The Requirement of Free Fatty Acids for the Fatty Liver of CCl_4 Intoxication* (32820)

I. WEINSTEIN,[1] L. WILLHITE, H. KLAUSNER,[2] AND M. HEIMBERG[3]
(Introduced by A. D. Bass)
Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37203

It has been postulated that the fatty liver which develops as a consequence of poisoning with CCl_4 results from inhibition of the biochemical mechanisms involved in the release of triglyceride (TG) by the liver into the serum; an extensive review of this problem has appeared recently (1). It was demonstrated, using the isolated perfused rat liver, that the release of tryglyceride into the perfusate and into the very low density lipoprotein (VLDL) in particular was depressed with livers from CCl_4 poisoned animals (2–4). It was observed also that CCl_4 acted directly on the liver to inhibit the release of triglyceride (5). The biosynthesis of the protein (6,7) and phospholipid (4) moieties of the VLDL is reduced in CCl_4 intoxication and may be responsible, in part, for the decreased release of triglyceride by the liver. Since the inhibition of release of triglyceride by the isolated perfused rat liver can be observed in poisoning with CCl_4, and since this is the presumed mechanism of production of the fatty liver, it would be most convincing if the accumulation of triglyceride could be demonstrated to occur in the liver *in vitro*. In some earlier experiments, it was shown that the decreased release of triglyceride in CCl_4 poisoning was associated with increased retention of palmitate-^{14}C in hepatic triglyceride (4); in retrospect, since only small amounts of free fatty acid were added to the medium, no net accumulation of triglyceride in the liver was detectable. Recently, we reported that the accumulation of triglyceride in perfused livers from normal rats was proportional to the concentration of free fatty acid in the medium (8); it is possible that a similar relationship also exists in CCl_4 intoxication. This premise was examined and the observation made that when perfused livers were exposed to concentrations of free fatty acid sufficient to induce accumulation of moderate amounts of triglyceride in the normal livers, under identical experimental conditions greater amounts of triglyceride were retained in livers poisoned with CCl_4.

Methods. The details of the procedures (9) and apparatus (10) which have been employed in the perfusion of the liver have been described previously. Normal male rats (obtained from the Holtzman Co., Madison, Wisconsin) were maintained on laboratory chow and water *ad libitum*. These animals served as the source of the liver, serum, and defibrinated blood for all experiments. Livers obtained from normal and CCl_4 poisoned animals were divided into three groups as follows: Group I, Control group: no further treatment was given; II, Carbon tetrachloride (0.25 ml/100 gm of body wt.) was administered to the rat *in vivo* by gastric intubation 3.5 hours prior to sacrifice of the animal and removal of the liver for perfusion (2); and III, Carbon tetrachloride (0.010 ml) was added directly to the perfusion medium via the portal cannula (5).

After the livers were removed from the animals, they were placed in the apparatus and were perfused for 20 min with a medium consisting of 33 ml of defibrinated rat blood, 15.

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[2] Postdoctoral trainee, Grant No. 1-F2-AM-25, 450-01 (PE), from the National Institutes of Health. Present Address: Atlas Chemical Industries, Biomedical Division, Wilmington, Delaware.

[3] This work was completed during the tenure of an Established Investigatorship of the American Heart Association.

ml of rat serum, 0.5 ml of heparin (500 IU), and sufficient Krebs-Henseleit bicarbonate buffer, pH 7.4, to make a volume of 60 ml (11). The first sample of perfusate was then taken for analysis; immediately thereafter, an infusion of a palmitate–serum complex was started (Groups I and II only). The livers of Group III were first treated with CCl_4 (5); 30 min later, the initial sample of serum was removed from the medium and the palmitic acid was infused.

The fatty acid–serum complex consisted of 75 mg (293 μmoles) of palmitic acid, 30 ml of rat serum low in triglyceride, and sufficient 0.9% NaCl to make a volume of 50 ml (2). A serum deficient in triglyceride was prepared by centrifugation of rat serum at 105,000g for 20 hours in a model L2-65 Spinco Ultracentrifuge (12); this serum was used in order to reduce the quantity of triglyceride which was added during the infusion of the palmitic acid. The very low density lipoprotein which floated to the top of the tube during centrifugation was removed and the volume was readjusted to its original value with 0.9% NaCl; 90–95% of the triglyceride was removed from the serum by this procedure. The fatty acid–serum complex was infused into the perfusion medium at the rate of 131.3 μmoles palmitate/hour (22.8 ml/hour).

