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TWO MECHANISMS OF CCl_4-INDUCED FATTY LIVER: LIPID PEROXIDATION OR COVALENT BINDING STUDIED IN CULTURED RAT HEPATOCYTES

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With cultured hepatocytes it was studied whether CCl_4-induced inhibition of secretion of VLDL and HDL from liver cells is a consequence of covalent binding of CCl_4 metabolites (i.e. $CCl_3^{\cdot}$; $CCl_3OO^{\cdot}$) to cell constituents or of membrane damage by lipid peroxidation. Comparing the kinetics of inhibition of lipoprotein secretion with that of CCl_4-bioactivation it was found, that covalent binding of (^{14}C)-CCl_4 occurred at early time points (5 min) after CCl_4 administration and inhibited the lipoprotein secretion. At 100 μM CCl_4 it was depressed by 53% within 60 min. Incubations of CCl_4-treated cells with increasing concentrations of vitamin E blocked lipid peroxidation, but lipoprotein secretion was still inhibited. Piperonyl butoxid, a radical scavenger, protected against CCl_4-induced inhibition of lipoprotein section, lipid peroxidation and covalent binding.

These results show that during the early phases of CCl_4 poisoning fat accumulation is the consequence of covalent binding of CCl_4 metabolities to cell structures.

KEY WORDS: CCl_4, cultured hepatocytes, lipid peroxidation, covalent binding of CCl_4-metabolites, lipoprotein secretion (VLDL, HDL).

INTRODUCTION

One of the earliest and most striking manifestations of carbon tetrachloride (CCl_4)-induced liver damage is hepatic accumulation of fat. Chronic CCl_4 intoxication is often associated with cirrhosis and cancer. Clinical investigations point to a causal relationship between toxic liver injury and long-standing occupational exposure to CCl_4 and related compounds.[1,2]

The mechanism of toxicity includes bioactivation of CCl_4. In this process short-lived reactive intermediates such as $CCl_3^{\cdot}$ and $CCl_3OO^{\cdot}$ are produced, which are thought to be responsible for the production of fatty liver and related pathological consequences.[3,5]

The reductive dehalogenation of CCl_4 has been demonstrated to be catalysed by the mixed function oxidase system; spin-trapping experiments proved directly the existence of free radicals.[6,7]

The exact mechanism of hyperlipemia is not fully clarified, but evidence gathered up to now indicates that liver steatosis is assumed to be a consequence of the inhibition of lipoprotein secretion from the liver cell. Some authors have studied this problem with the model of isolated hepatocytes.[8–10]

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As most noxious to the hepatocyte four effects have been considered:

i) The depletion of pyridinenucleotides,

ii) the possible role of an altered calcium homeostasis,

iii) covalent binding of CCl_4-metabolites to cell lipids and protein,

iv) lipid peroxidation, leading to oxidative destruction of membrane lipids.

As an initial test of the last two hypotheses we investigated CCl_4-induced inhibition of lipoprotein secretion using cultured hepatocytes.

We prefered cultured hepatocytes instead of isolated hepatocytes, because the latter have greatly impaired functions. For instance their protein turnover is catabolic, their synthesis of serum proteins is low and hormonal induction of some enzymes, i.e. inducibilities of tyrosine transaminase, formation of c-AMP etc., are poor.[11] The reason for these impaired functions is, that the cells membranes may be damaged by collagenase digestion. To avoid these disadvantages this study was, therefore, undertaken with cultured hepatocytes.

In the course of these experiments the doses of CCl_4 used were comparable to those concentrations which might be attained in vivo during exposure to CCl_4.[12]

MATERIALS AND METHODS

Chemicals

They were reagent grade and obtained from Sigma GmbH, München or Boehringer Mannheim GmbH. Carbon-14-labeled carbon tetrachloride, (1-^{14}C)-acetate and (2,3-^{3}H)-leucine were obtained from Amersham Buchler GmbH, Braunschweig.

