The Effect of Carbon Tetrachloride on the Lipoproteins of Rat-Liver Cell Sap and Serum

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(*Received* 15 *December* 1965)

1. The lipoproteins of the liver cell sap can be resolved by paper electrophoresis into five components. Almost two-thirds of the total lipid, cholesterol and phospholipid are present in the slowest- and the fastest-moving components. 2. There is a two- to three-fold increase in the lipid content of the liver cell sap after the administration of carbon tetrachloride. 3. The cholesterol and phospholipid contents of the serum α- and β-lipoproteins separated by paper electrophoresis fall after treatment with carbon tetrachloride, whereas the total lipid content of that fraction that remains at the origin rises.

Since the action of carbon tetrachloride in producing a fatty liver is thought to be due to its ability to inhibit either the formation or the release of serum lipoproteins by the liver (Seakins & Robinson, 1963; Rees & Shotlander, 1963*a*; Aiyar, Fatterpaker & Sreenivasan, 1964; Lombardi & Ugazio, 1965), its effect on the concentration of lipoproteins in liver cell sap has been investigated in the rat.

METHODS

Preparation of liver cell-sap and floating-fat fractions. Male or female Wistar-strain rats weighing 175–225g. and fed on a laboratory stock diet were used throughout. Administration of carbon tetrachloride (0·2ml./100g. body wt.) diluted with an equal volume of liquid paraffin was done by stomach tube. Control rats were given only liquid paraffin. The livers were perfused via the dorsal aorta (Cowdry, 1949) with chilled 0·9% NaCl after anaesthetizing the rats with Nembutal (35mg./kg. body wt.). The early stages of perfusion were completed before the heart stopped beating. After perfusion, which took 8–10min., the livers were quickly excised, immersed in 0·25M-sucrose at 0° and homogenized in a homogenizer with a Teflon pestle. The homogenate was made to a final volume of twice the weight of the liver with 0·25M-sucrose and centrifuged at 15000*g* at 2–4° for 20min. The resulting supernatant was centrifuged at 105000*g* at 2–4° for 60min. in an MSE ultracentrifuge and the clear cell sap was withdrawn with a syringe without disturbing the top fatty layer (1·5ml. of cell sap was obtained from 5g. of liver). The fatty layer was recovered separately by adding more 0·25M-sucrose solution and freeing the fat from the sides of the centrifuge tube carefully with a glass rod without disturbing the sediment. This fraction is referred to as the floating-fat fraction.

Collection of serum samples. Blood from the hepatic artery was allowed to clot at 37° for 30min. Serum was separated after centrifuging the blood at 3000rev./min. for 15min. in a Clay-Adam Clinical centrifuge.

Paper electrophoresis. Paper electrophoresis was carried out on samples of the liver cell sap at a constant voltage of 200v and a current density of 20mA in a horizontal type apparatus with borate buffer, pH8·6 and *I* 0·06 (Pellegrino & Caravaglios, 1957). Four to six strips of Whatman 3MM paper (38cm.×5cm.) were run at a time, 0·1ml. of the samples being spotted towards the cathodic end. The optimum period of run at 4–10° was found to be 12–14hr. After the run, the strips were dried in an air oven at 100° for 20min., stained for 4hr. with Sudan Black in 55% (v/v) ethanol (Swahn, 1952) and washed thrice in 55% ethanol for 20min. each time. The strips were then air-dried after blotting between sheets of Whatman no. 1 filter paper and the stain density was determined in a Photovolt densitometer. With strict adherence to this staining and washing procedure it was possible to obtain reproducible results in which the lipoproteins of the liver cell sap were fractionated into five distinct bands, instead of three as observed by Aiyar *et al.* (1964). The diffuse staining at the origin found by Aiyar *et al.* (1964) was avoided by the preliminary separation of the cell sap from the floating-fat fraction. All five lipoproteins were stainable with protein stains such as Bromophenol Blue and Amido Black (Jencks, Jetton & Durrum, 1955).

Paper electrophoresis of serum lipoproteins was carried out in veronal buffer, pH8·6 and *I* 0·1. Other details were the same as for the liver cell-sap lipoproteins. Three definite lipoprotein bands were observed.

Extraction and estimation of lipids. The liver cell sap (1·5ml.) was treated with an equal volume of 10% (w/v) trichloroacetic acid and total lipids in the sediment were extracted three times with chloroform–methanol (1:1, v/v). The use of this solvent eliminates interference caused by proteins that form an interphase layer when the proportion of methanol is less. The combined extracts were washed with 0·007% $MgCl_2$ and made to known volume (16ml.).