Aliquots of the perfusate were removed for analysis immediately preceding, and 60 and 120 min after the infusion of fatty acid was started. At the end of the experiment, the livers were removed and were perfused with ice-cold 0.9% NaCl to remove any medium residual in the sinuses. The livers were rapidly cleansed of nonhepatic tissues, blotted, weighed, and homogenized in 10–15 volumes of 95% ethanol. The lipids were extracted from the perfusate (13) or from the liver (4) as described previously. Aliquots of hepatic and perfusate lipids in $CHCl_3$ were separated into neutral and phospholipids by passage through a 3.0 gm silicic acid column (13). Triglycerides in the $CHCl_3$ eluates of the column were assayed by the method of Van Handel and Zilversmit (14), and free fatty acids were estimated as described by Duncombe (15). Phospholipids in the CH_3OH eluates of the column were measured by the procedure of King (16). The concentration of triglyceride and phospholipid in the livers at the start of the experiment was considered to be equal statistically to that quantity which was present in livers that were not perfused; such livers were removed from the animals as if they were to be perfused, but instead, were analyzed immediately. The flow of perfusate through the liver, the production of bile, and the appearance of the liver were considered as indices of hepatic viability during the 2-hour experimental period. The statistical significance of the differences between experimental groups was evaluated using a two-tailed table of Student's distribution for t (17).

Results and Discussion. It is well known that when animals are poisoned with CCl_4 the concentration of triglyceride increases in the livers of such animals (1) (Table I, A vs C). When livers from normal rats were perfused with the palmitate–serum complex, triglyceride accumulated in the tissue during the experiment (A vs B). When livers from CCl_4 poisoned animals were perfused *in vitro,* it is probable that more triglyceride accumulated in these livers (C vs D) than in livers from normal animals under identical conditions. The quantity of triglyceride which accumulated during perfusion of livers from normal rats treated with CCl_4 *in vitro* (A vs E) was significantly greater than that which accumulated in livers not treated with CCl_4, even though all livers were exposed to the same level of free fatty acid in the medium (B vs E).

The livers from rats poisoned with CCl_4 contained no more phospholipid than did livers from normal animals (A vs C). It is of interest that the hepatic concentration of phospholipid seemed to decrease during perfusion of livers from rats intoxicated with CCl_4 (C vs D) or during perfusion of livers from normal rats to which CCl_4 had been added *in vitro* (A vs E). The magnitude of the decrease in hepatic concentration of phospholopid is not large, and in earlier experiments was not observed to be statistically significant (4); the decrease, however, was consistent with conclusions derived from isotopic experiments that the biosynthesis of phospholipid was diminished in CCl_4 intoxication (4, 6, 18).

In agreement with earlier observations, the

TABLE I. Concentration of Triglyceride and Phospholipid in Liver.[a,b]

Group	Triglyceride (μmoles/gm of liver)	Phospholipid (μmoles/gm of liver)
I. Normal		
A. Nonperfused	(6) 5.48 ± 0.50	(4) 45.93 ± 2.04
B. Perfused	(6) 8.23 ± 0.94	(4) 39.56 ± 3.54
II. CCl_4 (*in vivo*)		
C. Nonperfused	(9) 13.18 ± 1.06	(5) 45.76 ± 2.42
D. Perfused	(8) 18.05 ± 0.57	(7) 36.79 ± 2.33
III. CCl_4 (*in vitro*)		
E. Perfused	(13) 12.29 ± 0.56	(5) 36.30 ± 2.29
Statistical analysis (p values):		
A vs B	<0.05	NS
A vs E	<0.001	<0.025
A vs C	<0.001	NS
B vs E	<0.001	NS
C vs D	<0.005	<0.05