Animals

Female Sprague-Dawley rats (180–200 g), from the Institution were used. The animals had free access to food and water.

Phenobarbitone Treatment

For the experiments involving binding of CCl_4-carbon to hepatocyte lipids rats were given daily one i.p. injection of phenobarbitone (100 mg/kg) for four days.

Vitamin E Treatment

Pretreatment of rats with vitamin E was performed as described by Pfeifer:[13] α-Tocopherolacetat was dissolved in 1 vol. of ethanol, followed by addition of 9 vol. of 16% (v/v) tween 80 in 0.9% NaCl. Portions (100 mg per kg body weight) of vitamin E were injected i.p. 15 h prior to the preparation of hepatocytes.[13]

Hepatocytes

They were prepared according to the method of Seglen[14] and plated on collagen-coated Nunc culture flasks (25 cm^2) in Ham's-F-12 medium containing fetal calf

serum (10% v/v) and dexamethasone (1 μM). Cell density at the time of plating was 5×10^6 cells/ml. The cells were incubated at 37°C for at least 4 hours and then washed once with Ham's-F-12 medium prior to the addition of medium.

Medium Concentration of CCl_4

CCl_4 was added to Ham's-F-12 medium as a 20% solution in dimentyl sulfoxide; this solution was sonified (Branson Sonifier, 50 watt, 10 sec) and equilibrated at 37°C for 15 min.

Lipoprotein Secretion

Cells were prelabeled in Ham's-F-12 medium containing 5 μCi (1-^{14}C)-acetate (58 mCi/mmol), 5 mM Na-acetate/8 ml medium, and 20 μCi (2,3-^{3}H)-leucine (50 Ci/mmol). After two hours the medium was changed and the hepatocytes were incubated in medium free of radioactivity, equilibrated with 100 μM CCl_4 or without CCl_4.

Incubations were terminated by the addition of ice-cold 0.9% NaCl solution and cell-free medium was assayed for radioactive labeled lipoproteins by the method of Burstein *et al.*[15] Aliquots of the resulting lipoprotein pellet were dissolved in NCS tissue solubilizer (Amersham Buchler, Braunschweig) and measured by scintillation spectrophotometry.

Malondialdehyde

Lipid peroxidation was estimated by measuring thiobarbituric acid reacting compounds.[16]

Measurement of Covalent Binding

Covalent binding of CCl_4 metabolites (Table I) was performed essentially as described by Dianzani and Poli.[17] Lipids were extracted from the individual cell fractions by the method of Bligh and Dyer[18] and, to determine protein bound radioactivity, an aliquot of each fraction was precipitated with 10% trichloroacetic acid and collected on glass fibre filters. After washing with methanol and ether the protein pellet was dissolved in NCS and the radioactivity measured.

Preparation of Fatty Acid Methyl Esters

The lipid extract of each cell fraction was subjected to transesterification according to the method of Smith.[19] Samples of the hexane extract of fatty acid methl esters were applied to thin-layer plates precoated with n-undecane (TLC plates silica gel 60, with concentrating zone). The plates were developed with acetonitril/acetic acid, (1:1, v/v). Labeled compounds were detected by t.l.c. radioactivity scanning and then scraped off from the plate and radioactivity was counted.

DNA-Content

In cultured hepatocytes it was determined according to Arndt-Iovin.[20]

TABLE I
Distribution analysis of (^{14}C)-CCl_4 metabolites among the cellular protein and lipid fractions

Cell fraction	% of total bound radioactivity	
	protein	lipid
25.000 × g pellet	34.5	65.5
100.000 × g pellet	30.9	69.1
cytosol	19.0	81.0
intracellular lipoproteins	51.5	48.5
extracellular lipoproteins	84.5	15.5
total cell	32.9	67.1

Distribution analysis of (^{14}C)-CCl_4 metabolites among the protein and lipid fractions. Hepatocytes were prepared from phenobarbitone-treated rats and incubated in Ham's-F-12 medium containing 250 μM (^{14}C)-CCl_4 for 1 h (37°C, 21% O_2, 5% CO_2). Lipids were extracted according to the method of Bligh and Dyer.[18] Unbound (^{14}C)-CCl_4 was removed by vacuum treatment at 45°C. The residue was dissolved in scintillation coctail and radioactivity was measured. Proteins of different cell fractions were isolated as previously described by Dianzani.[17]

RESULTS

To study the effect of CCl_4 on the secretion of lipoproteins from hepatocytes, cells were incubated with CCl_4 and radioactive VLDL and HDL were determined.

