with noncarbohydrate fuel, and of the uncertainties then current (106) regarding the importance of the flux of fatty acids through the liver and plasma triglyceride pools in the noncarbohydrate fuel-bearing processes. They asked an important question: What was the source of the astonishingly large amounts of triglyceride which accumulated in the plasma compartment after Triton administration? They recognized that by linking the triglyceride-sequestering effect of Triton with experimental removal of various tissues it should be possible to determine the site of origin of the plasma triglycerides. They showed first that in sham-operated rats, plasma triglyceride levels rose 20-fold within 6 hours after intravenous Triton (39). When liver and gastrointestinal tract were removed, there was no elevation of plasma triglyceride levels after intravenous Triton. With hepatectomy alone the increase was only 4- to 5-fold, indicating that the liver was the major source of the plasma triglyceride fraction. As indicated above, earlier workers had already noted the rapid movement of labelled liver triglycerides into the plasma triglyceride pool. However, the huge magnitude of the flux of fatty acids from liver triglycerides to plasma triglycerides was first fully evident in the Triton experiments of Byers and Friedman (39). In our laboratory the possible importance of the work of Byers and Friedman to the problem of pathological lipid accumulation was first recognized by Dr. B. Lombardi. With the knowledge that carbon tetrachloride was concentrated in the liver within the first several hours (257), that simultaneously liver triglycerides were rising rapidly (296), and that at the same time the endoplasmic reticulum was damaged both enzymatically (224, 258) and structurally (225), it was possible to formulate a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation (259). Furthermore, the Triton experiments of Byers and Friedman also provided the means to test the hypothesis. If carbon tetrachloride poisoned the liver in such a way that entrance of plasma fatty acids into the liver, and biosynthesis to triglycerides were essentially normal, and if at the same time exit of liver triglycerides to plasma was blocked, then, if experimental animals were first subjected to carbon tetrachloride intoxication, and subsequently challenged with intravenous Triton, they should exhibit a markedly reduced post-Triton triglyceridemia. This expectation was fully realized (259). Ninety minutes after Triton administration to control rats, plasma triglycerides were elevated 12-fold. But if rats had been given carbon tetrachloride only 2 hours previously, intravenous Triton administration resulted in only a 3-fold rise in plasma triglycerides. Evidently, carbon tetrachloride administration had very significantly blocked the movement of triglycerides from liver to plasma. Furthermore, in non-Tritonized rats given carbon tetrachloride, plasma triglyceride concentration fell from 24.4 mg% to 6.4 mg% within 3.5 hours after intoxication, a result that would be expected if plasma triglycerides were running off to peripheral sites while replenishment from the liver was curtailed.

The work of Byers and Friedman (39), which revealed the magnitude of the

triglyceride flux from liver to plasma, and the demonstration (259) that a hepatic triglyceride secretory mechanism was blocked shortly after carbon tetrachloride poisoning established the pathophysiological basis for carbon tetrachloride fatty liver. This work opened the door to a number of important questions. (1) Could a way be found to test the basic hypothesis by use of experimental designs not involving use of Triton? (2) Was the normal plasma fatty acid flux great enough to account for the hepatic triglyceride accumulation? (3) Is the normal rate of secretion of triglycerides by the liver sufficient to account for the rate of rise of liver triglycerides after carbon tetrachloride poisoning, on the assumption that the toxic agent blocks the exit pathway? (4) How effective is the action of Triton in blocking exit of triglycerides from the plasma compartment? (5) Did biochemical mechanisms in the hepatic parenchymal cells necessary for conversion of nonesterified fatty acids to triglycerides survive carbon tetrachloride poisoning? (6) Was blockade of hepatic triglyceride secretion peculiar to carbon tetrachloride poisoning or could pathological hepatic lipid accumulation following administration of other toxic agents, *e.g.*, ethionine, phosphorus, cerium, and ethanol, also be due to interference with hepatic triglyceride secretion? Was hepatic triglyceride accumulation in choline deficiency, protein malnutrition, and orotic acid feeding due to failure of triglycerides to leave the liver? An attempt will be made in what follows to review major developments along these several lines.

### *B. Further evidence that hepatic secretion of triglycerides is impaired after poisoning with carbon tetrachloride*

Marked lowering of post-Triton triglyceridemia in rats by previous administration of carbon tetrachloride (259) has since been confirmed in this laboratory on several occasions and in other laboratories as well (243, 265).

If carbon tetrachloride poisoning leads to a marked reduction in output of triglycerides from liver to plasma, while at the same time the normal exit of triglycerides out of the plasma compartment is unaffected, a reduction in plasma triglyceride levels should result. This result was reported by Recknagel *et al.* (259) and confirmed by Rees and Shotlander (265), by Poggi and Paoletti (243), and by Maling *et al.* (200). The latter authors were the first to demonstrate (104, 200), without the use of Triton, that after carbon tetrachloride poisoning there is a marked reduction in transfer of triglycerides from liver to plasma. On administration of palmitate-1-$C^{14}$ intravenously into rats poisoned with carbon tetrachloride, radioactivity appeared in the triglyceride fraction of the liver in good yield, but its appearance in the plasma triglyceride fraction was markedly attenuated. Shortly before the work of Maling *et al.* (200) appeared, it had been shown by Borgström and Olivecrona (21) that virtually no radioactivity recycles in the plasma triglyceride pool if palmitate-1-$C^{14}$ is injected intravenously into a hepatectomized rat. Havel and Goldfien (140) reported similar findings for the dog. A comparison of figure 2 of Maling *et al.* (200) with figure 2 of Borgström and Olivecrona (21) indicates clearly that with respect to hepatic triglyceride secretion, the carbon tetrachloride-poisoned rat is for all intents and purposes

functionally hepatectomized. Schotz *et al.* (293) reported that in the normal, fasting rat 0.15 mg of triglycerides per minute per 100 g rat body weight were being transferred from liver to plasma, and that 4 hours after carbon tetrachloride poisoning the above rate was reduced to 0.015 mg per minute. These quantitative data were made possible by application of a multicompartment model of hepatic lipid metabolism developed by Baker and Schotz (13).

We know of no experimental work indicating that intravenous administration of Triton interferes in any way with hepatic lipid metabolism. This is particularly important for the following reason. Since the isotope data of Maling *et al.* (200) and of Schotz *et al.* (293) confirmed the earlier conclusion of the Triton experiments (259), and since no evidence is available indicating any direct effects of Triton on hepatic lipid metabolism, the validity of the Triton test as a simple and rapid method of detecting an inhibition in hepatic triglyceride secretion is well enough established to warrant its use for at least a preliminary survey of other types of pathological condition leading to abnormal hepatic triglyceride accumulation.

Heimberg *et al.* (144) were able to confirm by a third method that carbon tetrachloride poisoning leads to inhibition of movement of triglycerides from liver to plasma. These workers monitored release of triglycerides by the isolated rat liver into perfusion fluid. With livers taken from normal fed rats the perfusion fluid became enriched with triglyceride as perfusion of the isolated liver proceeded, whereas, if the liver was taken from a rat 3.5 hours after carbon tetrachloride administration, triglycerides of the perfusion fluid were removed by the liver. This demonstration that prior administration of carbon tetrachloride alters the capacity of the isolated liver to secrete triglycerides is of course precisely the result one would have predicted on the basis of the earlier work. Nevertheless, use of the isolated liver preparation presents certain advantages for the study of hepatotoxicity. For example, it was shown (143) that injection of 5 to 10 $\mu$l of carbon tetrachloride directly into the portal venous cannula of perfused rat livers inhibited net outward movement of triglycerides. A direct effect of the toxic agent on the liver was thus demonstrated. However, the isolated perfused liver as employed by Heimberg and associates has one decided disadvantage, in that the method, as they used it, permits qualitative decisions only. In particular, it should be recognized that the maximum rate of net efflux of triglycerides by the isolated liver, as reported by Heimberg and associates (141–145) is of the order of 0.1 the rate at which the normal, fasted rat is releasing triglycerides into the plasma compartment (13, 293, and see below, VI C). With 0.9 of the normal rate of secretion *in vivo* unaccounted for, the demonstration that carbon tetrachloride reduced the secretory rate obviously has qualitative significance only. However, in the light of the Triton experiments (243, 259, 265) and the isotope experiments (13, 200, 293) the significance of the perfusion experiments is greatly strengthened. It may be pointed out that the low rates of hepatic triglyceride secretion as reported by Heimberg and associates can be greatly increased if the $d > 1.21$ plasma protein fraction is added to the perfusion medium, as reported by Eder and co-workers (89, 90) and Roheim *et al.* (279).

### *C. Quantitative considerations*

The discovery that carbon tetrachloride poisoning leads to marked inhibition of the normal transfer of triglycerides from liver to plasma (259) and its subsequent confirmation (243, 265) and extension (144, 200, 293) raised the question of the source of the fatty acids which accumulate. In fasting rats the diet cannot be a source. It has been known for many years that lipogenesis from glucose is markedly attenuated in the fasted rat (198, 203). Although it has not been demonstrated that this attenuation applies to the fasted rat given carbon tetrachloride, it would be highly unlikely that carbon tetrachloride poisoning would lift the restriction imposed on synthesis of fatty acids in the liver during fasting. According to Schotz *et al.* (293), in the normal, fasted, male rat 16% of newly formed hepatic triglycerides is derived directly from plasma triglycerides. If the plasma triglyceride pool is to serve as a source for the *net* transfer of triglycerides into the liver, then the plasma triglyceride pool would have to be supplied by some source other than the liver. Borgström and Olivecrona (21) studied recirculation of intravenously administered palmitate-1-$C^{14}$ into plasma lipids in glucose-fed rats. Considerable incorporation of label into plasma glycerides and phospholipids occurred in the intact animal, but after hepatectomy this recycling of plasma fatty acids into plasma lipids was almost completely abolished. These results indicate that when the diet is eliminated as a source, the liver is the only quantitatively significant source of plasma triglycerides. Hence net movement of triglycerides in the reverse direction (plasma to liver) can be eliminated. In addition to being the only quantitatively significant source of plasma triglycerides, in the fasted rat the liver is also the only source of plasma phospholipids (100, 123). Hence the plasma phospholipid pool is also ruled out as a source for net movement of fatty acids into the liver. These considerations lead to the conclusion that the albumin-bound fatty acids of the plasma are the sole source for the fatty acids which accumulate as triglycerides in the liver of the fasted rat poisoned with carbon tetrachloride. This important point was stated by Olivecrona (226), who said for the fasted rat that "... no 'net gain' of liver fatty acids can result from uptake of plasma esterified fatty acids."

These considerations raise 2 questions. (1) Is the plasma fatty acid flux in the normal animal enough to account for hepatic triglyceride accumulation? (2) Is the normal rate of secretion of triglycerides by the liver sufficient to account for the rate of accumulation of liver triglycerides, on the assumption that carbon tetrachloride has resulted in a block in the exit pathway?

*1. Rate of accumulation of liver triglycerides after carbon tetrachloride poisoning.* Schotz and Recknagel (296) reported that for the first 3 hours after intragastric administration of carbon tetrachloride the rate of hepatic triglyceride accumulation per 100 g body weight was 0.16 mg per minute. From the first to the third hour, the rate was 0.23 mg per minute. Other recorded rates in fasted male or female rats vary from 0.125 to 0.2 mg per minute (242, 265, 293, 320). The mean figure corresponds to 0.15 mg of fatty acids that would have to arrive *via* the blood stream per minute to account for the rate of triglyceride accumulation.

*2. Flux of fatty acids through the plasma pool.* Exit of palmitate-1-$C^{14}$ from the plasma compartment has been studied with the aid of the kinetic equation defining a single-compartment open system, with no return of label. For such a system, exit of material from the compartment is first-order with respect to concentration (19, 51, 105, 139) and flux is given by the product of pool size and fractional turnover rate. To estimate pool size, steady-state concentration and distribution volume are required. Estimation of steady-state concentration of plasma fatty acids is straightforward (82), but estimation of distribution volume of injected palmitate-1-$C^{14}$ is attended by uncertainties. Immediately after intravenous administration, the palmitate-1-$C^{14}$ albumin complex is rapidly mixed in the vascular compartment of the rat. This event is complete within 20 seconds. Following palmitate-1-$C^{14}$ addition to the plasma compartment, radioactivity appears in expired carbon dioxide within the first 2 minutes (207). The "miscible pool" (207), or "equilibration pool" (229) of fatty acids from which this carbon dioxide is derived is of the order of 100 to 250 times the plasma free fatty acids in circulation at any moment. The equilibration pool in the fasted rat consists largely of esterified fatty acids in liver and muscle, and in the unesterified fatty acid pool of adipose tissue (138, 185). The rapidity with which intravenously administered palmitate-1-$C^{14}$ is converted to carbon dioxide (207), and the rapid entry of plasma fatty acids into the equilibration pool (183, 331) attest the rapidity with which plasma fatty acids are leaving the plasma compartment. Therefore, the requirement of the isotope washout method that mixing of label with the *plasma* fatty acid pool be complete before significant loss of label to the equilibration pool occurs is not met in the rat. Data collected in our laboratory support this view. Mean distribution spaces for albumin-bound palmitate-1-$C^{14}$ were 8.9 and 8.8 ml per 100 g body weight, for control rats and for carbon tetrachloride-poisoned rats, respectively. Measurements were made 4 hours after carbon tetrachloride poisoning. These distribution spaces for intravenously administered palmitate-1-$C^{14}$ are about double the plasma volume. From these results it is evident that estimates of plasma fatty acid flux based on the assumption that the distribution space of intravenously administered palmitate-1-$C^{14}$ corresponds to the plasma volume will be minimum estimates, since the effective distribution space for the administered isotope may exceed the plasma volume.

The fractional turnover rate can be obtained from the washout curve for intravenously administered palmitate-1-$C^{14}$. Since the washout curve deviates from a single exponential within the first 2 minutes (186, 226, 294) it is necessary to make measurements within 2 minutes in order to estimate the initial disappearance rate of intravenously administered palmitate-1-$C^{14}$. Dole and Rizack (85) pointed out that estimates of total plasma fatty acid flux based on washout kinetics of a single, labelled fatty acid could only be approximations since the whole mixture of plasma long-chain fatty acids is involved in the flux. However, these workers were able to confirm in a clever way (85), independent of radioisotope flux data, that the fractional turnover rate in man was about 0.3 $min^{-1}$, a value which had been found earlier for man (105) as well as for the dog (19, 139) with the isotope method. The demonstration of Dole and Rizack (85) lends

credence to the isotope washout method. (For discussion of the theoretical basis of the method, see 51, p. 21; 334, p. 179; and 350.)

Fractional turnover rates derived from washout curves for intravenous palmitate-1-$C^{14}$ in the first 2 minutes are between 0.7 and 0.8 $min^{-1}$ (186, 226, 294). They are indistinguishable in normal rats or those given carbon tetrachloride 4 hours earlier (251, 294; see table 1). Since fatty acids are accumulating as triglycerides in the liver of the rat poisoned with carbon tetrachloride at a rate of only 0.15 mg per minute per 100 g body weight, the plasma fatty acid flux can easily supply the fatty acids required to account for the rate of hepatic triglyceride accumulation. About ⅓ of the plasma fatty acid flux enters the liver in the intact rat (22, 125, 138, 146, 185, 226, 227, 329). On the basis of the minimal values for the plasma fatty acid flux given in table 1, about 60 to 70% of total plasma fatty acid flux is being trapped in the liver triglyceride pool of rats poisoned with carbon tetrachloride. Maling *et al.* (200) suggested that in addition to failure of hepatic triglyceride secretion, there appeared to be a further hepatic disturbance of some kind leading to an accelerated incorporation of fatty acids into hepatic triglycerides and phospholipids after carbon tetrachloride poisoning. Schotz *et al.* (293) reported that the rate at which plasma fatty acids were esterified to hepatic triglycerides was 46% greater for carbon tetrachloride-treated rats than for normal animals. It is of interest that one of the findings which emerged from the study of Schotz *et al.* (293) was that in the fasted, carbon tetrachloride-poisoned rat only 40% of hepatic triglycerides came *directly* from plasma free fatty acids. This finding presents certain difficulties which have not been resolved. The complex, multicompartmental analysis employed by these workers led them to conclude that in the fasted, carbon tetrachloride-poisoned rat only 3.3% of newly formed hepatic triglycerides could have been derived directly from preformed plasma triglycerides, a finding in keeping with the markedly reduced input of triglycerides into the plasma triglyceride pool from the carbon tetrachloride-damaged liver. Since they found that only 40% of hepatic triglycerides came directly from the plasma fatty acid pool, they were forced to speculate that there must be some intermediate pool lying between the plasma fatty acid pool and the hepatic triglyceride pool which was capable of

TABLE 1

*Flux of fatty acids through the plasma compartment in intact and in $CCl_4$-poisoned rats*[a]

| | Controls | $CCl_4$ fed |
|---|---|---|
| Plasma volume,[b] ml/100 g body weight | 3.53 | 3.68 |
| Plasma fatty acid concentration, mg/ml of plasma | 0.095 | 0.085 |
| Pool size, mg/ 100 g body wt. | 0.34 | 0.31 |
| Fractional turnover rate, $min^{-1}$ | 0.74 | 0.74 |
| Fatty acid flux, mg/minute/100 g body wight[c] | 0.25 | 0.23 |

[a] Data of Recknagel and Ghoshal.

[b] Human serum albumin $I^{131}$ distribution space.

[c] 4 hours after mineral oil or $CCl_4$.

contributing in a quantitatively significant manner to hepatic triglyceride accumulation following carbon tetrachloride poisoning. They suggested hepatic phospholipids as such a source. From the analysis given above, the ultimate source of the fatty acids supplying such an intermediate lipid pool would have to be plasma fatty acids. Thus, although it is possible to conclude with certainty that the plasma fatty acid flux can easily deliver fatty acids to the liver at a rate adequate to account for the rate of hepatic triglyceride accumulation following carbon tetrachloride poisoning, neither the exact fraction of the plasma fatty acid flux delivered to the liver, nor the exact intrahepatic pathways followed by the entering plasma fatty acids *en route* to the eventual triglycerides which accumulate, have been established with certainty.

*3. Rate of hepatic triglyceride secretion.* The hypothesis put forward in 1960 (259) as a basis for understanding hepatic triglyceride accumulation following carbon tetrachloride poisoning suggested that the triglycerides that accumulated in the liver are those triglycerides which normally would have left the liver for the plasma *via* hepatic triglyceride secretion. If inhibition of this pathway is the only quantitatively significant aberration in hepatic triglyceride metabolism induced by the toxic agent, then the unidirectional efflux of triglycerides from liver to plasma in the normal rat would have to be large enough to account for the rate of rise of liver triglycerides following intoxication. This rate would have to be not less than 0.16 mg triglycerides per minute per 100 g body weight, which is a low estimate (see above). According to the multicompartmental analysis presented by Schotz *et al.* (293), the rate of hepatic triglyceride secretion in fasted male rats is 0.15 mg per minute. After carbon tetrachloride poisoning, the rate dropped to 0.015 mg per minute. This is the highest degree of suppression of hepatic triglyceride secretion by carbon tetrachloride which has been reported. Experiments based on depression of post-Triton triglyceridemia after carbon tetrachloride poisoning indicate a degree of suppression of the order of ⅓ to ⅕ normal. However, even given the degree of suppression reported by Scholtz *et al.* (293), inhibition of the normal rate of secretion cannot fully account for the rate of accumulation.

An estimate of the unidirectional efflux of triglycerides from liver to plasma can be obtained in another way. Following intravenous administration of Triton, exit of triglycerides from the plasma compartment is markedly reduced. If $A$ is the total plasma triglyceride pool, then following Triton administration, the rate of increase of the plasma triglyceride pool is given by

$$dA/dt = J - J' \tag{1}$$

in which $J$ is the unidirectional influx of triglycerides into the plasma compartment, and $J'$ is the unidirectional efflux. The quantity $dA/dt$ can be measured directly as the rate of increase of the plasma triglyceride pool following Tritonization. Since there is no reason to expect that triglycerides entering the plasma compartment as alimentary chylomicrons would not be subject to the sequestering action of Triton, estimates of hepatic triglyceride secretory rates based on

monitoring the rate of rise of plasma triglycerides following Triton administration must be carried out with fasted animals, or in animals ingesting completely lipid-free diets. The Triton method is based on the assumption that the liver is the only quantitatively significant source of plasma triglycerides (see 21, and discussion above). Furthermore, the presence of Triton in the plasma compartment is not known to affect the efflux of triglycerides from liver to plasma. If Triton affected this efflux, one might expect to find Triton-induced alterations in the triglyceride content of otherwise normal rats or of rats previously treated with various toxic agents leading to hepatic triglyceride accumulation. We have never observed such effects (196, and Recknagel, unpublished). It thus appears well founded that equation 1 can lead to a reliable estimate of the rate of secretion of triglycerides by the liver.