Lipid extracts of the floating-fat fraction and of serum were prepared without preliminary trichloroacetic acid treatment by using 5ml. portions of chloroform–methanol (2:1, v/v) as the extractant.

Determinations of total lipid, cholesterol and phospholipid were made on portions of the chloroform–methanol extracts that had been evaporated to dryness. Complete evaporation of the solvents was necessary since traces of chloroform interfered with the colour development. An oxidation method with $K_2Cr_2O_7$ (Bragdon, 1951) was employed for the estimation of total lipids. The volumes of dichromate reagent and water were reduced to one-fifth, thus increasing the sensitivity from 500–4000 μg. to 100–800 μg. of lipid. Cholesterol was estimated by an oxidation method with $FeCl_3$ (Crawford, 1959). Phospholipids were estimated by the method of Bartlett (1959), the factor 25 being employed for the conversion of the values for inorganic phosphorus.

For the determination of lipids in the lipoprotein fractions separated by electrophoresis, corresponding segments of three unstained strips that had been run together with a stained strip were extracted under reflux with chloroform–methanol (2:1, v/v) at 40° with an air condenser in a water bath for 30 min. The extracts were made to known volumes and suitable portions taken for estimation of the various lipids.

All results reported are means ± S.E.M. of at least four independent observations.

RESULTS

Lipids of the cell-sap and floating-fat fractions of rat-liver homogenate. When the cell particulate fractions are sedimented from rat-liver homogenate by centrifugation a fatty layer separates out at the surface of the cell sap. This fatty layer contains little cholesterol and phosphatide (Table 1) and presumably most of its lipid is present as triglyceride.

The lipids of the cell sap are precipitated by trichloroacetic acid and are presumably present in solution as lipoproteins. When the floating fat is redispersed in the cell sap and an equal volume of 10% trichloroacetic acid is added, although most of the cholesterol and phospholipid in the mixture are still precipitated, the bulk of the lipid, presumably the triglyceride of the floating fat, is not. It seems that either the triglycerides of the floating fat are not associated with protein or the amount of protein present is too small for the complexes to be centrifuged down.

Electrophoresis of the cell sap shows the presence of eight distinct protein bands stainable with Amido Black (Fig. 1*c*). The three with lowest mobility contain no lipid stainable with Sudan Black and, in the absence of the floating fat, no lipid stain is observed at the origin. All the other five protein fractions contain lipid. The one with the lowest mobility runs in the same position as the β-lipoprotein of serum (fraction 2, Fig. 1*a*), whereas serum α-lipoprotein (fraction 3, Fig. 1*a*) has a similar mobility to that of the fastest component. Analysis shows that, when expressed per g. fresh wt. of liver, 516 μg. of total lipid, 117 μg. of phospholipid and 108 μg. of cholesterol are present in these two lipoprotein fractions, which thus contain approximately two-thirds of the total lipids of the cell sap.

Effect of the administration of carbon tetrachloride on the lipids of the cell-sap and floating-fat fractions. At 3 hr. and at 24 hr. after a single dose of carbon tetrachloride the greatest increase in the lipid content of the non-particulate fraction of the rat liver occurs in the floating-fat fraction (Table 2). Although the cholesterol and phospholipid contents of this fraction rise several-fold, the absolute increases in the amounts of these lipids are too small to account for more than a small part of the increase in the total lipid. This is presumably mainly due to an increase in the triglyceride content.

The phospholipid and cholesterol contents of the cell sap also rise somewhat after carbon tetrachloride treatment. Analysis of the individual lipoprotein fractions of the cell sap after their separation by electrophoresis has shown that this increase occurs primarily in the lipoproteins of lowest and highest mobilities, both at 3 hr. and 24 hr. after carbon tetrachloride administration.

Effect of the administration of carbon tetrachloride on serum lipids. On paper electrophoresis serum lipoproteins separate into three fractions (Fig. 1*a*). In this study, normal values for cholesterol, phospholipid and total lipid in rat serum were respectively 687, 1061 and 2690 μg./ml. Nearly 60% of the total cholesterol was present in the β-lipoprotein band (fraction 2) with the remainder distributed almost equally between fractions 1 and 3. The distribution of the phospholipid in fractions 1, 2 and 3 was 53, 43 and 4% respectively. Corresponding values for total lipid were 14, 50 and 36%.