As can be seen from Fig. 1, in the controls both lipoproteins appear rather rapidly in the medium. Addition of CCl_4 (100 μM) almost completely inhibited VLDL-secretion, but only partly that of HDL. Figure 2 shows the effect of increasing CCl_4-concentrations on the VLDL- and HDL-secretion. It is evident that the export of the lipid moiety of VLDL is almost completely inhibited by high CCl_4-concentrations, while secretion of VLDL-protein is less affected. This is also the case with HDL, but here higher CCl_4-concentrations are necessary to exert this effect.

As mentioned in introduction, covalent binding of CCl_4-metabolites to cell constituents and/or lipid peroxidation and, as a consequence of this, membrane damage may be the two mechanisms leading to lowered secretion rates of lipoproteins. We have studied bioactivation of CCl_4 in terms of covalent binding of (^{14}C)-CCl_4-metabolites to the lipid fraction of cultured hepatocytes. The results are shown in Fig. 3. Binding of CCl_4-carbon to lipids occurred very rapidly and followed saturation kinetics. The binding of (^{14}C)-CCl_4 to lipids (Fig. 3) obviously correlates with the inhibition of VLDL-secretion (Fig. 1, panel A), but with HDL-secretion (Fig. 1 panel B) no close correlation can be seen. Hepatocytes from rats with increased cytochrome P_{450} level (phenobarbitone treated) have about a twofold higher capacity to produce lipid-binding CCl_4-intermediates.

Vitamin E is known to protect membrane lipids against lipid peroxidation by scavenging lipid peroxy radicals and possibly $CCl_3OO^{\cdot}$.[21] Incubation of CCl_4-treated hepatocytes with increasing concentrations of vitamin E reduced the extent of lipid