Recknagel *et al.* (259) reported that 90 minutes after Tritonization the rate of accumulation of triglycerides in the plasma compartment was 0.116 mg per minute per 100 g body weight (assuming 4 ml plasma). Other data obtained in this laboratory at various times over a period of several years indicated rates of 0.097 to 0.123 mg per minute per 100 g body weight. These values are for the first 2 hours following Tritonization of fasted male rats. Another series of experiments was carried out on rats ingesting a completely fat-free, semisynthetic diet supplemented with choline. The rise in plasma triglycerides was linear for the first 2 hours and decreased slightly over the next 3 hours. Plasma triglycerides increased at a rate of 0.134 mg per minute per 100 g body weight. From equation 1, the unidirectional influx of triglycerides from liver to plasma is the sum of the rate of change of plasma triglyceride content, and the efflux of triglycerides from the plasma compartment, both measured after Tritonization. The efflux of triglycerides from the plasma compartment following Triton administration was determined by use of an unpublished method described below and was found to be 0.011 mg triglycerides per minute per 100 g body weight. Therefore, for rats ingesting a fat-free diet, the liver is secreting triglycerides at a rate of 0.145 mg per minute. This value is virtually identical with the secretory rate of 0.15 mg per minute per 100 g body weight found by Schotz *et al.* (293) with an entirely different method.

The validity of the Triton method for estimating the rate of secretion of triglycerides by the liver was checked in another way as follows. For rats ingesting a fat-free diet, we found the plasma triglyceride pool size to be 1.52 mg triglycerides per 100 g body weight. If the liver is the only source supplying the plasma triglyceride pool, the turnover time for the pool is given by the ratio of the pool size over the influx rate, *i.e.*, the turnover time is 1.52/0.145, which equals 10.5 minutes. We determined the turnover time for the plasma triglyceride pool in rats ingesting a fat-free diet by monitoring washout of $C^{14}$-labelled plasma triglycerides. For 5 rats we obtained a mean turnover time of 10.5 $\pm$ 1.9 minutes (standard error). This value agrees well with values reported by Laurell (186) for glucose-fed rats.

It is evident from the above data that the efflux of triglycerides from the liver of normal, fasted rats or of rats ingesting a lipid-free diet is slightly less than the

rate at which triglycerides accumulate in the liver following carbon tetrachloride poisoning. A small fraction of the rate of hepatic triglyceride accumulation appears to be due to some aberration in hepatic lipid metabolism other than inhibition of the normal efflux of hepatic triglycerides. These considerations therefore again lead *via* a different route to the conclusion reached above that after carbon tetrachloride poisoning an abnormally large fraction of the flux of fatty acids through the plasma compartment is directed into the liver to be trapped there as triglycerides.

*4. Effectiveness of Triton in blocking exit of triglycerides from the plasma compartment.* The rate of hepatic triglyceride secretion can be obtained as the sum of the rate of accumulation of triglycerides in the plasma compartment and the exit rate of triglycerides from the plasma compartment, both measured in the Tritonized animal. This section presents a method for obtaining the latter rate. The experimental details have not been published, but since the point involved is critical to the arguments presented in the previous section, the method and the main conclusion derived from its application are presented.

French and Morris (108) found that prior administration of Triton reduced efflux of plasma triglycerides to about ⅓ normal. However, the method they used does not correctly describe the physiological system under investigation, and these workers considerably underestimated the effectiveness of Triton in blocking exit of triglycerides from the plasma compartment. They administered $C^{14}$-labelled chylomicrons intravenously into rats. Radioactivity was present almost exclusively as triglyceride-$C^{14}$. Blood samples were taken and the logarithms of the percent of initial radioactivity remaining in the plasma compartment were plotted against time. In non-Tritonized rats the isotopic washout data did not follow a single exponential decline, but the data could be resolved into a fast component with a half-life of about 8.9 minutes, which accounted for the removal of about 95% of administered label, and a residual slow component with a half-life of 66.5 minutes. This decomposition of a complex washout curve by "peeling off" of a slow component is equivalent to the assumption that the disappearance of label governed by the fast component is following the kinetics of a single-compartment, open system with no return of label. This assumption is probably justified for the normal rat. For the non-Tritonized rat, the rate of change of total residual plasma radioactivity will depend on the rate of efflux of triglycerides from the plasma compartment and the concentration of radioactivity in the plasma triglyceride pool, *i.e.*,

$$dC/dt = -J'C/A \tag{2}$$

in which $C$ is residual radioactivity in the plasma compartment, $J'$ is the outflux rate and $A$ is the pool size. The outflux rate is assumed to be constant, and for the normal rat the pool size is constant, and equation 2 can be integrated. However, prior administration of Triton changes the physiological system. The efflux drops to a new constant value, but the pool size, far from being constant, is increasing rapidly. Equation 2 no longer describes the system. However, the pool size $A$ can be defined as a linear function of time. For male rats ingesting a

fat-free diet, we found that the plasma pool of triglycerides after intravenous Triton administration followed a linear increase with time for the first 2 hours according to the equation $A = A_0 + kt$, in which $A_0$ is the initial pool size before Triton, and $k$ is the linear rate constant. Time $t$ is in minutes. Inserting this expression into equation 2, integrating between suitable limits and rearranging leads to the following expression,

$$\log C = -(J'/k) \log [(A_0 + kt)/A_0] + \log C_0 \quad (3)$$

in which $C_0$ = total initial plasma radioactivity; $C$ = plasma radioactivity at time $t$; $A_0$ = plasma triglyceride content before Triton (equal to 1.52 mg triglycerides per 100 g body weight in our experiment); and $k$ = the linear rate constant for the increase in plasma triglycerides after Triton (equal to 0.134 mg triglycerides per minute per 100 g body weight in our experiment). It is evident from equation 3 that if the logarithm of the residual radioactivity is plotted against the logarithm of the term $(A_0 + kt)/A_0$, the data should fall on a straight line of slope $-J'/k$.

We administered palmitate-1-$C^{14}$ intravenously to Tritonized donor rats and obtained serum after 45 minutes or after 6 hours. Radioactivity was distributed as follows: neutral lipid, 91.8%; phospholipid, 7.5%; nonesterified fatty acids, less than 0.7%. The radioactive serum was injected intravenously into rats which had been given Triton 30 minutes previously. Plasma samples were obtained at frequent intervals up to 4 hours. When plotted according to equation 3, the data fell nicely along a straight line. For 3 rats ingesting fat-free diets, efflux from the plasma compartment following intravenous Triton was 0.00921, 0.00963, and 0.0139 mg triglycerides per minute per 100 g body weight (mean 0.011 mg per minute per 100 g body weight).

For rats ingesting a fat-free diet, the liver is secreting triglycerides at a rate of 0.145 mg per minute per 100 g body weight (see above). For non-Tritonized rats, this is also the unidirectional efflux of triglycerides from the plasma compartment. Since the unidirectional efflux from the plasma compartment after Tritonization is 0.011 mg per minute per 100 g body weight, Tritonization reduces the efflux of triglycerides from the plasma compartment by more than 90%.

### *D. Maintenance of hepatic triglyceride biosynthesis after carbon tetrachloride poisoning*

Conversion of plasma fatty acids to liver triglycerides involves activation of the fatty acids to their fatty acid acyl coenzyme A derivatives, transfer to $\alpha$-glycerophosphate to yield phosphatidic acids, dephosphorylation of the phosphatidic acids to yield the corresponding $\alpha,\beta$-diglycerides, and finally formation of triglycerides by addition of a third fatty acid (173–175). Since hepatic triglyceride accumulation continues at a steady rate for at least 20 hours after intoxication, one would predict that this pathway is intact for this period.

In the fasting rat the liver is the major, if not the sole, source of plasma triglycerides (21, 39). Since plasma triglycerides in the fasting rat are derived from liver triglycerides and not *vice versa*, the fact that radioactivity administered

intravenously as palmitate-1-$C^{14}$ into carbon tetrachloride-poisoned rats is found in the liver triglyceride fraction in high yield (104, 200, 292, 293) shows that triglycerides are synthesized in the damaged liver. The supposition that the liver is the only quantitatively significant source of plasma triglycerides in the fasted rat is central to the above argument, and in this sense the evidence derived from these experiments is indirect.

Rees and Shotlander (265) measured incorporation of $C^{14}$-pyruvate into total lipids of liver slices obtained from rats 3 hours after carbon tetrachloride poisoning. They reported a depression of 44% in comparison with control liver slices. Data of Rossi and Zatti (282) show that during the first 4 hours after carbon tetrachloride administration to rats there is no statistically significant depression of conversion of fatty acids to their acyl coenzyme A derivatives by liver homogenates; depression was evident by 5 hours, but even by 7 hours the activation was depressed only 25%.

We studied the movement of palmitate-1-$C^{14}$ from a perfusion medium into the triglyceride fraction of isolated, perfused livers taken from carbon tetrachloride-poisoned or control rats (Recknagel, Ghoshal, and Ward, unpublished). Radioactivity recovered in the hepatic neutral lipid fraction, free of unesterified fatty acids, was 19.6% of added palmitate-1-$C^{14}$ for control rats and 20.5% and 26.8% of added palmitate-1-$C^{14}$, respectively, 4 hours and 12 hours after carbon tetrachloride poisoning. In all 3 cases, radioactivity of the triglyceride fraction accounted for almost all of the radioactivity of the neutral lipid fraction. By 24 hours after carbon tetrachloride poisoning, when the liver mitochondria have undergone extensive functional degeneration, the capacity of the isolated, perfused liver to incorporate palmitate-1-$C^{14}$ into liver triglycerides was markedly reduced. The perfusion experiments demonstrate unequivocally that the biosynthetic pathway from plasma long-chain fatty acids to liver triglycerides remains intact for at least the first 12 hours after carbon tetrachloride poisoning.

## VII. DEPRESSION OF PROTEIN SYNTHESIS AS A BASIS FOR CARBON TETRACHLORIDE HEPATOTOXICITY

In 1961, Smuckler and co-workers (311, 312) reported that after carbon tetrachloride administration to rats a widespread dislocation of ribonucleoprotein particles from the membranes of the rough endoplasmic reticulum occurred with no apparent alteration in mitochondrial structure. Functional derangements of the mitochondria were not demonstrable before 6 to 12 hours, but within 2.5 hours incorporation of intravenously administered glycine-1-$C^{14}$ into plasma albumin, fibrinogen, and $\gamma$-globulin were markedly depressed. Entrance of amino acids into the liver was not impaired, but incorporation of radioactivity into whole liver proteins was considerably depressed. The defect in protein synthesis did not involve the supernatant fraction of rat liver homogenates, which contains the amino acid-activating enzymes, transfer enzymes, and sRNA, but was confined to the microsome fraction (309). Ultracentrifugation data indicated that in carbon tetrachloride-treated rats, a disaggregation of the larger classes of hepatic ribonucleoprotein particles into smaller classes had occurred

(309). Similar results were reported for thioacetamide poisoning (307, 308). Subsequent work demonstrated that disaggregation of the 80S ribosomes occurs after administration of low doses of carbon tetrachloride (307), and that depression of protein synthesis occurs within 1 hour (310). It was suggested (310) that the primary lesion in carbon tetrachloride poisoning may be the disorganization of the ribosome particles, and the separation of the latter from the ergastoplasmic membranes. Smuckler *et al.* (312, 313) suggested that mitochondrial degeneration, which occurred under their experimental conditions 3 to 6 hours after the impairment in protein synthesis, might be due to impairment in replenishment of necessary protein-containing constituents of the mitochondria, and attributed cell death to altered cellular functions dependent on protein synthesis. This hypothesis appears to be an oversimplification, as will be pointed out shortly.

Depression of hepatic protein synthesis has also been invoked as the key to pathological hepatic lipid accumulation. It is self-evident that the liver could not extrude uncombined triglycerides into the aqueous interstitial fluid or into the plasma. Triglycerides leave the liver as lipoproteins. A possible key to the biochemical nature of the block in hepatic triglyceride secretion could be an early failure of the liver of the carbon tetrachloride-poisoned rat to supply the protein needed for lipoprotein formation. Seakins and Robinson (300) stated: "The inhibition of the synthesis of plasma lipoprotein-protein after the administration of carbon tetrachloride is of particular interest since it may explain the subsequent changes in the concentrations of lipid in plasma and in liver. It seems reasonable to suggest that reduced synthesis of plasma lipoprotein will result in a fall in the amount of lipid which is normally transported from the liver in combination with this protein and that this will lead both to a decrease in the concentration of neutral lipids in the plasma and to the accumulation of fat in the liver. The failure of the 'triglyceride secretory mechanism' of the liver . . . can thus be attributed to the inhibition of the formation of plasma lipoprotein-protein." Robinson and Seakins (275) predicted correctly that administration of puromycin, which inhibits hepatic protein synthesis, should induce a fatty liver. They also showed (300) that within 2 hours after carbon tetrachloride poisoning, the incorporation *in vivo* of leucine-1-$C^{14}$ into lipoproteins and residue proteins of the plasma, as well as into liver proteins, was reduced. Liver slices of rats dosed with carbon tetrachloride also converted less leucine-1-$C^{14}$ into plasma lipoproteins and liver proteins. Depression of hepatic protein synthesis by carbon tetrachloride has been confirmed (255, 264, 265).

It is now quite clear that depression of hepatic protein synthesis is an early consequence of carbon tetrachloride poisoning, but the data are not adequate to support the views that the primary event in carbon tetrachloride poisoning is the disaggregation of the ribosomes (310), that attendant depression in protein synthesis is the key to the necrotizing action of this hepatotoxin (312, 313), or that depression in protein synthesis is the key to the pathological disturbance leading to hepatic triglyceride accumulation (272, 300).

It is evident that pathological disorganization of polyribosomes, and separa-

tion of ribonucleoprotein particles from ergastoplasmic membranes, are structural alterations. For the structures involved, the pathological alterations observed may be primary, in the sense that no other observable structural alterations precede those under observation. However, from a molecular point of view it is evident that structural disorganization must be an expression of underlying chemical and physical changes which constitute the primary lesion.

Within 2 hours after ethionine administration to rats (95), the liver cells are seriously affected, with ATP levels only about 20% of normal and nucleic acid and protein synthesis only 10 to 20% of normal, but despite the persistence of these abnormalities for at least 24 hours the liver cell does not die (see also 163). Because of this separation of depression in protein synthesis from necrosis in the case of ethionine intoxication, as Farber *et al.* (95) pointed out, the necrosis due to carbon tetrachloride and dimethylnitrosamine cannot be attributed merely to inhibition of protein synthesis. McLean *et al.* (212) have called attention to another example. Actinomycin D in doses of 1 mg per kg completely inhibits RNA synthesis, inhibits amino acid incorporation into protein by about 80%, and causes death of the animal in about 48 hours, without producing liver necrosis. It appears that some factor in addition to depression of protein synthesis must be invoked for initiating the chain of events which leads to death of the cell.

The view (195, 300) that depression of hepatic protein synthesis is the key to the mechanism of hepatic triglyceride accumulation in carbon tetrachloride poisoning is based on 3 main considerations: (1) The knowledge that triglycerides leave the liver as low-density lipoproteins; (2) the extension to carbon tetrachloride poisoning of findings indicating that triglyceride accumulation in ethionine poisoning is due to depression of hepatic protein synthesis; and (3) the demonstration that hepatic protein synthesis is depressed within 1 hour after carbon tetrachloride administration. In the case of ethionine poisoning, hepatic triglyceride accumulation does appear to be due to failure of the poisoned liver to manufacture the lipoprotein apoprotein. Depression of hepatic protein synthesis has also been implicated as a basis for the fatty liver of phosphorus poisoning (15, 301, 307) and dimethylnitrosamine poisoning (264, 265). In the case of choline deficiency, failure to supply adequate phospholipid for lipoprotein biosynthesis may be the key defect responsible for the fatty liver. If hepatic triglyceride accumulation following carbon tetrachloride poisoning could be understood in terms of a breakdown in supply of lipoprotein protein, a unifying mechanism can be seen to emerge: all of these instances of toxic and nutritional fatty liver could be due to failure in lipoprotein biosynthesis (see 195). A number of facts stubbornly resist inclusion of carbon tetrachloride hepatotoxicity in this scheme.

The lipids of the plasma exist almost entirely as lipoproteins. In the fasted rat the triglyceride moiety of lipoproteins of very low density ($d < 1.019$) accounts for 65% of plasma triglycerides (197). These lipoproteins represent the major vehicle for transport of triglycerides away from the liver (96, 109, 137, 138, 197, 233). The liver is the immediate source of these plasma low-density

lipoproteins for the rabbit (138), rat (13, 315) and man (96, 109, 137). For the rat and for the rabbit the liver triglyceride pool that supplies the triglycerides of the lipoproteins is not the triglyceride-rich layer of fat which appears at the top of centrifuged rat liver homogenates. This so-called "free-floating" triglyceride pool is turning over slowly and appears to be an intrahepatic storage pool of triglycerides (13, 138, 315). The immediate precursor pool of the triglyceride moiety of the plasma lipoproteins of very low density is a small and much more active hepatic pool (13, 138, 315), probably located in the endoplasmic reticulum (315), where protein synthesis and undoubtedly also lipoprotein synthesis (195, 331) occur. A large flux of fatty acids passes through this triglyceride pool in the liver and the plasma lipoproteins of very low density (13, 96, 138, 293). Formation of the latter in the liver, and their subsequent movement into the plasma, constitutes the essential function of the hepatic triglyceride secretory mechanism.

In the rat (197) the protein moiety of the very low-density plasma lipoproteins, the so-called apoprotein of the lipoprotein, constitutes only about 9% of the mass of the lipoprotein molecule (see also 131). With all other conditions remaining unchanged, failure of the liver to supply this small amount of specialized apoprotein would sooner or later result in a drop in the plasma lipoproteins of very low density and accumulation of triglycerides in the liver. This is the central thesis of the protein synthesis hypothesis (195, 272, 300). What this hypothesis fails to take adequately into consideration is that the apoprotein, which combines with lipid in the liver to form lipoprotein, may recycle back to the liver from the plasma, and, that in addition to a breakdown in hepatic protein synthesis, failure of hepatic triglyceride secretion may be the result of either defects in combination of lipid with apoprotein or release of lipoprotein to the plasma (279).

In plasma lipoproteins of very low density the turnover of triglycerides is much faster than that of protein. In man, dog, and rabbit the protein has a half-life of 2 to 4 days (10, 121, 122, 234, 290, 337), whereas the triglyceride turns over in 2 to 4 hours in man (96, 109, 137, 193, 194) and in 10 to 17 minutes in the rabbit and dog (138, 290). In the rat most of the plasma triglyceride fraction is in lipoproteins of very low density (197) and it turns over at least once every 10 minutes (186). In view of the situation existing in other species it is likely that in the rat the lipoprotein apoprotein turns over much more slowly than lipoprotein triglyceride. The large difference in turnover rates implies that the apoprotein must return to the liver shorn of lipid, where it reenters the hepatic triglyceride secretory mechanism. It is possible that the returning apoprotein may undergo some metabolic alteration in the liver before it can be re-used for lipoprotein formation. If such alteration involved peptide bond synthesis the argument favoring a central role for protein synthesis in carbon tetrachloride fatty liver would be strengthened; but no data are available on this point.

Direct evidence now exists that the liver can couple preformed plasma protein with liver lipid to form plasma lipoproteins of very low density. The isolated

rat liver perfused with blood transfers cholesterol, cholesterol esters, and triglycerides into the perfusion blood as very low-density lipoproteins, but the transfer process into whole blood ceases after about 2 hours, when only a small fraction of available hepatic cholesterol has been transferred (89, 132, 277–279). These observations suggested (90) the exhaustion of some entity in the perfusion blood necessary for the lipid transfer process. This entity is a plasma protein, the so-called "lipid acceptor protein," or very low-density lipoprotein apoprotein, which is synthesized in the liver, and can be recovered in the high-density ($d > 1.21$) protein fraction of the plasma. The active material is a glycoprotein (90) recoverable in the globulin fraction (279). Isolated, perfused livers of rats fed cholesterol will secrete triglycerides, cholesterol, and phospholipids as lipoproteins at a very slow rate when the perfusion medium is composed of washed red cells in Krebs-Ringer bicarbonate buffer. When the lipid acceptor protein is added to the perfusion medium, the rate of secretion of lipids, and notably triglycerides, is greatly increased (279). The secreted lipids appear in the perfusion fluid almost exclusively as very low-density lipoproteins (279). $C^{14}$-labelled lipid acceptor protein, which enters the protein-poor perfusion medium as very high-density lipoprotein, is converted by the liver almost exclusively into very low-density lipoproteins (279). In the absence of the liver, incorporation of labelled protein into the very low-density lipoproteins is markedly reduced (279). The efficient conversion of the lipid acceptor protein into very low-density lipoprotein exhibits a marked temperature dependence (279). These experiments show that release of lipid into the plasma is a stepwise process. The lipoidal and protein moieties of the very low-density plasma lipoproteins are synthesized *de novo* by the liver, combined into the definitive very low-density lipoprotein macromolecules, and eventually extruded into the plasma. The lipid acceptor protein re-enters the system from the plasma and is coupled with lipid to form very low-density lipoprotein. This is extruded back into the plasma. Data are not yet available on the turnover time of this very low-density lipoprotein apoprotein in rat plasma. Such data are obviously crucial to the question as to whether this lipid acceptor protein must be synthesized *de novo* in secretion of triglycerides by the liver of the rat. However, on the basis of the argument given above, it is highly likely that the replacement time for the triglyceride moiety of rat plasma lipoproteins of very low density is much shorter than the replacement time for the lipid acceptor protein.