After carbon tetrachloride administration, progressive and marked decreases were observed in the concentrations of serum cholesterol and phospholipid, their values being 81 and 68% respectively of those in control animals at 3 hr. and 70 and 63% at 24 hr. Smaller decreases in the concentration of

Table 1. *Analysis of the lipids of the cell-sap and floating-fat fractions of normal rat-liver homogenates*

The isolation of cell-sap and floating-fat fractions and extraction and estimation of lipids were carried out as described in the text. The values for total lipid are based on analyses by a dichromate oxidation method and therefore include cholesterol and phospholipid.

	Composition (μg./g. fresh wt. of liver)		
Fraction	Cholesterol	Phospholipid	Total lipid
Cell sap	163 ± 19	181 ± 22	822 ± 136
Floating fat	15 ± 6	22 ± 5	1837 ± 197

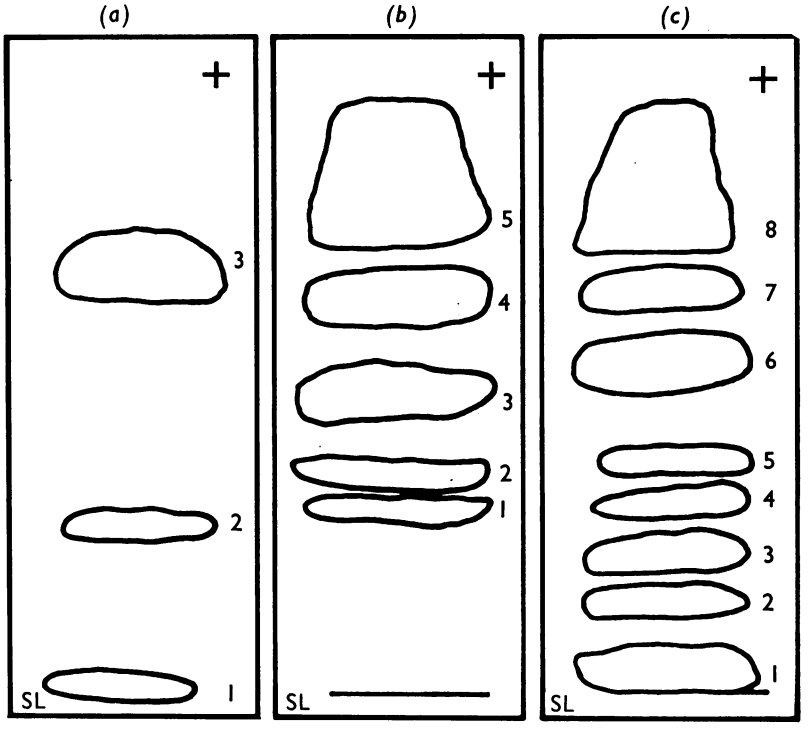

Fig. 1. Tracings of electrophoretic separations of liver cell-sap and serum proteins and lipoproteins. Samples of liver cell sap (0·1 ml. for lipoproteins and 0·025 ml. for proteins) and serum (0·1 ml.) were spotted at the starting line (SL). Electrophoresis was carried out for 12–14 hr. at 200 v and 20 mA at pH 8·6; borate buffer, I 0·06, and veronal buffer, I 0·1, were used for liver cell-sap and serum samples respectively. Staining for serum lipoproteins (*a*) and for liver cell-sap lipoproteins (*b*) was carried out with Sudan Black and for liver cell-sap proteins (*c*) with Amido Black. Tracings of bands are numbered in order of increasing mobility.

Table 2. *Effect of carbon tetrachloride administration on the lipids of the cell-sap and floating-fat fractions of rat-liver homogenates*

Rats were given a single intraperitoneal dose of carbon tetrachloride (0·2 ml./kg. body wt.) in paraffin oil and killed after 3 hr. or 24 hr. Control animals were given injections of paraffin oil alone. Cell-sap and floating-fat fractions were obtained from control and carbon tetrachloride-treated animals for extraction and estimation of lipids as described in the text and for Table 1.

	Composition (μg./g. fresh wt. of liver)					
	Cell sap			Floating fat		
Group	Cholesterol	Phospholipid	Total lipid	Cholesterol	Phospholipid	Total lipid
Control	183±19	200±22	973±83	20±6	20±6	1947±186
CCl_4-treated (3 hr.)	247±23	357±38	1612±163	40±13	37±12	15890±1350
CCl_4-treated (24 hr.)	350±31	592±53	2233±244	400±44	108±43	18120±1490

total lipid (13% after 3 hr. and 20% after 24 hr.) were found, however.