[a] All figures are means ± SE. Figures in parentheses indicate number of observations. The mean accumulation of triglyceride during perfusion may be considered to be 2.75, 4.87, and 6.81 μmoles triglyceride/gm of liver for livers from normal animals (B–A), or from animals poisoned with CCl_4 (D–C), and for livers treated with CCl_4 *in vitro* (E–A), respectively. The figure 6.81 is significantly greater than 2.75 ($p < 0.001$ by analysis of variance). The figure 4.87 is not statistically different from 2.75; however, this would seem to be a poor comparison, biologically. The degree to which triglyceride accumulates in nonperfused livers from animals poisoned with CCl_4 is extremely variable in response to a given oral dose of hepatotoxin and may be reflected in the lack of significance; it is also possible that accumulation of triglyceride in a liver already fatty may not proceed with the same rapidity as in a normal liver, when both are exposed to equal concentrations of fatty acid.

[b] Characteristics of perfused livers in each group were as follows:

	I	II	III
Liver wt. (wet, gm)	11.42 ± 0.63	11.65 ± 0.46	12.18 ± 0.29
Bile flow (ml/gm of liver/2 hours)	0.117 ± 0.005	0.084 ± 0.008	0.065 ± 0.007
Perfusate flow rate (ml/gm of liver/min), terminal value	2.9 ± 0.1	2.5 ± 0.2	2.0 ± 0.3

release of triglyceride by the liver into the perfusate was diminished by CCl_4 regardless of the method of administration of the hepatotoxin (2–5). In the present experiments more triglyceride was released than reported earlier; the increased release of triglyceride resulted, in part, from the availability of more free fatty acid to the liver (Figure 1A). Even so, inhibition of release of triglyceride in CCl_4 poisoning was evident. The degree of inhibition of release of triglyceride appeared to be more severe with livers from animals poisoned *in vivo*, than in livers which had been treated with CCl_4 *in vitro*.

It should be emphasized that in these experiments, the concentration of free fatty acid in the medium was kept relatively constant in all groups and at normal plasma levels during the perfusion experiments (Figure 1B). If we were to consider that the triglyceride which accumulated in the liver and perfusate arose from the added free fatty acid, the triglyceride formed by livers from normal rats could account for approximately 59% of the administered free fatty acid. When livers were removed from animals poisoned with CCl_4, 66% of administered free fatty acid, almost all of which was present as hepatic triglyc-

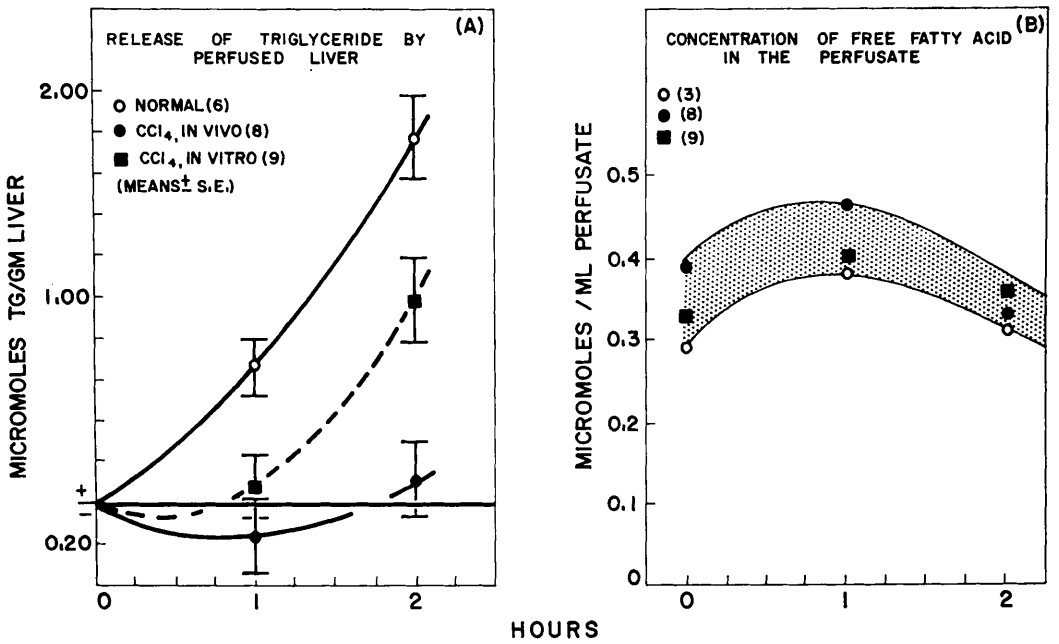

FIG. 1. Symbols indicate mean values ± S.E. Positive changes are indicative of net addition of triglyceride to the perfusate whereas negative changes are indicative of net loss of triglyceride from the medium. B. There were no statistically significant differences between groups; the symbols indicate mean values for perfusate concentration of free fatty acid in each group.