Roheim *et al.* (279) investigated the possibility that the lipoprotein apoprotein might be degraded in the liver into its constituent amino acids, which might then be preferentially incorporated into lipoproteins. Incorporation of labelled $d > 1.21$ proteins into lipoproteins of low density was not affected by the presence of a large pool of unlabelled amino acids; this indicates that the protein precursor does not break down to constituent amino acids in the liver, but rather is incorporated directly into lipoprotein by combination with lipid. The latter conclusion was also reached in experiments with puromycin (279).

If the triglyceride moiety of the very low-density plasma lipoprotein fraction in the rat turns over considerably faster than does the protein moiety, as ap-

pears likely, then coupling of preformed apoprotein with lipid would be expected to account for far more of the triglyceride flux from liver to plasma than would be expected if the protein moiety was synthesized *de novo* for each lipoprotein molecule extruded from the liver. This conclusion is significant for the study of toxic and nutritional fatty liver. In those instances of fatty liver disease in which failure of hepatic triglyceride secretion is the central pathophysiological defect, the way is now open to determine whether the biochemical basis of the failure lies in the depression of protein synthesis or in coupling of lipid acceptor protein to hepatic lipids and final passage of the lipoprotein into the plasma.

A convincing demonstration of the importance of recognizing the dual nature of hepatic triglyceride secretion has already been made in the case of the fatty liver induced by orotic acid (280). Orotic acid fatty liver was first reported in rats by Standerfer and Handler (314). The fat which accumulates is largely triglyceride (55). Plasma triglycerides, phospholipids, and cholesterol all begin to decline within less than 1 day after ingestion of orotic acid begins, and when the levels of these plasma lipids become depressed, liver triglycerides begin to accumulate (347). Post-Triton triglyceridemia is markedly depressed in rats fed orotic acid (347). Windmueller (347) concluded that the mechanism by which dietary orotic acid induces a fatty liver involves a defect in the synthesis or secretion of lipoproteins by the liver. Orotic acid feeding leads to profound disturbances in hepatic nucleotide metabolism (201, 338). Intrahepatic ATP levels are depressed in orotic acid fatty liver (283) and in ethionine fatty liver (95), and addition of adenine to the diet reverses the pathological consequences of both (95, 133).

Liver slices taken from rats fed either ethionine or orotic acid exhibit a marked depression in the normal capacity to incorporate leucine-1-$C^{14}$ into low-density serum lipoproteins (274, 283). This observation led to the suggestion that the defect in orotic acid fatty liver consisted in a failure to synthesize the protein required to transport neutral lipid from the liver (283). However, orotic acid feeding does not inhibit growth, and more specifically, hepatic protein synthesis as a whole is not inhibited in liver slices of rats fed orotic acid (283). This finding forced the rather unsatisfactory assumption that the defect in hepatic protein synthesis would have to be specific for the protein required to transport neutral lipid from the liver (283, see also discussion in 89, p. 230). The realization that hepatic triglyceride secretion can occur through coupling of preformed lipid acceptor protein with hepatic lipids provided a new approach to this problem. Roheim *et al.* (280) confirmed that orotic acid feeding did not depress the synthesis of liver proteins, and showed that in rats fed orotic acid there was only a slight depression in incorporation of mixed $C^{14}$-labelled amino acids into whole plasma proteins. By contrast, incorporation of $C^{14}$ into the protein moiety of the very low-density plasma lipoproteins, which comprises only a small fraction of plasma proteins as a whole, was depressed 97% in comparison with the isotope incorporation into the same fraction recorded for normal rats. As pointed out by Roheim *et al.* (280), there are 2 distinct possibilities as to why labelled amino

acids did not appear in the very low-density plasma lipoproteins of rats fed orotic acid. One of these is that orotic acid feeding results in inhibition of the synthesis of the lipid acceptor protein uniquely required for formation of plasma lipoproteins of very low density, without depression of hepatic protein synthesis in general. The other possibility is that no inhibition of protein synthesis occurs, but that steps in hepatic triglyceride secretion not involving *de novo* protein synthesis are inhibited. Roheim *et al.* (280) showed that the fatty liver of the rat fed orotic acid can synthesize the apoprotein of the very low density lipoproteins. However, when a fatty liver from a rat fed orotic acid was perfused with $C^{14}$-labelled d > 1.21 proteins prepared from normal rat plasma, incorporation of perfused labelled d > 1.21 proteins into very low-density lipoproteins was markedly depressed. The liver of the rat fed orotic acid, although capable of manufacturing the lipid acceptor protein, could not convert d > 1.21 proteins into very low-density lipoproteins. Roheim *et al.* (280) also showed that when the liver from a rat fed orotic acid was perfused with d > 1.21 proteins, no lipoprotein was released. When the pathological liver was replaced during the perfusion by a liver from a rat fed cholesterol, lipoproteins rapidly appeared in the perfusate. It is apparent from these experiments that orotic acid feeding results in an inhibition of the auxiliary coupling branch of the hepatic triglyceride secretory mechanism. Not only is over-all synthesis of liver proteins and plasma proteins apparently unaffected by orotic acid feeding, but synthesis of the apoprotein of the very low-density lipoproteins appears also to be relatively unaffected. It is apparent that in whole animal experiments, or in experiments with perfused livers or liver slices, failure of amino acid $C^{14}$ label to appear in very low-density plasma lipoproteins can be due to a primary failure in hepatic protein synthesis, but it can also be due to defects in coupling of lipid to lipoprotein apoprotein, or in extrusion of lipoproteins to plasma under circumstances in which hepatic protein synthesis may be unimpaired.

The liver of the rat fed cholesterol, when perfused with buffer solution containing red cells alone, transfers lipid to the perfusion medium at a very reduced rate. When lipid acceptor protein is added to the perfusion medium, the rate of transfer of lipid from liver to perfusion medium is increased 10-fold or more (279). This result supports the view given above that recycling of lipoprotein apoprotein probably accounts for a much greater share of the triglyceride flux from liver to plasma than does extrusion of lipoproteins formed from newly synthesized lipoprotein apoprotein. Similar experiments with the liver of the dog or the rabbit would be most interesting, since in these species the replacement time of the triglyceride moiety of the very low-density plasma lipoproteins appears to be very short in comparison to the replacement time for the lipoprotein apoprotein.

The dual nature of the hepatic triglyceride secretory mechanism, as revealed in the analysis of the orotic acid fatty liver, provides a framework for the analysis of other instances of fatty liver disease. Ethionine fatty liver is a case in point (see 92, 95, and 318 for reviews). Ethionine fatty liver is similar to orotic acid fatty liver and to carbon tetrachloride fatty liver insofar as triglycerides ac-

cumulate because of their failure to leave the liver (196, 226). Within 2 hours after administration of ethionine to female rats, liver ATP levels are reduced to 20% of control levels, and 1 hour after this dramatic fall in ATP levels there is a residuum of hepatic protein synthesis of less than 20% of normal (336). It is reasonable to suppose that such marked depression in hepatic protein synthesis would be reflected in a serious restriction in *de novo* synthesis of the apoprotein moiety of very low-density plasma lipoproteins. If movement of triglycerides from the liver depended exclusively on *de novo* synthesis of all parts of the triglyceride-carrying very low-density lipoproteins, one would expect that a marked depression in hepatic protein synthesis would result in an immediate onset in hepatic triglyceride accumulation. This does not appear to be the case. Hepatic triglyceride accumulation as shown by analysis of whole rat livers for triglycerides is not evident until about 6 hours after administration of ethionine (94, 196), after which it proceeds rapidly. The picture that emerges from these studies is the following: Ethionine poisoning leads primarily to a depression in hepatic protein synthesis. The depression in protein synthesis is not necrogenic (95); hence a wide variety of hepatocellular functions continue more or less normally. In particular, the auxiliary coupling branch of the hepatic triglyceride secretory mechanism continues to function after depression of protein synthesis sets in. It is for this reason that hepatic triglyceride accumulation is delayed after depression of protein synthesis has set in. However, the apoprotein of the plasma very low-density lipoprotein fraction has a finite lifetime. Once the supply of new lipid acceptor protein is cut off, the steady-state level of this plasma protein declines. After this phase of the process is essentially complete, a matter of about 3 to 4 hours after the depression of protein synthesis, hepatic triglyceride accumulation finally begins.

Villa-Trevino *et al.* (335, 336) showed that feeding of adenine reverses the inhibition in hepatic protein synthesis caused by ethionine feeding, and Farber *et al.* (94) showed that adenine or ATP feeding also prevented the fatty liver. This effect appears to involve maintenance of adequate levels of hepatic ATP, depleted by ethionine feeding (95). However, adenine also reverses the fatty liver of orotic acid feeding (133). It has also been reported that ATP administration protects against the hepatic triglyceride infiltration observed after administration of carbon tetrachloride, ethanol, and azaserine (164). The protective effects of adenine and ATP administration suggest that these various pathological conditions may have certain features in common which have not yet become apparent. Nevertheless, at the present time, the available data support the view that the fatty liver of ethionine feeding is most probably due to a primary failure in hepatic protein synthesis.

Regarding carbon tetrachloride poisoning, the view (195, 212, 275, 300, 311, 312) that depression of protein synthesis is the key to the hepatic lipid accumulation can now be critically examined. In the opinion of this reviewer the available evidence favors destruction of the auxiliary coupling phase of hepatic triglyceride secretion as the most probable initial event. The experimental work of Robinson and Harris (274) with puromycin has been cited in support

of the protein synthesis hypothesis, yet, as McLean *et al.* (212) have pointed out, actinomycin D markedly inhibits protein synthesis without producing fatty change. Thioacetamide also depresses hepatic protein synthesis, but does not lead to hepatic lipid infiltration (15, 264, 265). Hydrazine, on the other hand, like orotic acid, produces lipid infiltration without depressing hepatic protein synthesis (4, 5). It is clear from these examples that a dual effect of an agent such as carbon tetrachloride on depression of protein synthesis and hepatic triglyceride accumulation cannot be taken by itself as evidence that the lipid infiltration is caused by the depression in protein synthesis. Other aspects of the pathological situation, in particular the rapidity of the triglyceride accumulation, and the time of onset of the changes being monitored, must be taken into consideration.

As discussed above, in the intact animal coupling of preformed lipid acceptor protein with liver lipids probably accounts for a much larger share of the flux of triglycerides from liver to plasma than does flux of triglycerides dependent on *de novo* synthesis of lipid acceptor protein. For hepatotoxic agents or dietary conditions whose action is exclusively or mainly to suppress protein synthesis, but which have relatively little effect on the auxiliary coupling phase of hepatic triglyceride secretion, one would expect to find an initial period during which protein synthesis is suppressed with little or no hepatic triglyceride accumulation. During this phase, the auxiliary coupling branch of hepatic triglyceride secretion, which accounts for most of the triglyceride flux, would continue to function. Once the supply of lipid acceptor protein is exhausted, the rate of triglyceride accumulation should promptly increase. Outright destruction of the auxiliary coupling branch of the hepatic triglyceride secretory mechanism on the other hand would be expected to result in the prompt onset of hepatic triglyceride accumulation at a maximal or near maximal rate. A prompt onset of rapid hepatic triglyceride accumulation is well documented for carbon tetrachloride poisoning (197, 296, and see VI C, above). Furthermore, as shown with exceptional precision by Lombardi and Ugazio (197), after carbon tetrachloride poisoning in the rat there is a precipitous drop in the content of triglycerides in the plasma lipoproteins of very low density, as one would expect if the reaction of the liver to the toxic agent resulted in a sudden failure of the auxiliary coupling branch of the hepatic triglyceride secreting mechanism.

An analysis of the isotope data of Robinson and co-workers also supports the view presented here. Seakins and Robinson (300) studied the effect of carbon tetrachloride administration on incorporation of amino acids into liver proteins and serum lipoprotein proteins. When protein synthesis was tested with liver slices 2 hours after intoxication, incorporation of $C^{14}$-leucine into $d < 1.063$ lipoproteins was depressed to a much greater extent ($\frac{1}{20}$ of control levels) than was incorporation into liver proteins ($\frac{2}{3}$ of control levels). This result is what one would expect if the auxiliary coupling phase of the hepatic triglyceride secretory mechanism were nonfunctional while at the same time hepatic protein synthesis was only minimally affected. With the coupling and release mechanisms destroyed, the major pathway for movement of newly synthesized lipid acceptor protein into plasma very low-density lipoproteins is eliminated. New protein

may be synthesized, but if the auxiliary coupling phase of hepatic triglyceride secretion is nonfunctional, the newly formed protein will not appear in the low-density plasma lipoproteins.

In summary, depression of protein synthesis is a prominent feature of the liver damaged by carbon tetrachloride, but this depression of protein synthesis is not the primary lesion involved in the reaction of the liver to the toxic agent. A more basic chemical phenomenon of some sort must constitute the primary lesion. Depression of protein synthesis is probably not the key to the necrotizing action of carbon tetrachloride, since some hepatotoxins, *e.g.*, ethionine and actinomycin D, depress hepatic protein synthesis yet are not necrotizing. Finally, depression of protein synthesis is probably not the most important prerequisite for hepatic lipid accumulation of carbon tetrachloride poisoning. The protein synthesis hypothesis does not take into account the dual nature of the hepatic triglyceride secretory mechanism. Rather than a defect in hepatic protein synthesis, outright destruction of the auxiliary coupling phase of hepatic triglyceride secretion is most probably the key defect which accounts for the early onset and rapid rise of hepatic triglyceride content after carbon tetrachloride poisoning.

It should be pointed out that loss of function in the auxiliary coupling phase of hepatic triglyceride secretion may, and most probably does, involve loss of biosynthetic mechanisms of some kind necessary for coupling of lipid acceptor protein and lipid in the essential step which results in formation of very low-density lipoproteins. The essence of the analysis given here is that this biosynthetic mechanism, whatever its nature may be, does not involve the complete assembly of the lipid acceptor protein from its constituent amino acids. In the opinion of this reviewer, the problem of how lipid acceptor protein combines with lipid to form lipoproteins, and in particular the relationship of this process to the nature and integrity of hepatic cell cytoplasmic membranes, is one of the most important and most challenging problems existing at the present time in the study of toxic and nutritional fatty liver disease.

## VIII. LIPOPEROXIDATION AND THE MECHANISM OF ACTION OF CARBON TETRACHLORIDE

Recent work has projected study of the metabolism of carbon tetrachloride into the forefront. The central issues are the nature and location of mechanisms involved in cleavage of the carbon-to-chlorine bond and the possible consequences of this cleavage, including in particular the peroxidative destruction of structural lipids in the hepatic cells.

### *A. Timetable of major events in carbon tetrachloride-induced liver injury*

In the rat, hepatic lipid metabolism is profoundly disturbed within the first 30 minutes after intragastric administration of carbon tetrachloride (197). The rapid onset of disturbed hepatic lipid metabolism is accompanied and followed by a maze of altered structural and metabolic patterns. Change in appearance of the endoplasmic reticulum, depression of microsomal enzyme activity, and depression of hepatic protein synthesis are demonstrable within

the first hour after intoxication. Within 2 to 4 hours calcium content of liver mitochondria is already doubled and it rises rapidly thereafter, becoming 15 times normal by 40 hours (262, 270, 328). Associated with this abnormal movement of calcium into the liver cells, there are other disturbances in electrolyte distribution, and swelling of the liver cells (212). Liver glycogen is depleted after carbon tetrachloride poisoning (284). The lysosomes become disrupted between the fifth and the tenth hour (8, 65, 68, 256, 332). Intracellular enzymes appear in the plasma (262, 266, 267). Mitochondrial damage sets in about 10 hours after intoxication. Focal necrosis is evident as early as 6 hours after poisoning, and at first is midzonal (345). By 12 hours the centrilobular cells exhibit prenecrotic changes, and balloon cells are prominent in the midzonal region. By 24 hours there is marked centrilobular necrosis affecting up to half of the lobule (345).

In considering this array of pathological disturbances, 2 fundamental questions must be considered. (1) What is the initial event in the reaction of the liver to the toxic agent? (2) Of the whole set of effects in the reaction of the liver to the toxic agent, which are critical for hepatocellular necrosis? The subject of hepatocellular necrosis has been reviewed very recently by McLean *et al.* (212). The interesting review by these workers should also be consulted for literature on the hepatotoxicity of thioacetamide, dimethylnitrosamine, pyrrolizidine alkaloids, aflatoxin, cycasin, sporidesmin, the toxin of *Penicillium islandicum*, ethionine, and dietary liver necrosis (see also 165, 167, 262, 263). Popper and Schaffner (244) should be consulted for the literature on histopathology of carbon tetrachloride hepatotoxicity and its sequelae.

To the knowledge of this reviewer, no one has yet demonstrated which of the changes induced by carbon tetrachloride are the critical ones leading to the death of the cell. The complexity of the situation is evident from recent observations of Wigglesworth (345), who has emphasized that at least 2 separate series of changes appear to be taking place in cells of the midzonal region. One appears to be entirely cytoplasmic and is characterized by increased osmophilia. The other involves both cytoplasm and nucleus, and is characterized by gross vacuolization of the cytoplasm and increased osmophilia and pyknosis of the nucleus. Cells of the central zone, where necrosis eventually becomes most prominent, are involved in what appears to be a third series of pathological changes leading to cell death.

Whatever the changes may be that lead to death of the cell, triglyceride accumulation *per se* does not appear to be decisive. The triglyceride accumulation produced by ethionine or by choline deficiency is not necrogenic. Antihistaminics afford some protection against the necrogenic effects of carbon tetrachloride, but not against the fatty changes (267). Futhermore, some hepatotoxins such as thioacetamide produce cellular necrosis with little or no lipid accumulation. This point has been emphasized by Rees and Shotlander (264, 265), although Gupta (130) found lipids in the centrilobular areas 9 hours after thioacetamide poisoning.

Depression of protein synthesis does not appear to be the critical step pre-

ceding cell death (see Section VII). Regarding the lysosomes, McLean *et al.* (212) have pointed out that the occurrence of coagulative necrosis as opposed to liquefaction necrosis casts doubt on the notion that rupture of these organelles is indispensible in the pathogenesis of necrosis (see 62 for review).

The onset of mitochondrial damage would be expected to have the most serious of consequences (212). In this connection, the possible role in the necrogenic process of abnormal entrance of calcium into the cell has long been recognized. Calcium ions produce mitochondrial swelling (159), inhibit oxidations dependent on pyridine nucleotides (159), and activate mitochondrial ATPase (245). This suggests that mitochondrial involvement may be secondary to the entrance of calcium into the cell. If loss of mitochondrial function is the key event precipitating the changes leading to cell death, and if loss of mitochondrial function depends primarily on abnormal levels of intracellular calcium, then an early loss of the selective permeability of hepatocellular membranes would loom large as a key defect. Yet, some of the earlier sequelae of carbon tetrachloride poisoning do not appear to depend on entrance of calcium into the cell. For example, Smuckler (306) reported that very high levels of calcium (10 times normal) were necessary to depress amino acid incorporation significantly in a cell free system, whereas polysome-bound calcium ". . . seems instead to decrease in $CCl_4$ intoxication." Certainly, within the first hours after carbon tetrachloride poisoning, when hepatic protein synthesis is considerably depressed, hepatic cell calcium, although somewhat elevated, is nowhere near 10 times normal. In one experiment in our laboratory we could detect no depression of hepatic microsomal glucose-6-phosphatase activity *in vitro* on addition of calcium ions over a wide concentration range. In other unpublished work we could not "activate" particle-bound lysosomal acid phosphatase by addition of calcium ions *in vitro*. Glucose-6-phosphatase and the microsomal enzymes of protein synthesis are membrane bound. The activities of these enzymes are markedly depressed within the first 2 to 4 hours after carbon tetrachloride poisoning. The fact that these enzymes are little affected by addition of calcium ions *in vitro* speaks against the notion that these early changes could be due to a simple interaction between calcium ions and the catalytic sites involved. It would seem much more likely that a direct attack by carbon tetrachloride or its cleavage products on the integrity of the membranous components of the endoplasmic reticulum is involved. Furthermore, entrance of calcium into the liver cells of carbon tetrachloride-poisoned rats is only one index of what appears to be a more general breakdown in the selective permeability of liver cell membranes. Loss of liver cell enzymes to the plasma has been reported by a number of workers (80, 81, 212, 262, 266, 267). This loss of protein is undoubtedly accompanied by loss of cofactors of smaller molecular weight (305), intermediates, and ions (165–167, 212). It is clear that one of the earliest, and perhaps the most far-reaching event in the interaction of the liver cell with carbon tetrachloride involves a destructive attack on the membranous components of the cytoplasm. The most recent work points in this direction.