Analysis by paper electrophoresis showed that the main decreases occurred in the lipids of the α- and β-lipoproteins. The decreases in the cholesterol, phospholipid and total lipid contents of the α-lipoprotein (fraction 3) were respectively 30, 32 and 33% after 3 hr. and 60, 43 and 50% after 24 hr. of carbon tetrachloride administration. Corresponding decreases in the β-lipoprotein (fraction 2)

were 28, 42 and 43% after 3hr. and 56, 59 and 48% after 24hr. The cholesterol and phospholipid contents of the fraction that remained at the origin were unaltered, but the total lipid content of this fraction increased by 48% after 3hr. and 70% after 24hr. These findings presumably indicate an increase in the triglyceride content of this fraction.

DISCUSSION

In previous work from this Laboratory (Aiyar *et al.* 1964), a decrease in the uptake of Sudan Black B by the lipoproteins of rat-liver cell sap was observed after carbon tetrachloride administration and this was interpreted as reflecting a decrease in the lipoprotein content of the cell sap. In the present study, in which the lipids of the cell sap were chemically determined, a considerable increase as a result of carbon tetrachloride administration was observed. It has been reported that Sudan Black B stains predominantly lipids containing unsaturated fatty acids and their esters (Schjeide, Arthur & Yoshino, 1963), and therefore it may not serve as a reliable index of total lipid. Studies by Ghoshal & Recknagel (1965) indicate further that there is a considerable enhancement of lipoperoxidation and a consequent decrease in the amount of unsaturated fatty acid present in the livers of rats given carbon tetrachloride. These observations would invalidate the assumptions made in the previous work (Aiyar *et al.* 1964) that the ratio of unsaturated fatty acids to total fatty acids was unchanged by carbon tetrachloride treatment and that stain uptake was directly proportional to the total lipid content. The present findings can thus be reconciled with those obtained by Aiyar *et al.* (1964) if it is assumed that carbon tetrachloride causes a decrease in the unsaturated fatty acid content of the liver cell sap even though the total lipid content increases.

Getz *et al.* (1961) reported that triglycerides form over 90% of the total lipids in the floating-fat fraction and about 65% of those in the cell sap of normal rat liver. Our results (Table 1) are in good agreement with these values. Nearly 75% of the total triglyceride in rat liver is present in the combined cell-sap and floating-fat fractions (cf. Clement, Clement & Leberton, 1956) and, after carbon tetrachloride administration, the liver triglycerides may rise sevenfold (Stern, Furukawa & Brody, 1965). Our results (Table 2) show that this increase is localized in the cell-sap and floating-fat fractions. In the cell sap nearly two-thirds of the cholesterol, phospholipid and total lipid is present in two lipoproteins (fractions 1 and 5 on electrophoretic analysis), and it is to these two lipoproteins that almost all of the increase in lipid is confined. On the assumption that the accumulation of lipid signifies an accumulation of lipoprotein, these findings suggest that lipoprotein release, but not formation, is inhibited after carbon tetrachloride administration. On the other hand, the amount of protein in the lipoproteins was not estimated in the present study, and this could have fallen after carbon tetrachloride treatment even though the lipid content was increased. Further work is needed to distinguish between these possibilities.

Our findings that the cholesterol and phospholipid concentrations in serum decreased after carbon tetrachloride administration are in general agreement with those of other workers (Seakins & Robinson, 1963; Rees & Shotlander, 1963*b*; Poggi & Paoletti, 1964; Lombardi & Ugazio, 1965). However, Seakins & Robinson (1963) also reported that the total serum fatty acids decreased, Rees & Shotlander (1963*b*) found decreases in triglycerides and in total fatty acids and, in a detailed study, Lombardi & Ugazio (1965) found a fall in the triglyceride concentration in each of three plasma lipoprotein fractions, separated in the ultracentrifuge. Our results suggest that the concentration of total triglycerides in serum changes little after carbon tetrachloride treatment, and that those that are associated with the lipoproteins which remain at the origin on paper electrophoresis may actually increase in amount. This disagreement with the findings of other workers is unexplained, though it may be relevant that Ribeiro & MacDonald (1963), who used paper electrophoresis to study the serum lipid changes after carbon tetrachloride treatment, reported a rise in the ratio of β-lipoprotein to α-lipoprotein. Contrary to the present findings and those of Aiyar *et al.* (1964), however, Stern *et al.* (1965) have reported an increase in plasma triglycerides and free fatty acids after 18hr. of carbon tetrachloride administration.

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