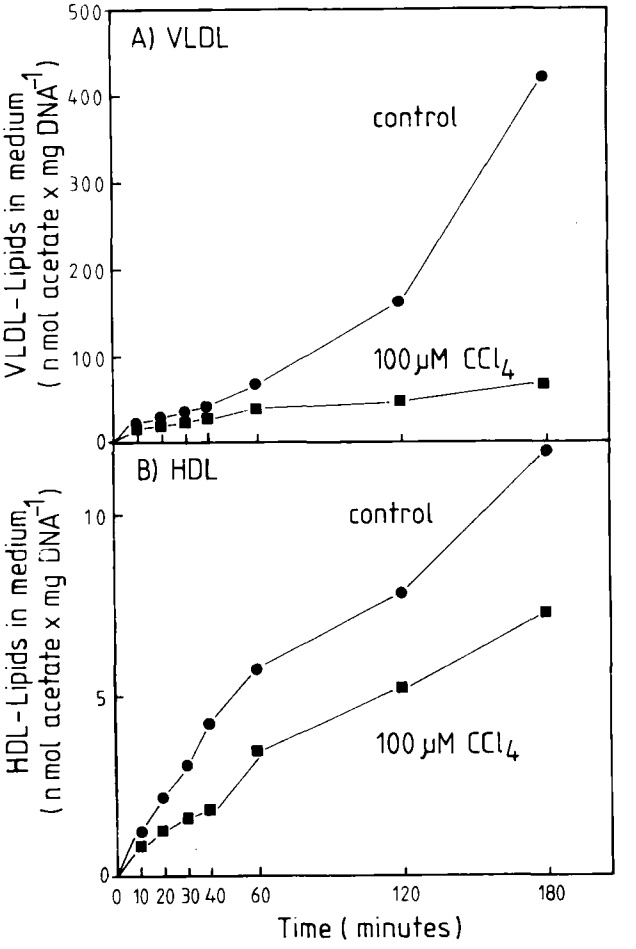

FIGURE 1 Effect of 100 μM CCl_4 on the secretion of VLDL (A) and HDL (B). Cultured hepatocytes were prelabeled in the medium containing 5 μ Ci (1-^{14}C)-acetate (58 mCi/mmol). 5 mM Na-acetate/8 ml medium. After two hours medium was changed and hepatocytes were incubated in radiocative-free medium for the indicated periods without (●) or with 100 μM CCl_4 (■). Radioactive labeled VLDL- and HDL-lipids were measured.

peroxidation to the control level (100% malondialdehyde in Fig. 4), but VLDL-secretion and covalent binding were unchanged by vitamin E.

The free radical scavenger and cytochrom P_{450} binding compound piperonyl butoxid,[22] however, abolished the CCl_4 effect: with increasing concentrations of this agent VLDL-secretion rises, while lipid peroxidation and covalent binding of (^{14}C)-CCl_4 dropped. Relatively high concentrations (up to 1 mM) of piperonyl butoxide are required to exert this effect. This was also found by other authors.[9]

These inhibition experiments show that the block of lipoprotein secretion is independent of lipid peroxidation. When malondialdehyde production is reduced to control levels (Fig. 4), VLDL-secretion is still inhibited. Piperonyl butoxid prevents covalent binding of CCl_4-metabolites and consequently VLDL-secretion increases.

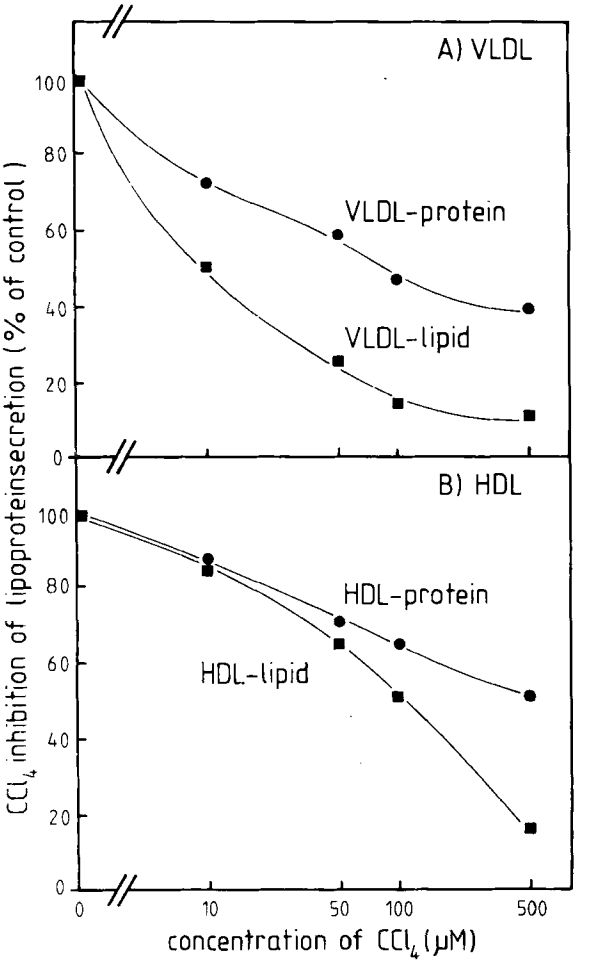

FIGURE 2 Effect of CCl_4 on protein – and lipid secretion of prelabeled hepatocytes. After prelabeling with 5 μ Ci (1-^{14}C)-acetate and 20 μCi (2,3-^{3}H)-leucine/8 ml medium for 2 hours, different amounts of CCl_4 (10–500 μM) were added to culture dishes and incubated for 60 minutes. The amounts of labeled VLDL- and HDL-lipids and VLDL- and HDL- apolipoproteins were measured in the medium.