### *B. Lipoperoxidation*

One approach to the elucidation of the mechanism of action of carbon tetrachloride has been through studies of conditions that ameliorate or exacerbate the toxic effects. Early work centered on the relationship of the nutritional state of animals to yellow atrophy of the liver after chloroform anesthesia (86, 87, 298) and the protective effect of drugs and other agents against poisoning by carbon tetrachloride and chloroform (58). In 1936, Forbes and Neale (102) described a preparation from hog liver that protected rats against hepatic necrosis resulting from carbon tetrachloride. The protective agent was xanthine (221). Protection by xanthine against liver injury induced by chloroform was reported by Vars *et al.* (333). However, the latter workers (248, 333) found that sodium ricinoleate given subcutaneously 24 hours before chloroform anesthesia protected rats from hepatic injury. Subcutaneous colloidal carbon (India ink) gave some protection. They ascribed these effects to a nonspecific inflammatory response, a conclusion which Calder (40) supported. It is interesting to note that Goldschmidt *et al.* (124) some time earlier had reported a protective action of oxygen inhalation against the necrotizing effect of chloroform and other anesthetics on the liver. This action of oxygen has never been explained in biochemical terms.

Leach and Forbes (187) reported that sulfanilamide, sulfathizole, and sulfapyridine, but not sulfanilic acid, protected against the lethal effects of carbon tetrachloride inhalation. This effect was confirmed by Wilson (346), who ruled out effects on intestinal flora or on the thyroid as factors. Miller and co-workers (216–218) found that methionine prevented liver damage in protein-depleted dogs even when given 3 to 4 hours after chloroform anesthesia, but methionine was of no additional benefit in dogs receiving adequate dietary protein. Drill and Loomis (88) found methionine to be ineffective against carbon tetrachloride liver damage. Brunschwig and co-workers (35, 36) reported on protection by various sulfhydryl compounds. Sodium thioglycollate was particularly effective.

In 1938, Campbell (45, 46) observed that a 5- to 7-day diet of raw carrots considerably lengthened the otherwise brief life span of rats subjected to lethal degrees of acute anoxia. The protective agent in carrots was not vitamin A. Campbell's observation was confirmed by Nelson *et al.* (222). Forbes and Taliaferro (103) then showed that a carrot diet afforded some protection against acute carbon tetrachloride toxicity. Hove *et al.* (158) confirmed that vitamin A was inactive against anoxic anoxia, and observed a protective effect by vitamin E. This set the stage for experimental work testing the effectiveness of vitamin E and other antioxidants in carbon tetrachloride poisoning. Hove (156) maintained rats on vitamin E-deficient diets that were also low in casein. In all such rats, 200 $\mu$l of carbon tetrachloride given intraperitoneally produced death within 4 days. With dietary supplements of theophylline or methionine, no deaths occurred within 4 days. $\alpha$-Tocopherol also afforded marked protection. Hove (156) pointed out that theophylline and methionine, as well as $\alpha$-tocopherol, were antioxidants *in vitro*. The antioxidant property of theophylline appears to de-

pend on the lability of the hydrogen atom at the 7-position in the imidazole nucleus. It is interesting to note that Hove found guanine to have a moderate protective effect. He also confirmed that xanthine was moderately effective. The latter 2 compounds also have replaceable hydrogens at the 7-position. Theobromine, with a methyl group at the 7-position, afforded no protection. However, hypoxanthine also was inactive. Methionine acts synergistically with primary antioxidants, such as $\alpha$-tocopherol, greatly increasing the antioxidant effectiveness of the latter (50, 299). Hove (157) noted a pro-oxidant effect of carbon tetrachloride in a nonbiological system.

Antioxidant protection against the hepatotoxic effects of carbon tetrachloride was rediscovered by Gallagher (114, 115). This appears to have come about in the following way. Childs and Siegler (48) reported in 1945 that thioacetamide prevented certain types of decay in oranges. Four years later Ambrose *et al.* (3) reported hepatic necrosis and death of rats after oral administration of thioacetamide. Thioacetamide hepatotoxicity was investigated more thoroughly by Gupta (130) and by Gallagher *et al.* (116). According to the latter workers, it was Ramchandra Rao who first suggested in 1933 that the centrilobular necrosis of carbon tetrachloride poisoning somewhat resembled the liver damage seen in prolonged shock. Since the latter was believed to be due to histamine, the release of histamine was implicated in toxic liver injury. The possibility that histamine release may have been a factor in thioacetamide poisoning led to experiments with antihistaminics. Pyrilamine, promethazine, and diphenhydramine all gave some protection against thioacetamide poisoning (116). These workers suggested that thioacetamide produced a primary loss of selective permeability of hepatic cell membranes. This was supposed to lead secondarily to entrance of calcium ions into the cell, which in turn was believed to precipitate a variety of biochemical disturbances leading to necrosis, autolysis, *etc.* The antihistaminics were thought to protect against membrane degeneration. In 1960, McLean (210) reported that promethazine or EDTA added *in vitro* prevented the early onset of a decline in oxygen uptake of slices taken from prenecrotic livers of rats fed Himsworth's vitamin E-deficient, necrogenic diet. Promethazine added to the diet delayed the onset of liver necrosis. McLean (211) shortly after described a test system for potassium reaccumulation by rat liver slices. After only 3 days on the necrogenic diet, the capacity of rat liver slices to regain lost potassium is markedly depressed, and promethazine *in vitro* prevents this depression. McLean suggested that a "chain of reactions" was set into motion by trauma to the liver, *e.g.*, by thioacetamide, or by manipulation, cutting, *etc.*, of the prenecrotic livers of rats on vitamin E-deficient diets. Promethazine in some way appeared to block the chain of reactions leading to death of the cells. In 1961, Rees *et al.* (267) reported that promethazine given to rats together with or 6 hours after carbon tetrachloride greatly diminished necrosis, assessed histologically at 24 hours. The work of McLean (210, 211), which showed that promethazine under certain circumstances could substitute for vitamin E, suggested that the antioxidant property of this phenothiazine derivative might be the key to its protective effects against carbon tetrachloride. This prompted experi-

mental work on the possible effectiveness of better known antioxidants against carbon tetrachloride toxicity. Gallagher (114, 115) found that $\alpha$-tocopherol given 40 hours before an otherwise lethal dose of carbon tetrachloride prevented death. Diphenyl-*p*-phenylenediamine (DPPD) given 48, 24, and 0 hours before carbon tetrachloride, also afforded complete protection. Selenium was moderately effective. Protection by selenium against carbon tetrachloride liver damage in rats has been reported by Fodor and Kemeny (101). On the basis of certain earlier experiences on protective effects achieved by intraperitoneal injection of nicotinic acid or tryptophane 2 days before carbon tetrachloride administration to sheep or rats (113, 117), Gallagher (114, 115) attempted to rationalize the protective effects of antioxidants in terms of maintenance of hepatocellular levels of oxidized pyridine nucleotides. Indeed, he found that administration of antioxidants before carbon tetrachloride preserved much higher levels of oxidized pyridine nucleotides than in rats given the same dose of carbon tetrachloride alone. An important question which has not yet been answered is whether the maintenance of oxidized levels of pyridine nucleotides is a specific biochemical effect related to the metabolic function of antioxidants, or whether a generalized protective effect by antioxidants against cell membrane destruction is involved.

DiLuzio (75) reported that intraperitoneal $\alpha$-tocopherol acetate or DPPD markedly reduced the hepatic triglyceride accumulation due to ethanol. It may be mentioned that Lieber and DeCarli (191) recently reported that addition of large amounts of antioxidants to nutritionally adequate diets containing 36% of calories as ethanol had no effect on ameliorating the long-term increase in liver triglycerides induced by ethanol. On this and other grounds, Lieber and DeCarli considered the acute and chronic effects of ethanol ingestion to involve different pathological mechanisms.

In 1963, Judah *et al.* (166), aware of the earlier work of Calder (40), which appeared to indicate a nonspecificity for protective agents given intraperitoneally, had cautioned against the interpretation that protection achieved by intraperitoneal administration of antioxidants implied an antioxidant mechanism of action for the protective agent. However, DiLuzio (76) reported that the commercial antioxidant G-50[R], a mixture of butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate in oil, given orally simultaneously with ethanol, reduced the hepatic triglyceride accumulation, an effect also reported for oral DPPD (78). Since antioxidants afforded some protection against hepatic lipid infiltration, induced by either ethanol or carbon tetrachloride, DiLuzio and coworkers (76–78) suggested that a common link in the acute effects induced by feeding these substances to rats may be formation of lipid peroxides accompanied by breakdown of function in the mitochondria, and in other membranous components of the liver cell (see 53, 69, 254, 304).

Peroxidative decomposition of membrane structural lipids engenders serious consequences (see 2, 325, 326 for reviews). Depending on the type of cell, and the particular membrane involved, lipoperoxidation results in hemolysis (37, 330) mitochondrial swelling and disintegration (152, 160–162, 327), loss of enzyme function in the endoplasmic reticulum (120), and lysosomal disinte-

gration (63). It is not known whether these changes of membrane properties are due mainly to alterations in orientation of membrane lipids consequent to lipoperoxidation, or to an attack by lipoperoxides on membrane proteins (64). According to DiLuzio, DPPD and $\alpha$-tocopherol, acting as intracellular antioxidants, would prevent the formation of lipid peroxides and would thus protect normal structure and enzyme activity of cellular membranes.

In essence, the lipoperoxidation hypothesis suggests that carbon tetrachloride poisoning initiates an intrahepatic process of destructive lipoperoxidation akin to the development of rancidity in stored fatty foods. In the food industry, rancidity is detected and quantified by a number of methods, one of the most sensitive being the thiobarbituric acid reaction for malonaldehyde. Dahle *et al.* (56), Day (60), and Lillard and Day (192) should be consulted regarding possible chemical mechanisms involved in the origin of malonaldehyde in autoxidizing lipids. Efforts to detect malonaldehyde in rat liver after carbon tetrachloride poisoning have not been successful (246, 252, 253). However, malonaldehyde can be metabolized. Holtkamp and Hill (154) reported an increased rate of oxygen consumption when malonaldehyde was added to whole rat liver homogenates, and Placer *et al.* (241) reported the rapid metabolism of malonaldehyde *in vivo* after its intraperitoneal administration to rats. Recknagel and Ghoshal (252, 253) showed by substrate disappearance studies that malonaldehyde is metabolized over mitochondrial pathways in rat liver. The fact that malonaldehyde is readily metabolized meant that failure to detect it *in vivo* could not be taken as evidence that lipoperoxidation had not occurred. This opened the door to the possibility that direct evidence for carbon tetrachloride-induced lipoperoxidation could be detected in some other way. Solution of the problem came in the form of an expansion of the lipoperoxidation hypothesis of DiLuzio and colleagues. This took into consideration recent work on the metabolism of carbon tetrachloride and a possible relation of the latter to peroxidative decomposition of structural lipids of hepatic cells.

The old idea that the toxicity of carbon tetrachloride depends in some way on its lipid solubility is not convincing (120, 254, 304). Many organic, nonpolar compounds with similar solubilities are not hepatotoxins, *e.g.*, nonchlorinated hydrocarbons and ethers. Even some haloalkanes with solubilities like that of carbon tetrachloride have little hepatotoxicity. Only about 150 cases of hepatic involvement have been reported among 50 million administrations of halothane for anesthesia (136). In low doses, carbon tetrachloride has a remarkably selective action on the liver of laboratory animals (see 263). The intestine, where it is absorbed (271), can be exposed to relatively high concentrations with impunity. Carbon tetrachloride is carried throughout the body by the circulation and is excreted largely *via* the lungs (236). Many cells in other organs are as richly endowed with lipoidal membranes as are the parenchymal cells of the liver, yet, with the exception of the kidney, they escape serious effects. The liver of the newborn rat is almost completely resistant to carbon tetrachloride (59) even if exposed to the same concentrations as the adult liver. The doses that affect the adult liver are low indeed. One $\mu$l of carbon tetrachloride per

100 g of body weight, fed to rats, depresses hepatic glucose-6-phosphatase activity and more than doubles liver triglycerides (254, 268). Such minute quantities could hardly damage the liver merely by dissolving in the lipoidal components of its cell membranes. These considerations suggest that the selective toxicity of carbon tetrachloride for the liver must depend in some way on its metabolism by the liver. That carbon tetrachloride is actually metabolized in the body has been known since the work of McCollister *et al.* (208) in 1951. Ten years later, Butler (38) showed that carbon tetrachloride is reduced to chloroform both *in vivo* and *in vitro*. He suggested that the carbon-chlorine bond in carbon tetrachloride and chloroform was subjected to homolytic cleavage, yielding the corresponding free radicals, which could then alkylate sulfhydryl groups of enzymes. In an important theoretical paper Wirtschafter and Cronyn (349) developed a general theory for solvent toxicity. According to their idea, the toxicity of solvents can best be understood by their reaction with free radicals that are normal intermediates in many important biological processes. Thus, a potentially toxic solvent A:B might be attacked by a pre-existing free radical, X·, to yield a new free radical B· and X:A. The new free radical could then react with sulfhydryl groups, as suggested by Butler. Wirtschafter and Cronyn (349) suggested that transfer constants be used as a guide to the understanding of solvent toxicity. Transfer constants are empirically derived in the field of polymer chemistry to indicate the relative rate of reaction of polymer chains with solvent molecules compared to their rate of reaction with new monomer units (340). For a series of solvents, the member with the highest transfer constant is most likely to be attacked by the free radical end of the growing polymer chain. Thus the transfer constant is a measure of the tendency of a solvent to undergo a homolytic cleavage under the conditions employed. The transfer constants for carbon tetrachloride, benzene, and cyclohexane (340, p. 152) indicate that carbon tetrachloride is much more prone to undergo homolytic cleavage than are benzene and cyclohexane, and the former is much more potent as a liver toxin (348, 349). On the other hand, some compounds, *e.g.*, ethyl thioglycollate, n-dodecyl mercaptan, n-butyl mercaptan, *etc.* (340, p. 153), have transfer constants many orders of magnitude greater than that for carbon tetrachloride, yet are not selectively toxic for the liver.

Recknagel and Ghoshal (254) pointed out that substances with high free-radical reactivity are also initiators of reactions catalyzed by free radicals. Furthermore, it has been known for many years (153) that the hydrogen atoms on the methylene bridges separating the double bonds in polyenoic fatty acids are particularly sensitive to free-radical attack. Following such an attack, the resulting organic free radical, either immediately or after a resonance shift of the free radical electron, rapidly forms the corresponding organic peroxide free radical, and eventually the corresponding organic peroxide. Molecular oxygen is paramagnetic and bears 2 unpaired electrons (118). Thus molecular oxygen is itself a di-free radical, and its reaction with organic free radicals to form the corresponding organic peroxide free radical occurs extremely rapidly (213). The organic peroxides are in general unstable compounds. They undergo a number

of very complex and still poorly understood reactions, leading to cleavage of the carbon chain, with formation of highly organoleptic aldehydes and ketones, malonaldehyde, and other products. Cleavage of the organic peroxides tends to be homolytic, yielding 2 new free radicals, which in turn can attack neighboring methylene bridges. The process, once initiated, is autocatalytic. This free radical-initiated, autocatalytic, peroxidative breakdown of unsaturated long-chain fatty acids is at the heart of the rancidity problem, of primary importance in the problem of food storage (153). Antioxidants protect stored foods against spoilage of rancidity by interrupting the self-propagating chains of peroxidative lipid breakdown. This theory of rancidity is the cornerstone of the version of the lipoperoxidation hypothesis advanced by Recknagel and coworkers (119, 120, 254). Rubinstein and Kanics (285) had shown that carbon tetrachloride was preferentially metabolized by the microsome fraction of rat liver, in the presence of the supernatant fraction. Recknagel and Ghoshal (254) pointed out that (a) the earlier work on the histopathology and biochemical pathology of carbon tetrachloride toxicity had clearly indicated that the endoplasmic reticulum of the hepatic parenchymal cell was the primary subcellular organelle involved in the early stages of liver damage, (b) the membranous components of the endoplasmic reticulum are thin, delicate, lipoprotein sheets, (c) the lipoidal centers of these sheets can be imagined to be composed of ordered arrangements of the fatty acid side chains of complex lipids, mostly phospholipids, (d) it can easily be imagined that functional properties of the endoplasmic reticulum could depend on maintenance of ordered lipoprotein structures, (e) a large fraction, of the order of 40%, of total liver phospholipid fatty acids are polyenoic, with a high content of arachidonic acid (16, 155), and (f) it was precisely in or near the lipoidal components of the endoplasmic reticulum, where a high density of methylene-bridge interrupted unsaturations prevailed, that carbon tetrachloride appeared to be metabolized. On the basis of these considerations the following hypothesis was advanced (254): "We envisage that the central and primary event in carbon tetrachloride hepatotoxicity is the homolytic cleavage of the toxic agent to trichloromethyl free radical and monatomic chlorine, as first suggested by Butler. This cleavage takes place in or near the endoplasmic reticulum of the hepatic parenchymal cells. Normally, the methylene bridges of the unsaturated fatty acid side chains are no doubt under constant threat of free radical attack, but the propagation of free radical catalyzed chains of destructive lipoperoxidation is prevented by $\alpha$-tocopherol, by other antioxidants, and no doubt by other means as well. We envisage that the sudden appearance of relatively high concentrations of free radicals, from the homolytic cleavage of carbon tetrachloride in or near the lipoidal centers of sheets of endoplasmic reticulum, initiates so many chains of autocatalytic, peroxidative breakdown of the unsaturated fatty acids, that normal levels of antioxidant protection are overwhelmed." The hypothesis envisages that morphological alteration of the hepatic endoplasmic reticulum (9, 17, 225, 312), loss of drug-metabolizing activitv (112, 214, 224), loss of glucose-6-phosphatase activity (258), loss of protein synthesis (300, 312), and loss of the capacity of the liver to form and excrete low-density

β-lipoproteins (197), all of which are early manifestations of carbon tetrachloride poisoning, are at least in part derivative phenomena, the resultants of a primary attack by the split products of carbon tetrachloride metabolism on the lipoidal elements of the endoplasmic reticulum.

One contribution of the hypothesis has been its usefulness in leading directly to experimental confirmation that lipoperoxidation is a factor in carbon tetrachloride hepatotoxicity. Free-radical attack on the methylene bridges of polyunsaturated fatty acids is the first step in their peroxidative breakdown. Following the initial attack, shift of the organic free radical electron results in the appearance of intense diene conjugation absorption (20). Recknagel and Ghoshal (254) were able to detect typical diene conjugation absorption in rat liver microsomal lipids as early as 90 minutes after carbon tetrachloride poisoning.

Since lipoperoxidation involves a destructive attack on polyenoic fatty acids, one would expect to find a marked reduction in polyunsaturated fatty acids of microsomal material subjected to extensive peroxidation *in vitro*. May *et al.* (206) reported marked decreases in arachidonic and docosahexaenoic acids in microsomal lipids peroxidized in the TPNH-dependent system of Hochstein and co-workers (149–151) and Orrenius *et al.* (235). Recknagel and Ghoshal (254) peroxidized a rat liver microsome system *in vitro* until the capacity of the system to produce malonaldehyde was exhausted. Subsequent gas-liquid chromatographic analysis revealed that the arachidonic acid of the peroxidized microsomal lipids was reduced to ⅕ of control levels, and that an unidentified fatty acid, probably docosahexaenoic acid, was reduced to 1/7 of control levels. Within 90 minutes after carbon tetrachloride administration *in vivo*, the arachidonic acid of rat liver microsomal lipids is reduced by 20% (254), and by 24 hours the percentage of arachidonic acid in total liver phospholipids is decreased by 33% (155). It is evident that the extension of such analytical studies to the fate of the structural lipids of the liver cell after poisoning by other hepatotoxins should be most interesting.

Another method used in the food industry for measuring rancidity is the direct estimation of lipoperoxides. Iodimetric (57, 344) and chromatographic procedures (215) are available. Milas (215) and Philpot (238) have emphasized the lability of organic peroxides as well as the readiness with which they appear artifactually in lipids of animal origin during extraction procedures. Furthermore, metal ion catalysis of organic peroxide decomposition is well known (18, p. 159). These considerations suggest that iodimetric titration of lipoperoxides might not be a reliable means for demonstrating that lipoperoxidation has occurred *in vivo*, and indeed Recknagel and Ghoshal (255) detected diene conjugation absorption in microsomal lipids 2 hours after carbon tetrachloride poisoning, but could not detect lipoperoxides. However, DiLuzio and Kalish (79) reported an increase in lipid peroxide levels in total liver lipids of the rat after acute ethanol intoxication. Comporti *et al.* (53) reported that up to 2 hours after administration of carbon tetrachloride to rats, the rate of malonaldehyde production by whole liver homogenates was increased; from 12 to 36 hours it was decreased, and it was increased over the control rate at 48 hours.