eride, could be accounted for. When livers from normal rats were treated with CCl_4 *in vitro,* the triglyceride which accumulated in the liver or was released into the perfusate accounted for all of the added palmitate. The reasons for these differences are not known. It is not inconceivable that esterification of free fatty acid to triglyceride may have been favored when CCl_4 was added *in vitro.*

The major conclusion that can be deduced from the results of these and previous experiments is that at least two factors are required simultaneously for the production of the fatty liver of CCl_4 intoxication. These factors are: (i) the availability of substrate free fatty acid to the liver, and (ii) a specific biochemical lesion induced by CCl_4. Free fatty acid must be supplied even to the livers in normal animals if a fatty liver rich in triglyceride is to be formed; presumably, free fatty acid would be supplied to the liver by mobilization from adipose tissue, or, alternatively, from intrahepatic biosynthesis of fatty acid, provided precursors are available in quantity. It can be shown, using the isolated perfused rat liver, that the accumulation of triglyceride by liver from normal animals is directly proportional to the concentration of free fatty acid in the medium (8); when the livers were poisoned with CCl_4, the retention of fatty acid, as triglyceride, was increased above normal.

It may be presumed that CCl_4 acts directly on the liver (5) to produce a specific biochemical lesion. The lesion produced prevents the normal formation and release by the liver of the lipoproteins concerned with triglyceride transport (1). Even though the biochemical injury to the liver is induced by CCl_4, a fatty liver may not become evident unless fatty acid is made available in sufficient quantity. When the rats were treated with CCl_4 *in vivo,* the flow of free fatty acid to the liver in the intact animal at the concentration normally found in plasma, was sufficient to induce accumulation of about 8 μmoles of triglyceride/gm of liver in 3.5 hours. It is of interest that when livers from normal animals were treated *in vitro* with CCl_4, the load of palmitate infused in these perfusion experiments resulted in the

deposition of a similar quantity of triglyceride in the liver within a period of 2 hours.

It has been observed that adrenergic blocking agents, cord section, adrenalectomy, and other factors reduce the magnitude of the fatty liver following poisoning with CCl_4 *in vivo* (19, 20). It is quite possible that these factors only reduce the mobilization of free fatty acid from adipose tissue to liver without necessarily altering in any way the initial injury to the liver by the chlorinated hydrocarbon.

Summary. Livers, isolated surgically from normal animals and from rats intoxicated with CCl_4, were perfused *in vitro* with a medium into which palmitic acid was infused continuously. Livers from normal rats were also treated with CCl_4 *in vitro* by direct addition of the chlorinated hydrocarbon to the medium. Under the conditions of these experiments, poisoning with CCl_4 resulted in inhibition of net release of triglyceride by the liver into the perfusate and simultaneous accumulation of triglyceride in the liver. These observations support the hypothesis that the fatty liver of CCl_4 intoxication results primarily from interference with the biochemical mechanisms involved in formation and release of the triglyceride in the very low density lipoprotein of the serum.

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Amyloid. I. Use of Freund's Adjuvant in Experimental Amyloidosis (32821)

J. SRI RAM, G. G. GLENNER, AND R. A. DELELLIS

Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

Amyloidosis, a disease of humans as well as animals, is characterized by the infiltration of various organs by an as yet poorly defined protein–carbohydrate material (1). A variety of methods involving the injection of foreign proteins and other substances have been used for inducing amyloid in experimental animals (2); of these the method of daily subcutaneous injection of a solution of casein for over 6 weeks has been most commonly used. These methods, however, are characterized by prolonged treatments and variable results. Rothbard and Watson (3) induced amyloidoisis in W-Swiss, H-line mice after ten weekly sub-