Binding of CCl_4-intermediates to cell constituents seems to be an important event in the block of lipoprotein secretion.[23] Therefore the distribution of (^{14}C)-CCl_4 to various cell fractions was studied in more detail.

In Table I the results of this distribution analysis are summarized. It is remarkable that the radioactive lable in the lipid fraction of extracellular lipoproteins is much lower than in that of intracellular lipoproteins. This is in agreement with the data from Fig. 2, panel A, where the secretion of VLDL-lipids is shown to be more affected than the secretion of VLDL-protein.

Finally the binding of (^{14}C)-CCl_4 metabolites to various fatty acids in the lipid fraction was investigated. For this purpose lipids were extracted from (^{14}C)-CCl_4-

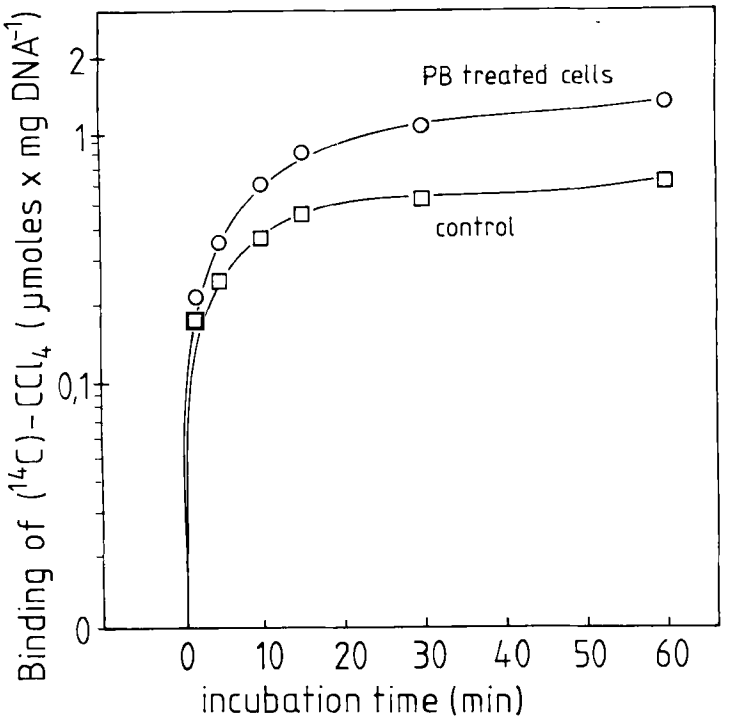

FIGURE 3 Kinetics of CCl_4 bioactivation. Covalent binding of (^{14}C)-CCl_4 to cellular lipids was used as an index. Ham's-F-12 medium + 10% foetal calf serum was equilibrated with 250 µM (^{14}C)-CCl_4 (0.14 µCi/ml medium, specific activity: 61 Ci/mmol). Cultured hepatocytes from control (□) and phenobarbitone (PB) treated rats (○) were incubated in the medium for the indicated times at: 37°C, 21% O_2, 5% CO_2. Lipids were extracted according to the method of Bligh and Dyer[18] and radioactivity was measured by scintillation spectrophotometry.

treated hepatocytes, and after esterification the fatty acid methyl esters were separated by chromatography. Radioactivity in the individual fatty acids was measured. More than 70% of the radioactive label was found to be bound to unsaturated fatty acids, and most was bound to oleic acid (C 18:1) and docosahexaenoic acid (C 22:6).

DISCUSSION

Poli *et al.*[8] and Pencil *et al.*[9] have studied the effect of CCl_4 on VLDL secretion in isolated hepatocytes. Using cultured hepatocytes, which have a number of advantages compared with isolated hepatocytes, we have confirmed the finding (Pencil *et al.*[9]) that bioactivation of CCl_4 is a prerequisite for the inhibition of VLDL secretion: Covalent binding of CCl_4-metabolites to lipids (Fig. 3) and to protein (not shown) occured very rapidly and CCl_4-induced inhibition of VLDL-secretion (Fig. 1) appears to be closely related to bioactivation and binding of CCl_4 intermediates. The CCl_4 effect is more pronounced with VLDL than with HDL, for reasons not yet known.