According to the lipoperoxidation hypothesis, carbon tetrachloride should act as a pro-oxidant for the peroxidative decomposition of structual lipids in liver cells. Comporti and co-workers (52, 53) and Dianzani *et al.* (69) reported a pro-oxidant effect for carbon tetrachloride on whole rat liver homogenates. Ghoshal and Recknagel (119, 120, 254) reported that in the microsome-supernatant fraction of rat liver as little as 0.3 $\mu$l of carbon tetrachloride accelerated production of malonaldehyde. Carbon tetrachloride had no pro-oxidant effect on lipoperoxidation either in the microsome fraction of rat liver in absence of the supernatant fraction, or in microsome-supernant fractions of rat brain or rat kidney (254). Heptane and diethyl ether were inert in the liver microsome-supernatant fraction, in which carbon tetrachloride exerted a pro-oxidant effect (254). These data are consistent with the hypothesis that the metabolism of carbon tetrachloride sets in motion a peroxidative decomposition of liver microsomal lipids. However, it should be stressed that no evidence has yet been reported that the pro-oxidant effect of carbon tetrachloride *in vitro* is in fact elicited only under conditions of cleavage of the carbon-to-chlorine bond.

The lipoperoxidation hypothesis has led to new evidence that carbon tetrachloride hepatotoxicity depends on carbon tetrachloride metabolism. Smuckler (306) and Smuckler and Benditt (310) observed that polysomes and ribosomes obtained from livers of intact rats by use of media containing carbon tetrachloride did not show alterations in ultrastructure and depression of amino acid incorporation characteristic of the same cell parts derived from rat livers damaged by carbon tetrachloride *in vivo*. These workers concluded that direct action by solvation of structural lipids could not be the explanation for the toxic action of carbon tetrachloride (see also 268, 304).

It has been known for many years that glucose-6-phosphatase activity is membrane-bound and very unstable. Ghoshal and Recknagel (120) showed that on incubation of rat liver microsomes (or the microsome-supernatant fraction) at neutral pH and in the presence of ascorbic acid, glucose-6-phosphatase activity was rapidly destroyed and at the same time microsomal lipids were peroxidized. However, if lipoperoxidation was prevented by addition of EDTA, $\alpha,\alpha'$-dipyridyl, or $\alpha$-tocopherol, there was little or no loss of enzyme activity. With glucose-6-phosphatase activity suitably protected by addition of either metal chelating agents or $\alpha$-tocopherol, it was possible to study the effects of added carbon tetrachloride under circumstances in which lipoperoxidation was prevented. An amount of carbon tetrachloride 20 times greater by weight than the entire quantity of microsomal lipid present in the *in vitro* system was required to destroy glucose-6-phosphatase activity in the absence of lipoperoxidation. Ghoshal and Recknagel (120) estimated that this ratio of carbon tetrachloride to microsomal lipid was at least 200 times greater than the absolute upper maximum that could conceivably be reached *in vivo*, even after administration of lethal doses. The fact that glucose-6-phosphatase activity of the liver of the rat poisoned with carbon tetrachloride is markedly depressed within the first 2 to 4 hours therefore must mean that carbon tetrachloride toxicity depends on its chemical reactivity and not on solvent action. It is interesting to note that Kondos and McClymont (177)

recently came to the conclusion that the anthelmintic action of carbon tetrachloride against *Fasciola hepatica* probably depends on the metabolism of carbon tetrachloride in the liver of the infected host.

The lipoperoxidation hypothesis places much of the earlier work into a new frame of reference. If the key to the toxic action of carbon tetrachloride consists in a breakdown of liver cell membrane structure and function brought about by a free-radical attack on membrane structural lipid, any compounds capable of reacting readily with free radicals, and capable of penetrating to the locus where the initial cleavage of the carbon-to-chlorine bond takes place, might be expected to afford some protection, since such compounds would short-circuit the expected attack on polyunsaturated long-chain fatty acids. Many of the agents for which protective effects have been claimed could act as free-radical scavengers. The lipid antioxidants belong to this class. Protection by molecular oxygen against hepatic involvement following chloroform anesthesia may involve a preferential reaction of free radicals with oxygen, rather than with constituents of the living cell. Phenothiazines (promazine, chlorpromazine, promethazine) contain a ring sulfur atom which can complex with free radicals. Sulfhydryl compounds (thioglycollate, methionine, mercaptoethylamine) function as free-radical scavengers. The recent monograph of Bacq (12) should be consulted for a discussion of the chemical mechanisms involved. Protective agents such as pyrilamine or diphenhydramine possess a benzyl carbon atom. Hydrogen on such a carbon atom may be available for reactions with free radicals. However, it would appear that not all the protective agents function as free-radical scavengers. For example, protection by cetyl trimethyl ammonium bromide (14) is not readily understood in these terms. The subject of protection against free radicals in biological systems is extraordinarily complex (12). This reviewer is not aware of any reasons for expecting *a priori* that mechanisms of protection against carbon tetrachloride hepatotoxicity should be either easier or more difficult to understand than mechanisms of protection against ionizing radiations. The point of interest is that the revelation of free-radical mechanisms in carbon tetrachloride poisoning means that the study of hepatic toxicology and the study of the effects of ionizing radiations appear to have come close together. Hopefully, the union will bear fruit.

It should be recognized, as Recknagel and Ghoshal (254) pointed out, that the lipoperoxidation hypothesis in its present form is much more a program of action than a theory of carbon tetrachloride hepatotoxicity. No direct evidence for a homolytic cleavage of carbon tetrachloride in the liver is available. The idea that free radicals arise is based on indirect evidence in the form of the appearance of diene conjugation absorption, loss of arachidonic acid, and an *in vitro* pro-oxidant effect of carbon tetrachloride. In this connection, it may be noted that Reynolds (268) reported a preferential incorporation of isotopic carbon and chlorine from labelled carbon tetrachloride into microsomal lipids. This result would be expected if carbon tetrachloride was cleaved homolytically in or near the lipoidal components of the endoplasmic reticulum and subsequently attacked the methylene bridges of unsaturated fatty acids of the structural phospholipids, as the lipoperoxidation hypothesis suggests. However, the extent of microsomal lipid

peroxidation, relative to total extractable microsomal lipid, appears to be small (255). This raises the interesting question as to whether destructive lipoperoxidation is perhaps only a minor manifestation of carbon tetrachloride hepatotoxicity, or whether destructive lipoperoxidation of a small number of strategically located structural lipids, especially rich in polyenoic fatty acids containing 3, 4, or more double bonds, may be of decisive importance for biochemical and physiological function resident in the endoplasmic reticulum, and other parts of the cell. Recknagel and Ghoshal (254) also stressed that the lipoperoxidation hypothesis does not exclude the suggestion of Butler (38) and of Wirtschafter and Cronyn (349) that free radicals arising from carbon tetrachloride cleavage may enter directly into a variety of reactions with labile groups of enzymes. Formation of abnormal cross-linkages within cell proteins following free-radical reactions may also occur. Thus, though obviously highly attractive and useful, the lipoperoxidation hypothesis in its present form is far from being a complete theory of haloalkane toxicity.

## IX. SUMMARY

During the past 20 years, study of the fatty liver induced by carbon tetrachloride has passed through 2 periods of revolutionary change, and at the present time a third revolutionary change is taking place. The dominant notion guiding most of the thinking before 1948 was that toxic and nutritional fatty liver disease could be understood in terms of failure in transport of fatty acids as phospholipids. As quantitative analyses of whole body phospholipid and neutral lipid metabolism became available through application of radioisotope technology, it became possible by 1953 to conclude that fatty acids were not transported in the plasma as phospholipids.

For the next 6 years work in this field was dominated by the mitochondrial hypothesis. Carbon tetrachloride was thought to damage the liver cell mitochondria. It was suggested that lipid accumulation was due to a failure of normal pathways of lipid oxidation, and that death of the liver cells resulted from interruption in energy-transducing mechanisms. By 1959 this hypothesis also proved untenable since accumulation of triglycerides and degeneration of the hepatocellular endoplasmic reticulum preceeded mitochondrial degeneration by many hours. The major contribution of the work of this period was the introduction into the study of experimental hepatic toxicology of methods of biochemical cytology.

A second revolutionary change took place in 1960. It became evident that carbon tetrachloride poisoning leads rapidly to cessation of movement of large quantities of triglycerides from the liver to the plasma. The blockade of hepatic triglyceride secretion by carbon tetrachloride accounts for the characteristic fatty liver. Earlier studies of rates of replacement of the different moieties of the plasma lipoproteins had demonstrated that low-density lipoprotein triglycerides were replaced much faster than the protein moiety. This work, as well as more recent studies combining use of the isolated, perfused liver with methods for separation of plasma protein and lipoprotein fractions, has made possible a most important

new insight into the nature of hepatic triglyceride secretion. The latter can best be understood as a dual mechanism. One part of the mechanism involves hepatic biosynthesis of the various moieties of plasma low-density lipoproteins, coupling of these to form definitive lipoprotein molecules, and their extrusion to the plasma compartment. An auxiliary mechanism provides for re-entry into the system of triglyceride-free lipoprotein apoprotein. Movement of triglycerides from liver to plasma depends largely on the continuous functioning of the second, or auxiliary arm of the cycle. The knowledge that hepatic triglyceride secretion involves a dual mechanism has provided a powerful new guide for the analysis of mechanisms underlying fatty liver disease. In the case of carbon tetrachloride poisoning, the rapid onset of liver triglyceride accumulation most probably results mainly from cessation in function of the auxiliary coupling phase of hepatic triglyceride secretion, and less significantly from a breakdown in hepatic protein synthesis.

During this period, *ca.* 1960 to 1965, highly provocative experimental findings led to an attempt to rationalize the hepatocellular necrosis and the triglyceride accumulation of carbon tetrachloride poisoning in terms of massive discharge of the sympathetic nervous system. The main evidence regarding the pathogenesis of hepatocellular necrosis was based on the observation that rats whose spinal cord had been divided were remarkably immune to the toxic agent. A review of the evidence does not support the contention that hepatocellular necrosis is a consequence of catecholamine discharge.

The catecholamine hypothesis suggested that hepatic lipid accumulation is due to an oversupply of fatty acids mobilized from peripheral adipose tissue depots. Activation of the hypophyseal-adrenocortical axis has also been invoked in support of the peripheral oversupply hypothesis. A central requirement of this hypothesis must be that if oversupply of fatty acids is to be invoked as a significant factor in the pathogenesis of fatty liver, then the oversupply must be of sufficient magnitude and duration to account for the time course of the hepatic lipid accumulation. From this point of view the evidence offered in support of the peripheral oversupply hypothesis is not convincing. In particular, for carbon tetrachloride poisoning, at a time when liver triglycerides are increasing rapidly, there is no increase in flux of fatty acids through the plasma compartment.

The most recent work in this field has inaugurated a third revolution in the study of carbon tetrachloride hepatotoxicity. Its essential feature is the recognition that carbon tetrachloride toxicity depends on cleavage of the carbon-to-chlorine bond. At the same time, the long-held view that the toxic action of carbon tetrachloride resided in its effectiveness as a lipid solvent has finally been discarded. An important link has been established between the metabolism of carbon tetrachloride and the peroxidative decomposition of cytoplasmic membrane structural lipids. Sufficient data are not yet available to decide whether the latter effect is a major or minor consequence of the metabolism of carbon tetrachloride. The important point is that study of this problem has reached the organic chemical level of organization. This augurs well for the immediate future, which should witness further interesting new developments in the study of haloalkane toxicity.

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## REFERENCES

1. Aiyar, A. S. and Sreenivasan, A.: Intracellular distribution and biosynthesis of ubiquinone in rat liver in carbon tetrachloride liver injury. Biochem. J. **82**: 179–182, 1962.
2. Alfin-Slater, R. B. and Morris, R. S.: Vitamin E and lipid metabolism. Adv. Lipid Res. **1**: 183–210, 1963.
3. Ambrose, A. M., DeEds, F. and Rather, L. J.: Toxicity of thioacetamide in rats. J. Ind. Hyg. Toxicol. **31**: 158–161, 1949.
4. Amenta, J. S. and Johnston, E. H.: Hydrazine-induced alterations in rat liver. A correlation of the chemical and histologic changes in acute hydrazine intoxication. Lab. Invest. **11**: 956–962, 1962.
5. Amenta, J. S. and Johnston, E. H.: The effects of hydrazine upon the metabolism of amino acids in the rat liver. Lab. Invest. **12**: 921–928, 1963.
6. Armstrong, D. T., Steele, R., Altszuler, N., Dunn, A., Bishop, J. S. and DeBodo, R. C.: Regulation of plasma free fatty acid turnover. Amer. J. Physiol. **201**: 9–15, 1961.
7. Artizzu, M., Baccino, F. M. and Dianzani, M. U.: The action of carbon tetrachloride on mitochondria *in vitro*. Biochim. Biophys. Acta **78**: 1–11, 1963.
8. Artizzu, M. and Dianzani, M. U.: The changes in the mitochondria and lysosomes in the fatty livers of rats fed with carbon tetrachloride and their independence of ribonuclease activation. Biochim. Biophys. Acta **63**: 453–464, 1962.
9. Ashworth, C. T., Luibel, F. J., Sanders, E. and Arnold, N.: Hepatic cell degeneration: Correlation of fine structure with chemical and histochemical changes in hepatic cell injury produced by carbon tetrachloride in rats. Arch. Pathol. **75**: 212–225, 1963.
10. Avigan, J., Eder, H. A. and Steinberg, D.: Metabolism of the protein moiety of rabbit serum lipoproteins. Proc. Soc. Exp. Biol. Med. **95**: 429–433, 1957.
11. Baccino, F. M., Satta, G. and Mameli, L.: Distribution of $CCl_4$ among liver cell fractions. Biochim. Biophys. Acta **90**: 606–608, 1964.
12. Bacq, Z. M.: Chemical Protection against Ionising Radiation, pp. 216–226, Charles C Thomas, Springfield, Ill., 1965.
13. Baker, N. and Schotz, M. C.: Use of multicompartment models to measure rates of triglyceride metabolism in rats. J. Lipid Res. **5**: 188–197, 1964.
14. Bangham, A. D., Rees, K. R. and Shotlander, V. L.: Penetration of lipid films by compounds preventing liver necrosis in rats. Nature (London) **193**: 754–756, 1962.
15. Barker, E. A., Smuckler, E. A. and Benditt, E. P.: Effects of thioacetamide and yellow phosphorus poisoning on protein synthesis *in vivo*. Lab. Invest. **12**: 955–960, 1963.
16. Bartley, W.: Lipids of intracellular organelles. In: Metabolism and Physiological Significance of Lipids, ed. by R. M. C. Dawson and D. N. Rhodes, 369–381, John Wiley and Sons, New York, 1964.
17. Bassi, M.: Electron microscopy of rat liver after carbon tetrachloride poisoning. Exp. Cell Res. **20**: 313–323, 1960.
18. Bateman, L.: Olefin oxidation. Quart. Rev. **8**: 147–167, 1954.
19. Bierman, E. L., Schwartz, I. L. and Dole, V. P.: Action of insulin on release of fatty acids from tissue stores. Amer. J. Physiol. **191**: 359–362, 1957.
20. Bolland, J. L. and Koch, H. P.: The course of autoxidation reactions in polyisoprenes and allied compounds. IX. The primary thermal oxidation product of ethyl linoleate. J. Chem. Soc. p. 445, 1945.
21. Borgström, B. and Olivecrona, T.: The metabolism of palmitic acid-1-$C^{14}$ in functionally hepatectomized rats. J. Lipid Res. **2**: 263–267, 1961.
22. Bragdon, J. H. and Gordon, R. S.: Tissue distribution of $C^{14}$ after the intravenous injection of labelled chylomicrons and unesterified fatty acids in the rat. J. Clin. Invest. **37**: 574–578, 1958.
23. Brauer, R. W.: Liver circulation and function. Physiol. Rev. **43**: 115–213, 1963.
24. Brauer, R. W., Balam, R. W., Bond, H. E., Carroll, H. W., Grisham, J. W. and Pessotti, R. L.: Reversible and irreversible changes in liver at temperatures approaching critical upper level. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **22**: 724–728, 1963.
25. Brauer, R. W., Leong, G. F. and Holloway, R. J.: Liver injury in isolated perfused rat liver preparation exposed to chloroform. Amer. J. Physiol. **200**: 548–550, 1961.
26. Bridges, W. F., Abele, D. and Hunter, F. E., Jr.: Action of carbon tetrachloride on liver mitochondria. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **16**: 158, 1957.
27. Brodie, B. B., Butler, W. M., Jr., Horning, M. G., Maickel, R. P. and Maling, H. M.: Alcohol-induced triglyceride deposition in liver through derangement of fat transport. Amer. J. Clin. Nutr. **9**: 432–435, 1961.
28. Brodie, B. B. and Maickel, R. P.: Role of the sympathetic nervous system in drug-induced fatty liver. Ann. N. Y. Acad. Sci. **104**: 1049–1058, 1963.

29. Brodie, B. B., Maling, H. M., Horning, M. G. and Maickel, R. P.: Triglyceride deposition in rat liver through derangement of fat transport by alcohol and other chemical agents. In: Drugs Affecting Lipid Metabolism, ed. by S. Garatini and R. Paoletti, pp. 104–111, Elsevier, New York, 1961.
30. Brody, T. M.: In: Discussion on Judah, J. D., and Rees, K. R., Mechanism of action of carbon tetrachloride. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 18: 1017–1019, 1959.
31. Brody, T. M: Some aspects of experimental carbon tetrachloride-induced hepatotoxicity. In: Hepatotoxicity of Therapeutic Agents, ed. by H. E. Ticktin. Ann. N. Y. Acad. Sci. 104: 1065–1073, 1963.
32. Brody, T. M. and Calvert, D. N.: Release of catechol amines from the adrenal medulla by $CCl_4$. Amer. J. Physiol. 198: 682–685, 1960.
33. Brody, T. M., Calvert, D. N. and Schneider, A. F.: Alteration of carbon tetrachloride-induced pathologic changes in the rat by spinal transection, adrenalectomy and adrenergic blocking agents. J. Pharmacol. Exp. Therap. 131: 341–345, 1961.
34. Brown, R. K., Boyle, E. and Anfinson, C. B.: Enzymatic transformation of lipoproteins. J. Biol. Chem. 204: 423–434, 1953.
35. Brunschwig, A., Johnson, C. and Nichols, S.: Carbon tetrachloride injury of the liver. The protective action of certain compounds. Proc. Soc. Exp. Biol. Med. 60: 388–391, 1945.
36. Brunschwig, A., Nichols, S., Bigelow, R. and Miles, J.: Sulfhydryl protection of the liver. Arch. Pathol. 40: 81–83, 1945.
37. Bunyan, J., Green, J., Edwin, E. E. and Diplock, A. T.: Studies on vitamin E. 5. Lipid peroxidation in dialuric acid-induced hemolysis of vitamin E-deficient erythrocytes. Biochem. J. 76: 47–51, 1960.
38. Butler, T. C.: Reduction of carbon tetrachloride *in vivo* and reduction of carbon tetrachloride and chloroform *in vitro* by tissues and tissue constituents. J. Pharmacol. Exp. Therap. 134: 311–319, 1961.
38a. Byers, S. O., Cady, P., Friedman, M. and Felton, L.: Effect of triton on triolein-$I^{131}$ distribution in atherosclerotic rabbit tissues. Amer. J. Physiol. 197: 1053–1055, 1959.
39. Byers, S. O. and Friedman, M.: Site of origin of plasma triglyceride. Amer. J. Physiol. 198: 629–631, 1960.
40. Calder, R. M.: The protective action of xanthine and other insoluble substances on the liver. J. Pathol. Bacteriol. 54: 369–373, 1942.
41. Calvert, D. and Brody, T. M.: Biochemical alterations of liver function by the halogenated hydrocarbons. I. *In vitro* and *in vivo* changes and their modification by ethylenediamine tetraacetate. J. Pharmacol. Exp. Therap. 124: 273–281, 1958.
42. Calvert, D. N. and Brody, T. M.: Role of the sympathetic nervous system in $CCl_4$ hepatotoxicity. Amer. J. Physiol. 198: 669–676, 1960.
43. Calvert, D. N. and Brody, T. M.: Further studies on $CCl_4$ hepatotoxicity. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 20: 434, 1961.
44. Calvert, D. N. and Brody, T. M.: The effects of thyroid function upon carbon tetrachloride hepatotoxicity J. Pharmacol. Exp. Therap. 134: 304–310, 1961.
45. Campbell, J. A.: Increase of resistance to oxygen want in rats on a carrot diet. J. Physiol. 93: 31P, 1938.
46. Campbell, J. A.: Resistance to oxygen want. J. Physiol. 95: 1P, 1939.
47. Chaikoff, I. L., Zilversmit, D. B. and Entenman, C.: Turnover rate of plasma phospholipids in completely depancreatized dogs. Proc. Soc. Exp. Biol. Med. 68: 6–9, 1946.
48. Childs, J. F. L. and Siegler, E. A.: Compounds for control of orange decays. Science (N. Y.) 102: 68, 1945.
49. Christie, G. S. and Judah, J. D.: Mechanism of action of carbon tetrachloride on liver cells. Proc. Roy. Soc., Ser. B 142: 241–257, 1954.
50. Clausen, D. F., Lundberg, W. O. and Burr, G. O.: Some effects of amino acids and certain other substances on lard containing phenolic antioxidants. J. Amer. Oil Chem. Soc. 24: 403–404, 1947.
51. Comar, C. L.: Radioisotopes in Biology and Agriculture, p. 21, McGraw-Hill, New York, 1955.
52. Comporti, M. and Saccocci, C.: Effetto stimolante sulla perossidasione lipidica di omogenati di fegato di ratto da parte di piccole quantita di $CCl_4$ *in vitro*. Boll. Soc. Ital. Biol. Sperim. 41: 1066–1068, 1965.
53. Comporti, M., Saccocci, C. and Dianzani, M. U.: Effect of $CCl_4$ *in vitro* and *in vivo* on lipid peroxidation of rat liver homogenates and subcellular fractions. Enzymologia 29: 185–204, 1965.
54. Cornforth, J. W., D'Arcy Hart, P., Rees, R. J. W. and Stock, J. A.: Antituberculosis effect of certain surface-active polyoxyethylene ethers in mice. Nature (London) 168: 150–153, 1951.
55. Creasey, W. A., Hankin, L. and Handschumacher, R. E.: Fatty livers induced by orotic acid. I. Accumulation and metabolism of lipids. J. Biol. Chem. 236: 2064–2070, 1961.
56. Dahle, L. K., Hill, E. G. and Holman, R. T.: The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. Arch. Biochem. 98: 253–261, 1962.
57. Dahle, L. K. and Holman, R. T.: Modified iodometric method for semimicrodetermination of lipid peroxides. Anal. Chem. 33: 1960–1961, 1961.
58. Davis, N. C. and Whipple, G. H.: The influence of drugs and chemical agents on the liver necrosis of chloroform anesthesia. Arch. Intern. Med. 23: 636–654, 1919.
59. Dawkins, M. J. R.: Carbon tetrachloride poisoning in the liver of the new-born rat. J. Pathol. Bacteriol. 85: 189–196, 1963.
60. Day, E. A.: Autoxidation of milk lipids. J. Dairy Sci. 43: 1360–1365, 1960.
61. Decsi, L., Méhes, J. and Varga, F.: Über die Veränderungen des Leberzellstoff-wechsels bei $CCl_4$-Vergiftung. Arch. Exp. Pathol. (Berlin) 231: 235–245, 1957.
62. deReuck, A. V. S. and Cameron, M. P., eds. Ciba Foundation Symposium on Lysosomes. Little, Brown and Company, Boston, Mass., 1963.

63. Desai, I. D., Sawant, P. L. and Tappel, A. L.: Peroxidative and radiation damage to isolated lysosomes. Biochim. Biophys. Acta 86: 277–285, 1964.
64. Desai, I. D. and Tappel, A. L.: Damage to proteins by peroxidised lipids. J. Lipid Res. 4: 204–207, 1963.
65. Dianzani, M. U.: Uncoupling of oxidative phosphorylation in mitochondria from fatty livers. Biochim. Biophys. Acta 14: 514–532, 1954.
66. Dianzani, M. U.: Content and distribution of pyridine nucleotides in fatty livers. Biochim. Biophys. Acta, 17: 391–405, 1955.
67. Dianzani, M. U.: The content of adenosine polyphosphates in fatty livers. Biochem. J. 65: 116–124, 1957.
68. Dianzani, M. U.: Lysosome changes in liver injury. In: Ciba Foundation Symposium on Lysosomes, ed. by A. V. S. deReuck and M. P. Cameron, pp. 335–351, Little, Brown and Co., Boston, 1963.
69. Dianzani, M. U., Baccino, F. M. and Comporti, M.: The direct effect of carbon tetrachloride on subcellular particles. Lab. Invest. 15: 149–156, 1966.
70. Dianzani, M. U. and Bahr, G. F.: Electron microscope investigation of mitochondria isolated from normal and steatotic livers by differential centrifugation. Acta Pathol. Microbiol. Scand. 35: 25–38, 1954.
71. Dianzani, M. U. and Dianzani Mor, M. A.: Displacement of thiamine pyrophosphate from swollen mitochondria. Biochim. Biophys. Acta 24: 564–568, 1957.
72. Dianzani, M. U. and Marinari, U.: The octanoate oxidation by mitochondria from fatty livers. Biochim. Biophys. Acta 48: 552–561, 1961.
73. Dianzani, M. U. and Scuro, S.: The effects of some inhibitors of oxidative phosphorylation on the morphology and enzyme activities of mitochondria. Biochem. J. 62: 205–215, 1956.
74. Dianzani, M. U. and Viti, I.: The content and distribution of cytochrome c in the fatty liver of rats. Biochem. J. 59: 141–145, 1955.
75. DiLuzio, N. R.: Prevention of the acute ethanol-induced fatty liver by antioxidants. Physiologist, 6: 169, 1963.
76. DiLuzio, N. R.: Prevention of the acute ethanol-induced fatty liver by the simultaneous administration of antioxidants. Life Sci. 3: 113–118, 1964.
77. DiLuzio, N. R. and Costales, F.: Inhibition of the carbon tetrachloride induced fatty liver by anti-oxidants. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 23: 520, 1964.
78. DiLuzio, N. R. and Costales, F.: Inhibition of the ethanol and carbon tetrachloride induced fatty liver by antioxidants. Exp. Mol. Pathol. 4: 141–154, 1965.
79. DiLuzio, N. R. and Kalish, G. H.: Enhanced peroxidation of lipid in the pathogenesis of acute ethanol-induced liver injury, p. 4. Amer. Assoc. for the Study of Liver Diseases, 16th Annual Meeting, Chicago, Ill., Nov., 1965.
80. Dinman, B. D., Fox, C. F., Frajola, W. J. and Rabor, A.: Serum enzyme and $B_{12}$ changes in $CCl_4$ hepatotoxicity. Arch. Environ. Health 4: 168–182, 1962.
81. Dinman, B. D., Hamdi, E. A., Fox, C. F. and Frajola, W. J.: $CCl_4$ toxicity. Arch. Environ. Health 7: 630–646, 1963.
82. Dole, V. P.: A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest. 35: 150–154, 1956.
83. Dole, V. P.: Transport of non-esterified fatty acids in plasma. In: Chemistry of Lipids as Related to Atherosclerosis, ed. by I. M. Page, pp. 189–198, Charles C Thomas, Springfield, Ill., 1958.
84. Dole, V. P. and Hamlin, J. T., III: Particulate fat in lymph and blood. Physiol. Rev. 42: 674–701, 1962.
85. Dole, V. P. and Rieack, M. A.: On the turnover of long-chain fatty acids in plasma. J. Lipid Res. 2: 90–91, 1961.
86. Drill, V. A.: Hepatotoxic agents: Mechanism of action and dietary interrelationship. Pharmacol. Rev. 4: 1–42,1952.
87. Drill, V. A.: Pharmacology of hepatotoxic agents. In: Hepatotoxicity of Therapeutic Agents, ed. by H. E. Ticktin. Ann. N. Y. Acad. Sci. 104: 858–874, 1963.
88. Drill, V. A. and Loomis, T. A.: Methionine therapy in experimental liver injury produced by carbon tetrachloride. J. Pharmacol. Exp. Therap. 90: 138–149, 1947.
89. Eder, H. A., Roheim, P. S., Gidez, L. I. and Switzer, S.: Regulation of lipid transport in the liver. In: Lipid Transport, ed. by H. C. Meng, pp. 202–209, Charles C Thomas, Springfield, Ill., 1964.
90. Eder, H. A., Roheim, P. S. and Switzer, S.: An apoprotein of the lipoproteins. Trans. Ass. Amer. Phys. 77: 259–267, 1964.
91. Entenman, C., Chaikoff, I. L. and Zilversmit, D. B.: Removal of plasma phospholipids as a function of the liver. The effect of exclusion of the liver on the turnover rate of plasma phospholipids as measured with radioactive phosphorus. J. Biol. Chem. 166: 15–23, 1946.
92. Farber, E.: Ethionine carcinogenesis. Adv. Cancer Res. 7: 383–474, 1963.
93. Farber, E., Koch-Weser, D., Szanto, P. B. and Popper, H.: Correlation between cytoplasmic basophilia and nucleic acid content of the liver. A.M.A. Arch. Pathol. 51: 399–408, 1951.
94. Farber, E., Lombardi, B., and Castillo, A. E.: The prevention by adenosine triphosphate of the fatty liver induced by ethionine. Lab. Invest. 12: 873–883, 1963.
95. Farber, E., Shull, K. H., Villa-Trevino, S., Lombardi, B. and Thomas, M.: Biochemical pathology of acute hepatic adenosinetriphosphate deficiency. Nature (London) 203: 34–40, 1964.
96. Farquhar, J. W., Gross, R. C., Wagner, R. M. and Reaven, G. M.: Validation of an incompletely coupled two-compartment nonrecycling catenary model for turnover of liver and plasma triglyceride in man. J. Lipid Res. 6: 119–134, 1965.
97. Feigelson, E. B., Pfaff, W. W., Karmen, A. and Steinberg, D.: Role of plasma free fatty acids in development of fatty liver. J. Clin. Invest. 40: 2171–2179, 1961.
98. Feinberg, H., Rubin, L., Hill, R., Entenman, C. and Chaikoff, I. L.: Reduction of serum lipids and lipoproteins by ethionine feeding in the dog. Science (N. Y.) 120: 317–318, 1954.

99. FINE, M. B. AND WILLIAMS, R. H.: Effect of fasting, epinephrine, and glucose and insulin on hepatic uptake of nonesterified fatty acids. Amer. J. Physiol. 199: 403–411, 1960.
100. FISHLER, M. C., ENTENMAN, C., MONTGOMERY, M. L. AND CHAIKOFF, I. L.: The formation of phospholipid by the hepatectomized dog as measured with radioactive phosphorus. J. Biol. Chem. 150: 47–55, 1943.
101. FODOR, G. AND KEMENY, G. L.: On the hepato-protective effect of selenium in carbon tetrachloride poisoning in albino rats. Experientia 21: 666–667, 1965.
102. FORBES, J. C. AND NEALE, R. C.: A new preparation protecting against rat liver necrosis. Proc. Soc. Exp. Biol. Med. 34: 319–320, 1936.
103. FORBES, J. C. AND TALIAFERRO, I.: Increased resistance of carrot-fed rats to carbon tetrachloride. Proc. Soc. Exp. Biol. Med. 59: 27–29, 1945.
104. FRANK, A., HORNING, M. G. AND MALING, H. M.: Decreased hepatic release of triglycerides in $CCl_4$-treated rats. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 20: 277, 1961.
105. FREDRICKSON, D. S. AND GORDON, R. S.: The metabolism of albumin-bound $C^{14}$-labelled unesterified fatty acids in normal human subjects. J. Clin. Invest. 37: 1504–1515, 1958.
106. FREDRICKSON, D. S. AND GORDON, R. S., JR.: Transport of fatty acids. Physiol. Rev. 38: 585–630, 1958.
107. FREDRICKSON, D. S., MCCOLLESTER, D. L., HAVEL, R. J. AND ONO, K.: The early steps in transport and metabolism of exogenous triglyceride and cholesterol. In: Chemistry of Lipides as Related to Atherosclerosis, ed. by I. H. Page, pp. 205–218, Charles C Thomas, Springfield, Ill., 1958.
108. FRENCH, J. E. AND MORRIS, B.: The removal of $^{14}C$-labeled chylomicron fat from the circulation in rats. J. Physiol. 138: 326–339, 1957.
109. FRIEDBERG, S. J., KLEIN, R. F., TROUT, D. L., BOGDONOFF, M. D. AND ESTES, E. H., JR.: The incorporation of plasma free fatty acids into plasma triglycerides in man. J. Clin. Invest. 40: 1846–1855, 1961.
110. FRIEDMAN, M. AND BYERS, S. O.: The mechanism responsible for the hypercholesteremia induced by Triton WR-1339. J. Exp. Med. 97: 117–130, 1953.
111. FRIEDMAN, M. AND BYERS, S. O.: Mechanism underlying hypercholesteremia induced by Triton WR-1339. Amer. J. Physiol. 190: 439–445, 1957.
112. FUJIMOTO, J. M. AND PLAA, G. L.: Effect of ethionine and carbon tetrachloride on urethane and phenobarbital induced changes in hexobarbital action. J. Pharmacol. Exp. Therap. 131: 282–286, 1961.
113. GALLAGHER, C. H.: The effect of precursors of pyridine nucleotides on poisoning by carbon tetrachloride. Aust. J. Exp. Biol. Med. Sci. 38: 251–260, 1960.
114. GALLAGHER, C. H.: Protection by antioxidants against lethal doses of carbon tetrachloride. Nature (London) 192: 881–882, 1961.
115. GALLAGHER, C. H.: The effect of antioxidants on poisoning by carbon tetrachloride. Aust. J. Exp. Biol. Med. Sci. 40: 241–254, 1962.
116. GALLAGHER, C. H., GUPTA, D. N., JUDAH, J. D. AND REES, K. R.: Biochemical changes in liver in acute thioacetamide intoxication. J. Pathol. Bacteriol. 72: 193–201, 1956.
117. GALLAGHER, C. H. AND SIMMONDS, R. A.: Prophylaxis of poisoning by carbon tetrachloride. Nature (London) 184: 1407–1408, 1959.
118. GERSCHMAN, R.: The biological effects of increased oxygen tension. In: Man's Dependence on the Earthly Atmosphere, ed. by Karl E. Schaefer, p. 171, The Macmillan Co., New York, 1958.
119. GHOSHAL, A. K. AND RECKNAGEL, R. O.: Positive evidence of acceleration of lipoperoxidation in rat liver by carbon tetrachloride: *In vitro* experiments. Life Sci. 4: 1521–1530, 1965.
120. GHOSHAL, A. K. AND RECKNAGEL, R. O.: On the mechanism of carbon tetrachloride hepatotoxicity: Co-incidence of loss of glucose-6-phosphatase activity with peroxidation of microsomal lipid. Life Sci. 4: 2195–2209, 1965.
121. GITLIN, D. AND CORNWELL, D.: Plasma lipoprotein metabolism in normal individuals and in children with the nephrotic syndrome. J. Clin. Invest. 35: 706, 1956.
122. GITLIN, D., CORNWELL, D. G., NAKASATO, D., ONCLEY, J. L., HUGHES, W. L., JR. AND JANEWAY, C. A.: Studies on the metabolism of plasma proteins in the nephrotic syndrome. II. The lipoproteins. J. Clin. Invest. 37: 172–184, 1958.
123. GOLDMAN, D. S., CHAIKOFF, I. L., REINHARDT, W. O., ENTENMAN, C. AND DAUBEN, W. G.: Site of formation of plasma phospholipids studied with $C^{14}$-labeled palmitic acid. J. Biol. Chem. 184: 727–733, 1950.
124. GOLDSCHMIDT, S., RAVDIN, I. S. AND LUCKE, B.: The protective action of oxygen against the necrotizing effect of certain anesthetics on the liver. J. Pharmacol. Exp. Therap. 59: 1–14, 1937.
125. GØRANSSON, G.: The incorporation of palmitic acid and oleic acid into liver lipids by the rat. Biochem. J. 92: 41P, 1964.
126. GORDON, R. S., JR. AND CHERKES, A.: Unesterified fatty acid in human blood plasma. J. Clin. Invest. 35: 206–212, 1956.
127. GORDON, R. S. AND CHERKES, A.: Production of unesterified fatty acids from isolated rat adipose tissue incubated *in vitro*. Proc. Soc. Exp. Biol. Med. 97: 150–151, 1958.
128. GORDON, R. S., JR., CHERKES, A. AND GATES, H.: Unesterified fatty acids in human blood plasma. II. The transport function of unesterified fatty acid. J. Clin. Invest. 36: 810–815, 1957.
129. GROSSMAN, M. L., PALM, L., BECKER, G. H. AND MOELLER, H. C.: Effect of lipemia and heparin on free fatty acid content of rat plasma. Proc. Soc. Exp. Biol. Med. 87: 312–315, 1954.
130. GUPTA, D. N.: Acute changes in the liver after administration of thioacetamide. J. Pathol. Bacteriol. 72: 183–192, 1956.
131. GURD, F. R. N.: Some naturally occurring lipoprotein systems. In: Lipide Chemistry, ed. by D. J. Hanahan, pp. 260–325, John Wiley & Sons, Inc., New York, 1960.
132. HAFT, D. E., ROHEIM, P. S., WHITE, A. AND EDER, H. A.: Plasma lipoprotein metabolism in perfused rat livers. I. Protein synthesis and entry into the plasma. J. Clin. Invest. 41: 842–849, 1962.