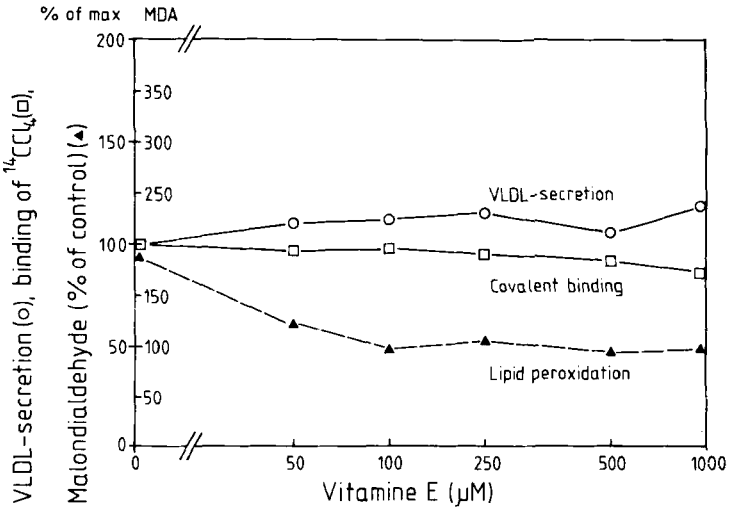

FIGURE 4 Effect of vitamin E on binding of CCl_4-carbon, lipid peroxidation and CCl_4-induced inhibition of VLDL secretion. Cultured hepatocytes were preincubated with 5 μCi (1-^{14}C)-acetate (58 mCi/mmol), 5 mM Na-acetate/8 ml medium, except that, in experiments where (^{14}C)-CCl_4 binding was measured, acetate was not labeled. After prelabeling for 2 h, vitamin E was added to the concentrations indicated. After 30 min cells were washed and Ham'sF-12 medium was added containing 250 μM CCl_4 for 30 min. Secreted and radioactive labeled lipoproteins, covalent binding of ^{14}C-CCl_4 to lipids and malondialdehyde as indicator for lipid peroxidation were measured. Means of three experiments are shown.

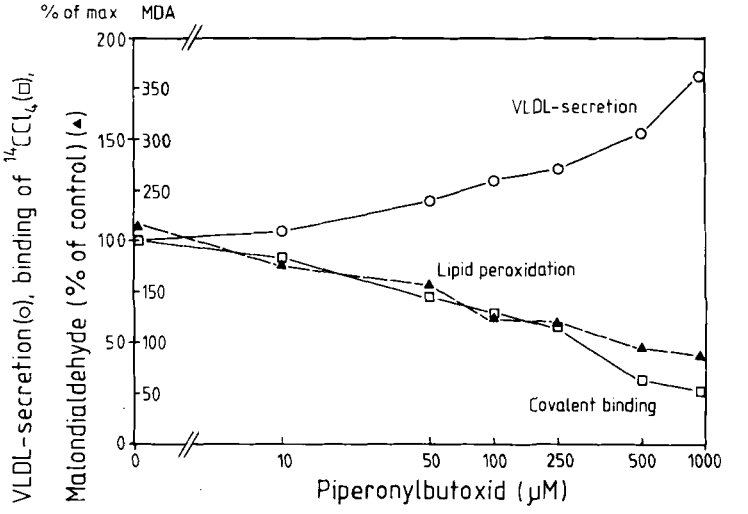

FIGURE 5 Effect of piperonyl butoxid on binding of CCl_4-carbon, lipid peroxidation and CCl_4-induced inhibition of VLDL secretion. For further details see legend to Fig. 4. Piperonyl butoxid was used instead of vitamin E.