133. HANDSCHUMACHER, R. E., CREASEY, W. A., JAFFE, J. J., PASTERNAK, C. A., AND HANKIN, L.: Biochemical and nutritional studies on the induction of fatty livers by dietary orotic acid. Proc. Natn. Acad. Sci. U. S. A. 46: 178–186, 1960.
134. HARDIN, B. L.: Carbon tetrachloride poisoning—a review. Ind. Med. Surg. 23: 93–105, 1954.
135. HARPER, P., NEAL, W. AND HLAVACEK, G.: Lipid synthesis and transport in the dog. Metabolism 2: 69–80, 1953.
136. HAUBRICH, W. S.: Liver injury following halothane anesthesia, p. 14, Amer. Assoc. for the Study of Liver Diseases, 16th Annual Meeting, Chicago, Ill., Nov., 1965.
137. HAVEL, R. J.: Conversion of plasma free fatty acids into triglycerides of plasma lipoprotein fractions in man. Metabolism 10: 1031–1034, 1961.
138. HAVEL, R. J., FELTS, J. M. AND VAN DUYNE, C. M.: Formation and fate of endogenous triglycerides in blood plasma of rabbits. J. Lipid Res. 3: 297–308, 1962.
139. HAVEL, R. J. AND FREDRICKSON, D. S.: The metabolism of chylomicra. I. The removal of palmitic acid-1-$C^{14}$ labeled chylomicra from dog plasma. J. Clin. Invest. 35: 1025–1032, 1956.
140. HAVEL, R. J. AND GOLDFIEN, A.: The role of the liver and of extrahepatic tissues in the transport and metabolism of fatty acids and triglycerides in the dog. J. Lipid Res. 2: 389–395, 1961.
141. HEIMBERG, M. AND FIZETTE, N. B.: Action of norepinephrine on transport of fatty acids and triglycerides by the isolated perfused rat liver. Biochem. Pharmacol. 12: 392–394, 1963.
142. HEIMBERG, M., FIZETTE, N. B. AND KLAUSNER, H.: Action of adrenal hormones on hepatic transport of triglycerides and fatty acids. J. Amer. Oil Chem. Soc. 41: 774–779, 1964.
143. HEIMBERG, M., WATKINS, M. L. AND TOOKER, R.: Carbon tetrachloride hepatotoxicity: Direct action of $CCl_4$ on the liver. J. Pharmacol. Exp. Therap. 145: 92–101, 1964.
144. HEIMBERG, M., WEINSTEIN, I., DISHMON, G. AND DUNKERLEY, A.: The action of carbon tetrachloride on the transport and metabolism of triglycerides and fatty acids by the isolated perfused rat liver and its relationship to the etiology of fatty liver. J. Biol. Chem. 237: 3623–3627, 1962.
145. HEIMBERG, M., WEINSTEIN, I., KLAUSNER, H. AND WATKINS, M. L.: Release and uptake of triglycerides by isolated perfused rat liver. Amer. J. Physiol. 202: 353–358, 1962.
146. HILLYARD, L. A., CORNELIUS, C. E., AND CHAIKOFF, I. L.: Removal by the isolated rat liver of palmitate-1-$C^{14}$ bound to albumin and of palmitate-1-$C^{14}$ and cholesterol-4-$C^{14}$ in chylomicrons from perfusion fluid. J. Biol. Chem. 234: 2240–2245, 1959.
147. HIMSWORTH, H. P.: Lectures on the liver and its diseases, pp. 19–47, Harvard University Press, Cambridge, Mass., 1947.
148. HIRSH, R. L. AND KELLNER, A.: The pathogenesis of hyperlipemia induced by means of surface active agents II. Failure of exchange of cholesterol between the plasma and the liver in rabbits given Triton WR-1339. J. Exp. Med. 104: 15–24, 1956.
149. HOCHSTEIN, P.: Microsomal peroxidation of lipids and its possible role in cellular injury. In: Ciba Found. Sym. on Cellular Injury, ed. by A. V. S. deReuck and J. Knight, pp. 123–135, Little, Brown and Co., Boston, Mass., 1964.
150. HOCHSTEIN, P. AND ERNSTER, L.: ADP-activated lipid peroxidation coupled to the TPNH oxidase system of microsomes. Biochem. Biophys. Res. Commun. 12: 388–394, 1963.
151. HOCHSTEIN, P., NORDENBRAND, K. AND ERNSTER, L.: Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and mitochondria. Biochem. Biophys. Res. Commun. 14: 323–328, 1964.
152. HOFFSTEN, P. E., HUNTER, F. E., JR., GEBICKI, J. M. AND WEINSTEIN, J.: Formation of "lipid peroxide" under conditions which lead to swelling and lysis of rat liver mitochondria. Biochem. Biophys. Res. Commun. 7: 276–280, 1962.
153. HOLMAN, R. T.: Autoxidation of fats and related substances. In: Prog. in the Chemistry of Fats and Other Lipids, vol. 2, ed. by R. T. Holman, W. O. Lundberg and T. Malkin, p. 51, Academic Press, New York, 1954.
154. HOLTKAMP, D. E. AND HILL, R. M.: Comparison of malonate and malondialdehyde in *in vitro* oxygen uptake studies. Arch. Biochem. Biophys. 34: 216–218, 1951.
155. HORNING, M. G., EARLE, M. J. AND MALING, H. M.: Changes in fatty acid composition of liver lipids induced by carbon tetrachloride and ethionine. Biochim. Biophys. Acta 56: 175–177, 1962.
156. HOVE, E. L.: Interrelation between $\alpha$-tocopherol and protein metabolism. III. The protective effect of vitamin E and certain nitrogenous compounds against $CCl_4$ poisoning in rats. Arch. Biochem. 17: 467–473, 1948.
157. HOVE, E. L.: The toxicity of tri-o-cresyl phosphate for rats as related to dietary casein level, vitamin E and vitamin A. J. Nutr. 51: 609–622, 1953.
158. HOVE, E. L., HICKMAN, K. AND HARRIS, P. L.: Effect of tocopherol and of fat on the resistance of rats to anoxic anoxia. Arch. Biochem. 8: 395–404, 1945.
159. HUNTER, F. E., JR. AND FORD, L.: Inactivation of oxidative and phosphorylative systems in mitochondria by preincubation with phosphate and other ions. J. Biol. Chem. 216: 357–369, 1955.
160. HUNTER, F. E., JR., SCOTT, A., HOFFSTEN, P. E., GEBICKI, J. M., WEINSTEIN, J. AND SCHNEIDER, A.: Studies on the mechanism of swelling, lysis, and disintegration of isolated liver mitochondria exposed to mixtures of oxidized and reduced glutathione. J. Biol. Chem. 239: 614–621, 1964.
161. HUNTER, F. E., JR., SCOTT, A., HOFFSTEN, P. E., GUERRA, F., WEINSTEIN, J., SCHNEIDER, A., SCHUTZ, B., FINK, J., FORD, L. AND SMITH, E.: Studies on the mechanism of ascorbate-induced swelling and lysis of isolated liver mitochondria. J. Biol. Chem. 239: 604–613, 1964.
162. HUNTER, F. E., SCOTT, A., WEINSTEIN, J. AND SCHNEIDER, A.: Effects of phosphate, arsenate, and other substances on swelling and lipid peroxide formation when mitochondria are treated with oxidized and reduced glutathione. J. Biol. Chem. 239: 622–630, 1964.

163. Hutterer, F., Fitschen, W. and Popper, H.: Mitochondrial damage and hepatic steatosis in ethionine intoxication. Brit. J. Exp. Pathol. **42**: 187–194, 1961.
164. Hyams, D. E. and Isselbacher, K. J.: Prevention of fatty liver by administration of adenosine triphosphate. Nature (London) **204**: 1196–1197, 1964.
165. Judah, J. D.: Protection against cellular injury by drugs. In: Ciba Foundation Symposium on Enzyme and Drug Action, ed. by J. L. Mongar and A. V. S. deReuck, pp. 359–383, Little, Brown and Co., Boston, 1962.
166. Judah, J. D., Ahmed, K. and McLean, A. E. M.: Action of drugs on hepatic organelles. Ann. N. Y. Acad. Sci. **104**: 926–938, 1963.
167. Judah, J. D., Ahmed, K. and McLean, A. E. M.: Possible role of ion shifts in liver injury. Ciba Foundation Symposium on Cellular Injury, ed. by A. V. S. deReuck, and J. Knight, pp. 53–66, Little, Brown and Company, Boston, 1964.
168. Judah, J. D. and Rees, K. R.: Mechanism of action of carbon tetrachloride. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **18**: 1013–1016, 1959.
169. Kasbekar, D. K., Lavate, W. V., Rege, D. V. and Sreenivasan, A.: A study of vitamin $B_{12}$ protection in experimental liver injury in the rat by carbon tetrachloride. Biochem. J. **72**: 384–389, 1959.
170. Kasbekar, D. K. and Sreenivasan, A.: Lability of intramitochondrial components in experimental liver injury. Nature (London) **178**: 989–990, 1956.
171. Kellner, A.: Serum phospholipids in experimental atherosclerosis. Amer. J. Pathol. **26**: 732, 1950.
172. Kellner, A., Correll, J. W. and Ladd, A. T.: Sustained hyperlipemia induced in rabbits by means of intravenously injected surface active agents. J. Exp. Med. **93**: 373–385, 1951.
173. Kennedy, E. P.: Metabolism of lipides. Annu. Rev. Biochem. **26**: 119–148, 1957.
174. Kennedy, E. P.: The biosynthesis of phospholipids. Amer. J. Clin. Nutr. **6**: 216–221, 1958.
175. Kennedy, E. P.: Biosynthesis of complex lipids. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **20**: 934–940, 1961.
176. Kiessling, K-H. and Tilander, K.: Thiamine and thiamine diphosphate in liver and brain from rats treated with $CCl_4$. Acta Chem. Scand. **14**: 841–845, 1960.
177. Kondos, A. C. and McClymont, G. L.: Indirect anthelmintic action of carbon tetrachloride against Fasciola hepatica. Nature (London) **206**: 846–847, 1965.
178. Kutob, S. D. and Plaa, G. L.: The effect of acute ethanol intoxication on chloroform-induced liver damage. J. Pharmacol. Exp. Therap. **135**: 245–251, 1962.
179. Larson, R. E. and Plaa, G. L.: Effect of spinal cord transection on $CCl_4$ hepatotoxicity. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **22**: 189, 1963.
180. Larson, R. E. and Plaa, G. L.: Spinal cord transection and $CCl_4$-toxicity. Experientia **19**: 604, 1963.
181. Larson, R. E. and Plaa, G. L.: A correlation of the effects of cervical cordotomy, hypothermia, and catecholamines on carbon tetrachloride-induced hepatic necrosis. J. Pharmacol. Exp. Therap. **147**: 103–111, 1965.
182. Larson, R., Plaa, G. L. and Brody, M. J.: Immunological sympathectomy and $CCl_4$ hepatotoxicity. Proc. Soc. Exp. Biol. Med. **116**: 557–560, 1964.
183. Larson, R. E., Plaa, G. L. and Crews, L. M.: The effect of spinal cord transection on carbon tetrachloride hepatotoxicity. Toxicol. Appl. Pharmacol. **6**: 154–162, 1964.
184. Laurell, S.: Turnover rate of unesterified fatty acids in human plasma. Acta Physiol. Scand. **41**: 158–167, 1957.
185. Laurell, S.: Distribution of $C^{14}$ in rats after intravenous injection of non-esterified palmitic acid-1-$C^{14}$. Acta Physiol. Scand. **46**: 97–106, 1959.
186. Laurell, S.: Recycling of intravenously injected palmitic acid-1-$C^{14}$ as esterified fatty acid in the plasma of rats and turnover rate of plasma triglycerides. Acta Physiol. Scand. **47**: 218–232, 1959.
187. Leach, B. E. and Forbes, J. C.: Sulfonamide drugs as protective agents against carbon tetrachloride poisoning. Proc. Soc. Exp. Biol. Med. **48**: 361–363, 1941.
188. Leduc, E. H. and Wilson, J. W.: Injury to liver cells in carbon-tetrachloride poisoning. Arch. Pathol. **65**: 147–157, 1958.
189. Leevy, C.: Fatty liver: A study of 270 patients with biopsy proven fatty liver and a review of the literature. Medicine **41**: 249–278, 1962.
190. Levi-Montalcini, R.: Growth control of nerve cells by a protein factor and its antiserum. Science (N.Y.) **143**: 105–110, 1964.
191. Lieber, C. S. and DeCarli, L. M.: Study of agents for the prevention of the fatty liver produced by prolonged alcohol intake, p. 6, Amer. Assoc. for the Study of Liver Diseases, 16th Annual Meet., Chicago, Ill., Nov. 1965.
192. Lillard, D. A. and Day, E. A.: Degradation of monocarbonyls from autoxidising lipids. J. Amer. Oil Chem. Soc. **41**: 549–552, 1964.
193. Lipsky, S. R., Haavik, A., Hopper, C. L. and McDivitt, R. W.: The biosynthesis of the fatty acids of the plasma of man. I. The formation of certain chromatographically separated higher fatty acids of the major lipid complexes from acetate-1-$C^{14}$. J. Clin. Invest. **36**: 233–244, 1957.
194. Lipsky, S. R., McGuire, J. S., Jr., Bondy, P. K. and Man, E. B.: The rates of synthesis and the transport of plasma fatty acid fractions in man. J. Clin. Invest. **34**: 1760–1765, 1955.
195. Lombardi, B.: Pathogenesis of fatty liver. Fed. Proc. Fedn. Amer. Socs. Exp. Biol. **24**: 1200–1205, 1965.
196. Lombardi, B. and Recknagel, R. O.: Interference with secretion of triglycerides by the liver as a common factor in toxic liver injury, with some observations on choline deficiency fatty liver. Amer. J. Pathol. **40**: 571–586, 1962.
197. Lombardi, B. and Ugazio, G.: Serum lipoproteins in rats with carbon tetrachloride-induced fatty liver. J. Lipid Res. **6**: 498–505, 1965.
198. Lyon, I., Masri, M. S. and Chaikoff, I. L.: Fasting and hepatic lipogenesis from $C^{14}$-acetate. J. Biol. Chem. **196**: 25–32, 1952.

199. MALAMED, S., LITTERIA, M. AND RECKNAGEL, R. O.: Swelling of mitochondria produced by $CCl_4$. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **16**: 359, 1957.
200. MALING, H. M., FRANK, A. AND HORNING, M. G.: Effect of carbon tetrachloride on hepatic synthesis and release of triglycerides. Biochim. Biophys. Acta **64**: 540–545, 1962.
201. MARCHETTI, M., PUDDU, P. AND CALDARERA, C. M.: Metabolic aspects of 'orotic acid fatty liver': Nucleotide control mechanisms of lipid metabolism. Biochem. J. **92**: 46–51, 1964.
202. MARTIN, G. H., BUNTING, C. H. AND LOEVENHART, A. S.: The morphologic changes in the tissues of the rabbit as a result of reduced oxidations. J. Pharmacol. Exp. Therap. Proc. **8**: 112–113, 1916.
203. MASORO, E. J., CHAIKOFF, I. L., CHERNICK, S. S. AND FELTS, J. M.: Previous nutritional state and glucose conversion to fatty acids in liver slices. J. Biol. Chem. **185**: 845–856, 1950.
204. MAXIMCHUK, A. J. AND RUBINSTEIN, D.: Alterations in tissues following carbon tetrachloride poisoning. Ann. Occup. Hyg. **4**: 49–61, 1961.
205. MAXIMCHUK, A. J. AND RUBINSTEIN, D.: Lipid mobilization following carbon tetrachloride administration. Can. J. Biochem. Physiol. **41**: 525–528, 1963.
206. MAY, H. E., POYER, J. L. AND MCCAY, P. B.: Lipid alterations occurring in the microsomes during the enzymic oxidation of TPNH. Biochem. Biophys. Res. Commun. **19**: 166–170, 1965.
207. MCCALLA, C., GATES, H. S., JR. AND GORDON, R. S., JR.: $C^{14}O_2$ excretion after intravenous administration of albumin-bound palmitate-1-$C^{14}$ to intact rats. Arch. Biochem. Biophys. **71**: 346–351, 1957.
208. MCCOLLISTER, D. D., BEAMER, W. H., ATCHISON, G. J. AND SPENCER, H. C.: Distribution and elimination of radioactive $CCl_4$ by monkeys upon exposure to low vapor concentration. J. Pharmacol. Exp. Therap. **102**: 112–124, 1951.
209. MCELROY, W. T., JR., SIEFERT, W. K. AND SPITZER, J. J.: Relationship of hepatic uptake of free fatty acids to plasma concentration. Proc. Soc. Exp. Biol. Med. **104**: 20–23, 1960.
210. MCLEAN, A. E. M.: Phenergan and Versene in dietary liver necrosis. Nature (London) **185**: 191, 1960.
211. MCLEAN, A. E. M.: Intracellular potassium in dietary liver necrosis. Nature (London) **185**: 936–937, 1960.
212. MCLEAN, A. E. M., MCLEAN, E. AND JUDAH, J. D.: Cellular necrosis in the liver induced and modified by drugs and other agents. Int. Rev. Exp. Pathol. **4**: 127–157, 1965.
213. MCMILLAN, G. R. AND CALVERT, J. G.: Gas phase photo-oxidation. Oxidation Combustion Rev. **1**: 84–135, 1965.
214. MEGIRIAN, R.: Influence of $CCl_4$ on thiopental sleeping time and distribution in the rat. J. Pharmacol. Exp. Therap. **144**: 331–336, 1964.
215. MILAS, N. A.: Detection, separation and identification of organic peroxides. Radiat. Res. Suppl. **3**: 71–92, 1963.
216. MILLER, L. L., ROGERS, C. A., LAMSON, B. G. AND LAMBIE, M. W.: Protein depletion and hepatic function in the dog: Rationale of methionine in therapy. Amer. J. Med. Sci. **214**: 84–88, 1947.
217. MILLER, L. L., ROSS, J. F. AND WHIPPLE, G. H.: Methionine and cystine, specific protein factors preventing chloroform liver injury in protein-depleted dogs. Amer. J. Med. Sci. **200**: 739–756, 1940.
218. MILLER, L. L. AND WHIPPLE, G. H.: Liver injury, liver protection, and sulfur metabolism: methionine protects against liver injury even when given after anesthesia. J. Exp. Med. **76**: 421–435, 1942.
219. MOORE, K. E. AND BRODY, T. M.: Functional changes in liver mitochondria following *in situ* anoxia. Amer. J. Physiol. **198**: 677–681, 1960.
220. MYREN, J.: Injury of liver tissue in mice after single injections of carbon tetrachloride. Acta Pathol. Microbiol. Scand., Suppl. **116**: 64 pp., 1956.
221. NEALE, R. C. AND WINTER, H. C.: Identification of the active crystalline substance from liver which protects against liver damage due to chloroform or carbon tetrachloride; and a study of related compounds. J. Pharmacol. Exp. Therap. **62**: 127–148, 1938.
222. NELSON, D., GOETZL, S., ROBINS, S. AND IVY, A. C.: Carrot diet and susceptibility to acute anoxia. Proc. Soc. Exp. Biol. Med. **52**: 1–2, 1943.
223. NESTEL, P. J. AND STEINBERG, D.: Fate of palmitate and of linoleate perfused through the isolated rat liver at high concentrations. J. Lipid Res. **4**: 461–469, 1963.
224. NEUBERT, D. AND MAIBAUER, D.: Vergleichende Untersuchungen der oxydativen Leistungen von Mitochondrien und Mikrosomen bei experimenteller Leberschädigung. Arch. Exp. Pathol. Pharmakol. **235**: 291–300, 1959.
225. OBERLING, CH. AND ROUILLER, CH.: Les effets de l'intoxication aiguë au tetrachlorure de carbone sur le foie du rat. Ann. Anat. Pathol. **1**: 401–427, 1956.
226. OLIVECRONA, T.: The metabolism of 1-$C^{14}$-palmitic acid in rats with ethionine induced fatty livers. Acta Physiol. Scand. **54**: 287–294, 1962.
227. OLIVECRONA, T.: The metabolism of 1-$C^{14}$-palmitic acid in the rat. Acta Physiol. Scand. **54**: 295–305, 1962.
228. OLIVECRONA, T.: Kinetics of fatty acid transport. An experimental study in the rat, p. 10, Håkan Ohlssons Boktryckeri, Lund, 1962.
229. OLIVECRONA, T., GEORGE, E. P. AND BORGSTRÖM, B.: Chylomicron metabolism. Fed. Proc. **20**: 928–933, 1961.
230. OLSON, R. E.: Effect of dietary protein upon fat transport. Diabetes **7**: 202–210, 1958.
231. OLSON, R. E.: Atherosclerosis—a primary hepatic or vascular disorder? Perspect. Biol. Med. **2**: 84–121, 1958.
232. OLSON, R. E., JABLONSKI, J. R. AND TAYLOR, E.: Effect of dietary protein, fat, and choline upon the serum lipids and lipoproteins of the rat. Amer. J. Clin. Nutr. **6**: 111–118, 1958.
233. OLSON, R. E. AND VESTER, J. W.: Nutrition-endocrine interrelationships in the control of fat transport in man. Physiol. Rev. **40**: 677–733, 1960.
234. ONCLEY, J. C.: Plasma lipoproteins. In Chemistry of Lipides as Related to Atherosclerosis, ed. by I. H. Page, pp. 114–128, Charles C Thomas, Springfield, Ill., 1958.
235. ORRENIUS, S., DALLNER, G. AND ERNSTER, L.: Inhibition of the TPNH-linked lipid peroxidation of liver microsomes by drugs undergoing oxidative demethylation. Biochem. Biophys. Res. Commun. **14**: 329–334, 1964.