Vitamin E, an antioxidant, could prevent lipid peroxidation as measured by malondialdehyde formation, but under these circumstances covalent binding and VLDL-secretion remained unchanged (Fig. 4). This indicates that membrane damages caused by lipid peroxidation obviously are not the main reason(s) for inhibition of VLDL secretion.

Piperonyl butoxid however, a radical scavenger used also by Pencil *et al.*[9] abolished both, lipid peroxidation and covalent binding (Fig. 5). Prevention of covalent binding of CCl_4-metabolites results in an increase in VLDL secretion. One has thus to conclude that inhibition of VLDL secretion is to a much greater extent (if not exclusively) the consequence of binding of CCl_4 metalbolites to cell constituents than of lipid peroxidation.

The analysis of the binding pattern of CCl_4-metabolites to the bulk of fatty acids supports the above findings. It is evident from Fig. 6 that more than 70% of (^{14}C)-CCl_4 radioactivity is bound to unsaturated fatty acids. It is, however, not yet possible, to decide to which cell constituents these fatty acids belong. Recently Marinari *et al.*[24] discussed that the Golgi apparatus, being involved in the transportation of lipoproteins, might be affected by CCl_4-poisoning of hepatocytes.

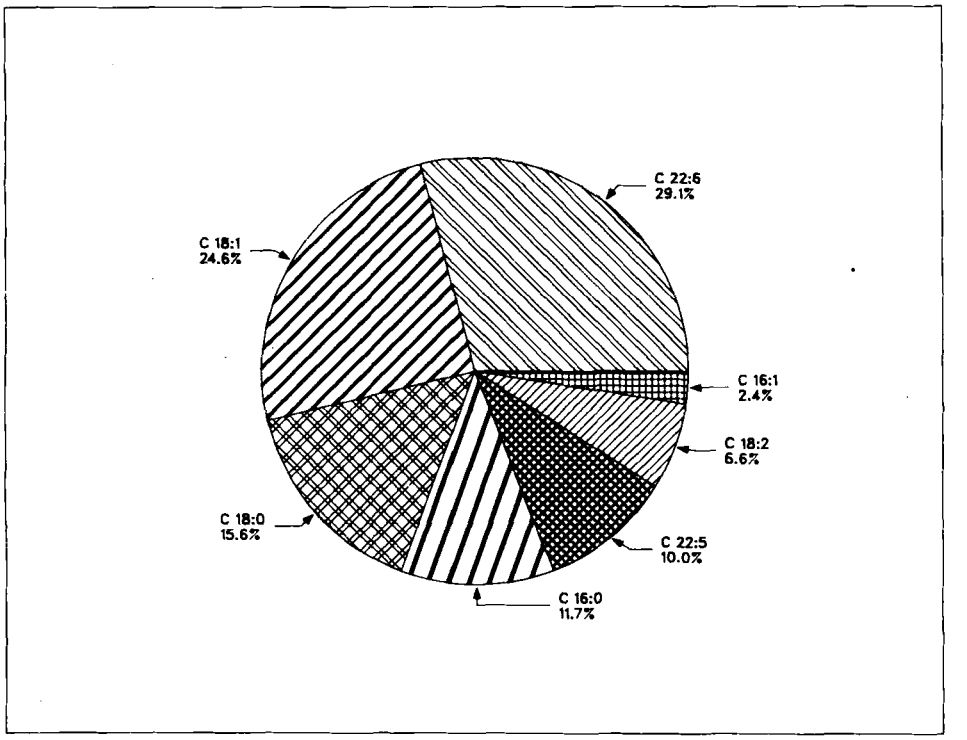

FIGURE 6 Covalent binding of CCl_4 reactive intermediates on fatty acids. The hepatocytes were incubated in Ham's-F-12 medium as described in the legend to Table I. The lipid extract of each cell fraction was subjected to transesterification according to Smith.[19] Separation and detecting of fatty acids was done as described in Materials and Methods.

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