236. PAUL, B. B. AND RUBINSTEIN, D.: Metabolism of carbon tetrachloride and chloroform by the rat. J. Pharmacol. Exp. Therap. 141: 141–148, 1963.
237. PETERS, J. P. AND VANSLYKE, D. D.: Quantitative Clinical Chemistry: Interpretations, ed. 2, Williams & Wilkins Co., Baltimore, 1946.
238. PHILPOT, J. ST. L.: The estimation and identification of organic peroxides. Radiat. Res. Suppl. 3: 55–70, 1963.
239. PIHL, A., BLOCH, K. AND ANKER, H. S.: The rates of synthesis of fatty acids and cholesterol in the adult rat studied with the aid of labeled acetic acid. J. Biol. Chem. 183: 441–450, 1950.
240. PLAA, G. L. AND LARSON, R. E.: Carbon tetrachloride induced liver damage. Arch. Environ. Health 9: 536–543, 1964.
241. PLACER, Z., VESELKOVA, A. AND RATH, R.: Kinetic des Malondialdehydes im Organismus. Experientia 21: 19, 1965.
242. POGGI, M., FUMAGALLI, R., SABATINI-PELLEGRINI, A. AND PAOLETTI, R.: Investigations on the early effects of $CCl_4$ on lipid transport. In: Drugs and Enzymes, pp. 363–370, Proceedings of the 2nd International Pharmacological Meeting, Prague, Aug. 20–23, 1963, Pergamon Press, New York, 1965.
243. POGGI, M. AND PAOLETTI, R.: A new insight on carbon tetrachloride effect on lipid transport. Biochem. Pharmacol. 13: 949–954, 1964.
244. POPPER, H. AND SCHAFFNER, F.: Liver: Structure and Function, The Blakiston Division, McGraw-Hill Book Company, Inc. New York, 1957.
245. POTTER, V. R., SIEKEVITZ, P. AND SIMONSON, H. C.: Latent adenosine triphosphatase activity in resting rat liver mitochondria. J. Biol. Chem. 205: 893–908, 1953.
246. PRIEST, R. E., SMUCKLER, E. A., ISERI, O. A. AND BENDITT, E. P.: Liver lipid peroxide levels in carbon tetrachloride poisoning. Proc. Soc. Exp. Biol. Med. 111: 50–51, 1962.
247. RAMEY, E. R. AND GOLDSTEIN, M. S.: The adrenal cortex and the sympathetic nervous system. Physiol. Rev. 37: 155–195, 1957.
248. RAVDIN, I. S., VARS, H. M. AND GOLDSCHMIDT, S.: Non-specificity of suspensions of sodium xanthine in protecting liver against injury by chloroform and probable cause of its action. J. Clin. Invest. 18: 633–640, 1939.
249. RECKNAGEL, R. O. AND ANTHONY, D. D.: Effects of $CCl_4$ on enzyme systems of rat liver mitochondria. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 16: 456, 1957.
250. RECKNAGEL, R. O. AND ANTHONY, D. D.: Biochemical changes in carbon tetrachloride fatty liver: Separation of fatty changes from mitochondrial degeneration. J. Biol. Chem. 234: 1052–1059, 1959.
251. RECKNAGEL, R. O. AND GHOSHAL, A. K.: No accelerated mobilization of peripheral lipid in $CCl_4$ hepatotoxicity. Gastroenterology 48: 509, 1965.
252. RECKNAGEL, R. O. AND GHOSHAL, A. K.: Acceleration of lipoperoxidation by carbon tetrachloride. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 24: 299, 1965.
253. RECKNAGEL, R. O. AND GHOSHAL, A. K.: New data on the question of lipoperoxidation in carbon tetrachloride poisoning. Exp. Mol. Pathol. 5: 108–117, 1966.
254. RECKNAGEL, R. O. AND GHOSHAL, A. K.: Lipoperoxidation as a vector in carbon tetrachloride hepatotoxicity. Lab. Invest. 15: 132–146, 1966.
255. RECKNAGEL, R. O. AND GHOSHAL, A. K.: Quantitative estimation of peroxidative degeneration of rat liver microsomal and mitochondrial lipids after carbon tetrachloride poisoning. Exp. Mol. Pathol. 5: 413–426, 1966.
256. RECKNAGEL, R. O. AND LITTERIA, M.: Activation of acid phosphatase in carbon tetrachloride fatty liver. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 18: 125, 1959.
257. RECKNAGEL, R. O. AND LITTERIA, M.: Biochemical changes in carbon tetrachloride fatty liver. Concentration of carbon tetrachloride in liver and blood. Amer. J. Pathol. 36: 521–531, 1960.
258. RECKNAGEL, R. O. AND LOMBARDI, B.: Studies of biochemical changes in subcellular particles of rat liver and their relationship to a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. J. Biol. Chem. 236: 564–569, 1961.
259. RECKNAGEL, R. O., LOMBARDI, B. AND SCHOTZ, M. C.: A new insight into the pathogenesis of carbon tetrachloride fat infiltration. Proc. Soc. Exp. Biol. Med. 104: 606–610, 1960.
260. RECKNAGEL, R. O. AND MALAMED, S.: The osmotic nature of mitochondrial swelling produced by carbon tetrachloride and inorganic phosphate. J. Biol. Chem. 232: 705–713, 1958.
261. RECKNAGEL, R. O., STADLER, J. AND LITTERIA, M.: Biochemical changes accompanying development of fatty liver. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 17: 129, 1958.
262. REES, K. R.: Cellular injury by drugs. In: Ciba Foundation Symposium on Enzymes and Drug Action, ed. by J. L. Mongar and A. V. S. deReuck, pp. 344–358, Little, Brown and Co., Boston, 1962.
263. REES, K. R.: Mechanism of action of certain exogenous toxic agents in liver cells. In: Ciba Foundation Symposium on Cellular Injury, ed. by A. V. S. deReuck and J. Knight, pp. 53–66, Little, Brown and Company, Boston, 1964.
264. REES, K. R. AND SHOTLANDER, V. L.: Fat accumulation in acute liver injury. Ann. N. Y. Acad. Sci. 104: 905–915, 1963.
265. REES, K. R. AND SHOTLANDER, V. L.: Fat accumulation in acute liver injury. Proc. Roy. Soc., Ser. B 157: 517–535, 1963.
266. REES, K. R. AND SINHA, K. P.: Blood enzymes in liver injury. J. Pathol. Bacteriol. 80: 297–307, 1960.
267. REES, K. R., SINHA, K. P. AND SPECTOR, W. G.: The pathogenesis of liver injury in carbon tetrachloride and thioacetamide poisoning. J. Pathol. Bacteriol. 81: 107–118, 1961.
268. REYNOLDS, E. S.: Comparison of the acute toxic effects of $CCl_4$, $CHCl_3$, ethanol, diethyl ether, thioacetamide, and dimethylnitrosamine. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 24: 166, 1965.

269. Reynolds, E. S. and Thiers, R. E.: 51st annual meeting, Am. Soc. Clin. Invest., p. 55, Atlantic City, N. J., May, 1959.
270. Reynolds, E. S., Thiers, R. E. and Vallee, B. L.: Mitochondrial function and metal content in carbon tetrachloride poisoning. J. Biol. Chem. **237**: 3546–3551, 1962.
271. Robbins, B. H.: The absorption, distribution and excretion of carbon tetrachloride in dogs under various conditions. J. Pharmacol. Exp. Therap. **37**: 203–216, 1929.
272. Robinson, D. S.: The uptake and release of lipids by the liver. In: Lipid Transport, ed. by H. C. Meng, pp. 194–201, Charles C Thomas, Springfield, Ill., 1964.
273. Robinson, D. S. and French, J. E.: Heparin, the clearing factor lipase, and fat transport. Pharmacol. Rev. **12**: 241–263, 1960.
274. Robinson, D. S. and Harris, P. M.: Ethionine administration in the rat. 2. Effects on the incorporation of ($^{32}$P) orthophosphate and DL-(1-$^{14}$C) leucine into the phosphatides and proteins of liver and plasma. Biochem. J. **80**: 361–369, 1961.
275. Robinson, D. S. and Seakins, A.: The development in the rat of fatty livers associated with reduced plasma-lipoprotein synthesis. Biochim. Biophys. Acta **62**: 163–165, 1962.
276. Rodahl, K. and Issekutz, B.: Fat as a Tissue, McGraw-Hill Book Co., New York, 1964.
277. Roheim, P. S. and Eder, H. A.: The formation of lipoproteins by the isolated perfused rat liver. Circulation **24**: 1101, 1961.
278. Roheim, P. S., Haft, D. E., Gidez, L. I., White, A. and Eder, H. A.: Plasma lipoprotein metabolism in perfused rat livers. II. Transfer of free and esterified cholesterol into the plasma. J. Clin. Invest. **42**: 1277–1285, 1963.
279. Roheim, P. S., Miller, L. and Eder, H. E.: The formation of plasma lipoproteins from apoprotein in plasma. J. Biol. Chem. **240**: 2994–3001, 1965.
280. Roheim, P. S., Switzer, S., Girard, A. and Eder, H. A.: The mechanism of inhibition of lipoprotein synthesis by orotic acid. Biochem. Biophys. Res. Commun. **20**: 416–421, 1965.
281. Rosin, A. and Doljanski, L.: Pyroninophilic structures of liver cells in carbon tetrachloride poisoning. Proc. Soc. Exp. Biol. Med. **62**: 62–64, 1946.
282. Rossi, F. and Zatti, M.: Activation of fatty acids in the liver in carbon tetrachloride poisoning. Experientia **16**: 513–514, 1960.
283. Rubin, R. J. and Pendleton, R. G.: Biochemical events in fatty livers induced by orotic acid. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **23**: 126, 1964.
284. Rubinstein, D.: Epinephrine release and liver glycogen levels after carbon tetrachloride administration. Amer. J. Physiol. **203**: 1033–1037, 1962.
285. Rubinstein, D. and Kanics, L.: The conversion of carbon tetrachloride and chloroform to carbon dioxide by rat liver homogenates. Can. J. Biochem. Physiol. **42**: 1577–1585, 1964.
286. Rubinstein, B. and Rubinstein, D.: The effect of carbon tetrachloride on hepatic lipid metabolism. Can. J. Biochem. Physiol. **42**: 1263–1273, 1964.
287. Rudman, D., Girolamo, M. D., Kendall, F. E., Wertheim, A. R., Seidman, F., Reid, M. B. and Bern, S.. Further observations on the effect of pituitary gland extracts upon the serum lipids of the rabbit. Endocrinology **67**: 784–800, 1960.
288. Scanu, A. and Oriente, P.: Triton hyperlipemia in dogs. I. *In vitro* effects of the detergent on serum lipoproteins and chylomicrons. J. Exp. Med. **113**: 735–757, 1961.
289. Scanu, A., Oriente, P., Szajewski, J. M., McCormack, L. J. and Page, I. H.: Triton hyperlipemia in dogs. II. Atherosclerosis, diffuse lipidosis, and depletion of fat stores produced by prolonged administration of the non-ionic surface active agent. J. Exp. Med. **114**: 279–294, 1961.
290. Scanu, A. and Page, I. H.: Plasma transport of lipids and lipoprotein proteins in dogs treated with Triton WR-1339. J. Clin. Invest. **41**: 495–504, 1962.
291. Schneider, W. C., Striebich, M. J., and Hogeboom, G. H.: Cytochemical studies. VII. Localization of endogenous citrate in rat liver fractions. J. Biol. Chem. **222**: 969–977, 1956.
292. Schotz, M. C.: Rate of triglyceride secretion into plasma of normal and $CCl_4$-treated rats. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **21**: 292, 1962.
293. Schotz, M. C., Baker, N. and Chavez, M. N.: Effect of carbon tetrachloride ingestion on liver and plasma triglyceride turnover rates. J. Lipid Res. **5**: 569–577, 1964.
294. Schotz, M. C., Baker, N. and Chavez, M. N.: Plasma free fatty acid turnover in carbon tetrachloride treated rats. Metabolism **14**: 1023–1026, 1965.
295. Schotz, M. C. and Recknagel, R. O.: Effect of carbon tetrachloride on release of free fatty acids by rat adipose tissue. Proc. Soc. Exp. Biol. Med. **103**: 398–400, 1960.
296. Schotz, M. C. and Recknagel, R. O.: Rapid increase of rat liver triglycerides following carbon tetrachloride poisoning. Biochim. Biophys. Acta **41**: 151–152, 1960.
297. Schotz, M. C., Scanu, A. and Page, I. H.: Effect of triton on lipoprotein lipase of rat plasma. Am. J. Physiol. **188**: 399–402, 1957.
298. Schwarz, K. (ed.): Nutritional factors and liver diseases. Ann. N. Y. Acad. Sci. **57**: 615–692, 1954.
299. Schwarz, K.: Lipids and antioxidants in dietary necrotic liver degeneration. In: Symposium on Foods: Lipids and Their Oxidation, ed. by H. W. Schults, pp. 387–408, The AVI Publishing Co., Westport, Conn., 1962.
300. Seakins, A. and Robinson, D. S.: The effect of the admnistration of carbon tetrachloride on the formation of plasma lipoproteins in the rat. Biochem. J. **86**: 401–407, 1963.
301. Seakins, A. and Robinson, D. S.: Changes associated with the production of fatty livers by white phosphorus and by ethanol in the rat. Biochem. J. **92**: 308–312, 1964.

302. SHARE, L. AND RECKNAGEL, R. O.: Effect of carbon tetrachloride poisoning on potassium, sodium, and water content of liver mitochondria. Amer. J. Physiol. **197:** 121–125, 1959.
303. SHERRINGTON, C. S.: Notes on temperature after spinal transection, with some observations on shivering. J. Physiol. **58:** 404–424, 1924.
304. SLATER, T. F.: Necrogenic action of carbon tetrachloride in the rat: A speculative mechanism based on activation. Nature (London) **209:** 36–40, 1966.
305. SLATER, T. F., STRÄULI, U. D. AND SAWYER, B. C.: Changes in liver nucleotide concentrations in experimental liver injury. Biochem. J. **93:** 260–266, 1964.
306. SMUCKLER, E. A.: Polysome changes in acute toxic ($CCl_4$, DMNA, TIA) liver injury requires the intact cell. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **24:** 556, 1965.
307. SMUCKLER, E. A. AND BARKER, E. A.: Ribosomal changes in thioacetamide and $CCl_4$ poisoned rat liver and absence of changes in yellow phosphorus poisoning. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **23:** 335, 1964.
308. SMUCKLER, E. A., BARKER, E. A. AND BENDITT, E. P.: Defects induced in liver microsomes by acute carbon tetrachloride and thioacetamide poisoning. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. **22:** 369, 1963.
309. SMUCKLER, E. A. AND BENDITT, E. P.: Carbon tetrachloride poisoning in rats: Alteration in ribosomes of the liver. Science (N.Y.) **140:** 308–310, 1963.
310. SMUCKLER, E. A. AND BENDITT, E. P.: Studies on carbon tetrachloride intoxication. III. A subcellular defect in protein synthesis. Biochemistry **4:** 671–679, 1965.
311. SMUCKLER, E. A., ISERI, O. A. AND BENDITT, E. P.: Studies on carbon tetrachloride intoxication. I. The effect of carbon tetrachloride on incorporation of labeled amino acids into plasma proteins. Biochem. Biophys. Res. Commun. **5:** 270–275, 1961.
312. SMUCKLER, E. A., ISERI, O. A. AND BENDITT, E. P.: An intracellular defect in protein synthesis induced by carbon tetrachloride. J. Exp. Med. **116:** 55–72, 1962.
313. SMUCKLER, E. A., ISERI, O. A. AND BENDITT, E. P.: Studies on carbon tetrachloride intoxication. II. Depressed amino acid incorporation into mitochondrial protein and cytochrome C. Lab. Invest. **13:** 531–538, 1964.
314. STANDERFER, S. B. AND HANDLER, P.: Fatty liver induced by orotic acid feeding. Proc. Soc. Exp. Biol. Med. **90:** 270–271, 1955.
315. STEIN, Y. AND SHAPIRO, B.: Assimilation and dissimilation of fatty acids by the rat liver. Amer. J. Physiol. **196:** 1238–1241, 1959.
316. STEINBERG, D.: Fatty acid mobilization-mechanisms of regulation and metabolic consequences. In: The Control of Lipid Metabolism, Biochem. Society Symp. **24:** ed. by J. K. Grant, pp. 111–145, Academic Press, New York, 1963.
317. STEINBERG, D.: The fate of plasma free fatty acids and their effects on tissue metabolism. Metabolism **13:** 1264–1272, 1964.
318. STEKOL, J. A.: Biochemical basis for ethionine effects on tissues. Adv. Enzymol. **25:** 369–394, 1963.
319. STERN, P. H. AND BRODY, T. M.: Catecholamine excretion following carbon tetrachloride administration. J. Pharmacol. Exp. Therap. **141:** 65–73, 1963.
320. STERN, P. H., FURUKAWA, T. AND BRODY, T. M.: Rat liver and plasma lipids after carbon tetrachloride administration. J. Lipid Res. **6:** 278–286, 1965.
321. STETTIN, D., JR. AND BOXER, G. E.: Studies in carbohydrate metabolism. I. The rate of turnover of liver and carcass glycogen, studied with the aid of deuterium. J. Biol. Chem. **155:** 231–236, 1944.
322. STETTEN, D., JR. AND SALCEDO, J.: The source of extra liver fat in various types of fatty liver. J. Biol. Chem. **156:** 27–32, 1944.
323. STONER, H. B. AND MAGEE, P. N.: Experimental studies on toxic liver injury. Brit. Med. Bull. **13:** 102–106, 1957.
324. TAMURA, U., TSUCHIYA, T., HARADA, T., KUROIWA, K. AND KITANI, T.: The effect of vitamin E upon the liver of rats intoxicated with $CCl_4$. Med. J. Osaka Univ. **10:** 91–98, 1959.
325. TAPPEL, A. L.: Vitamin E and selenium as inhibitors in *in vivo* lipid oxidation. In: Lipids and Their Oxidation, ed. by H. W. Schultz, pp. 367–386, The AVI Publishing Co., Westport, Conn., 1962.
326. TAPPEL, A. L.: Vitamin E as the biological lipid antioxidant. Vitamins Hormones **20:** 493–510, 1962.
327. TAPPEL, A. L. AND ZALKIN, H.: Lipid peroxidation in isolated mitochondria. Arch. Biochem. Biophys. **80:** 326–332, 1959.
328. THIERS, R. E., REYNOLDS, E. S. AND VALLEE, B. L.: The effect of carbon tetrachloride poisoning on subcellular metal distribution in rat liver. J. Biol. Chem. **235:** 2130–2133, 1960.
329. TROUT, D. L. AND ESTES, E. H., JR.: Factors affecting liver uptake of serum free stearic acid-1-$C^{14}$. Amer. J. Physiol. **203:** 1024–1028, 1962.
330. TSEN, C. C. AND COLLIER, H. B.: The protective action of tocopherol against hemolysis of rat erythrocytes by dialuric acid. Can. J. Biochem. Physiol. **38:** 957–964, 1960.
331. TZUR, R. AND SHAPIRO, B.: Dependence on microsomal lipid synthesis on added protein. J. Lipid Res. **5:** 542–547, 1964.
332. UGAZIO, G., ARTIZZU, M., PANI, P. AND DIANZANI, M. U.: The changes in some hydrolytic enzymes in carbon tetrachloride induced fatty livers. Biochem. J. **90:** 109–116, 1964.
333. VARS, H. M., RAVDIN, I. S. AND GOLDSCHMIDT, S.: The non-specificity of suspensions of sodium xanthine in protecting the liver against injury by chloroform, and the probable cause of its action. Am. J. Physiol. **126:** 646, 1939.
334. VEALL, N. AND VETTER, H.: Radioisotope Techniques in Clinical Research and Diagnosis, p. 178, Butterworths, London, 1958.
335. VILLA-TREVINO, S., FARBER, E., STAEHELIN, T., WETTSTEIN, F. O. AND NOLL, H.: Breakdown and reassembly of rat liver ergosomes after administration of ethionine or puromycin. J. Biol. Chem. **239:** 3826–3833, 1964.

336. VILLA-TREVINO, S., SHULL, K. H. AND FARBER, E.: The role of adenosine triphosphate deficiency in ethionine-induced inhibition of protein synthesis. J. Biol. Chem. 238: 1757–1763, 1963.
337. VOLWILER, W., GOLDSWORTHY, P. D., MACMARTIN, M. P., WOOD, P. A., MACKAY, I. R. AND FREMONT-SMITH, K.: Biosynthetic determination with radioactive sulfur of turnover rates of various plasma proteins in normal and cirrhotic man. J. Clin. Invest. 34: 1126–1146, 1955.
338. VON EULER, L. H., RUBIN, R. J. AND HANDSCHUMACHER, R. E.: Fatty livers induced by orotic acid. II. Changes in nucleotide metabolism. J. Biol. Chem. 238: 2464–2469, 1963.
339. WADSTRÖM, L. B.: Lipolytic effect of the injection of adrenalin on fat depots. Nature (London) 179: 259–260, 1957.
340. WALLING, C.: Free Radicals in Solution, p. 150, John Wiley and Sons, Inc., New York, 1957.
341. WANG, C., PARONETTO, F., SOHAR, E. AND ADLERSBERG, D.: Effects of ethionine administration in rabbits and dogs. A. M. A. Arch. Pathol. 65: 279–285, 1958.
342. WEINMAN, E. O., CHAIKOFF, I. L., ENTENMAN, C. AND DAUBEN, W. G.: Turnover rates of phosphate and fatty acid moieties of plasma phospholipids. J. Biol. Chem. 187: 643–649, 1950.
343. WELLS, H. G., Chemical Pathology, ed. 5, pp. 623–627, W. B. Saunders, Philadelphia, 1925.
344. WHEELER, D. H.: Peroxide formation as a measure of autoxidative deterioration. Oil and Soap 9: 89–97, 1932.
345. WIGGLESWORTH, J. S.: The use of the osmium-ethyl gallate technique in the study of carbon tetrachloride liver injury. J. Pathol. Bacteriol. 87: 333–339, 1964.
346. WILSON, J. W.: Protective action of sulfa drugs against injury to liver of mouse by $CCl_4$. Fedn. Proc. Fedn. Amer. Socs. Exp. Biol. 11: 433–434, 1952.
347. WINDMUELLER, H. G.: An orotic acid-induced, adenine-reversed inhibition of hepatic lipoprotein secretion in the rat. J. Biol. Chem. 239: 530–537, 1964.
348. WIRTSCHAFTER, Z. T. AND CRONYN, M. W.: Relative hepatotoxicity. Arch. Environ. Health 9: 180–185, 1964.
349. WIRTSCHAFTER, Z. T. AND CRONYN, M. W.: Free radical mechanism for solvent toxicity. Arch. Environ. Health 9: 186–191, 1964.
350. ZILVERSMIT, D. B.: The design and analysis of isotope experiments. Amer. J. Med. 29: 832–848, 1960.
351. ZILVERSMIT, D. B., ENTENMAN, C., FISHLER, M. C. AND CHAIKOFF, I. L.: Turnover rate of phospholipids in the plasma of the dog as measured with radioactive phosphorus. J. Gen. Physiol. 26: 333–341, 1943.