The Role of Lipid Peroxidation in the Pathogenesis of Carbon Tetrachloride-Induced Liver Injury

DAVID H. ALPERS, MICHAEL SOLIN, AND KURT J. ISSELBACHER

Department of Medicine, Harvard Medical School, and the Medical Services (Gastrointestinal Unit), Massachusetts General Hospital, Boston, Massachusetts 02114

(Received May 16, 1968)

SUMMARY

The effect of carbon tetrachloride on lipid peroxidation and protein synthesis has been studied in rat liver. In liver microsomes, no correlation could be found *in vitro* between the degree of lipid peroxidation and protein synthesis. Stimulation of peroxidation by CCl_4, ultraviolet irradiation, or ascorbic acid, or inhibition of peroxidation by vitamin K, did not affect the rate of protein synthesis. Despite large changes in the degree of lipid peroxidation *in vitro*, ribosomes remained bound to microsomal membranes.

The administration of antioxidants *in vivo*, such as *N,N'*-diphenyl-*p*-phenylenediamine or α-tocopherol, prevented the fatty liver and necrosis produced by CCl_4, but did not reverse the inhibition of protein synthesis in liver slices or isolated polyribosomes derived from such animals. Since the formation and release of hepatic lipoproteins involve many steps, including triglyceride synthesis, interaction with apoprotein, and release or secretion of lipoprotein from the liver, it is possible that antioxidants act at some stage other than apoprotein synthesis per se.

It is concluded that the demonstration of lipid peroxidation *in vitro* may not always imply functional damage to the subcellular component so oxidized. In particular, while CCl_4 administered *in vivo* clearly decreases protein synthesis, there may not be a direct relationship between this effect and the degree of lipid peroxidation.

INTRODUCTION

Until now the pathogenesis of the induction of fatty livers by carbon tetrachloride has not been elucidated. Many investigators have searched for a single or major factor to explain the pronounced increase in hepatic fat accumulation. Hove was the first investigator to discover the pro-oxidant effect of CCl_4 (1). Later, Gallagher (2) demonstrated that when animals were first treated by injection with antioxidants, the induction of fatty livers by CCl_4 was prevented. Kalish and Di Luzio confirmed these findings and suggested that increased lipid peroxidation might be an important factor in the production of fatty livers both by CCl_4 and by ethanol (3), perhaps by damaging the lipid-containing membranes of the subcellular organelles.

Lysosomes (4) and mitochondria (5) have been shown to lose both structural integrity and function when lipid peroxidation is stimulated. Microsomes are a subcellular fraction implicated in the production of fatty livers by CCl_4, and Smuckler and Benditt have shown that protein synthesis in isolated microsomes is decreased (6). Ghoshal and Recknagel have demonstrated that CCl_4 promotes lipid peroxidation in isolated microsomes and that the activity of a microsomal enzyme, glucose 6-phosphatase, is decreased

These studies were carried out with support from The John A. Hartford Foundation, Inc., and Grants AM-01392, AM-03014, and AP-4501 from the National Institutes of Health.

during lipid peroxidation *in vitro* (7). On the other hand, we have recently shown that CCl_4 has a marked effect on the integrity of lysosomal membranes without causing an increase in lipid peroxidation (8). In addition we have shown (9) that CCl_4 inhibits synthesis and polyribosome formation in the intestinal mucosa, a tissue which does not peroxidize lipids *in vitro* (10). We have therefore attempted to determine whether or not CCl_4 diminishes protein synthesis in the liver by primarily increasing lipid peroxidation and secondarily decreasing protein synthesis. The results suggest that there is no direct relationship between the effects of CCl_4 on protein synthesis and lipid peroxidation. A preliminary report of this work has appeared (11).

EXPERIMENTAL PROCEDURES

Materials. *N,N'*-Diphenyl-*p*-phenylenediamine was obtained from Eastman Organic Chemicals. α-Tocopherol acetate (vitamin E) and CCl_4 were products of Fisher Scientific Company. Menadione sodium bisulfite, a synthetic analogue of vitamin K, was purchased from Abbott Laboratories. Thiobarbituric acid was purchased from Sigma Chemical Company. Uniformly labeled L-leucine-^{14}C was a product of New England Nuclear Corporation. Sprague-Dawley rats (CD strain) were purchased from Charles River Laboratories.

Methods. Rat livers were homogenized in a Dounce homogenizer in medium containing 0.25 M sucrose, 0.06 M Tris-HCl at pH 7.8, 0.025 M KCl, and 0.005 M $MgCl_2$. Mitochondria were removed by centrifugation at $10{,}000 \times g$ for 10 min, and microsomes were isolated at $105{,}000 \times g$ for 90 min. The microsomal pellet was washed once and resuspended in 0.1 volume of homogenizing medium. Polyribosomes were isolated by the method of Wettstein *et al.* (12). Sucrose gradients were made by the method of Britten and Roberts (13).

Lipid peroxidation was measured by the thiobarbituric acid reaction (14) with the modification of Fortney and Lynn (15) to avoid interference by sucrose. Amino acid incorporation studies were performed *in vitro* in a final volume of 3 ml containing 2 mg of microsomal protein, 2 mg of postmicrosomal supernatant protein, 750 μmoles of sucrose, 150 μmoles of Tris-HCl (pH 7.8), 6 μmoles of ATP, 0.6 μmole of GTP, 30 μmoles of creatine phosphate, 100 μg of creatine phosphokinase, 30 μmoles of $MgCl_2$, 180 μmoles of KCl, 0.12 μC of L-leucine-^{14}C (20 μC/μmole), and 0.3 μmole of each of the other 19 L-amino acids. Incubations were performed at 37° with constant shaking. Protein was precipitated and analyzed as previously described (8).

Lipid peroxidation *in vitro* was performed under ultraviolet light with a wavelength of 3600 A. The light source was 10 cm from the incubating medium, and the flasks were shaken and oxygenated during the experiments. *N,N'*-Diphenyl-*p*-phenylenediamine and α-tocopherol were administered to animals intraperitoneally according to the schedule of Di Luzio and Costales (16), except that the final dose, given 2 hr before beginning the experiment, was omitted. Control rats received corn oil intraperitoneally. Rats were fasted only overnight before being killed. CCl_4 was given via stomach tube to unanesthetized rats in the amount of 0.125 ml/100 g of body weight, mixed with an equal volume of mineral oil. Control rats received mineral oil alone.

Liver triglycerides were measured according to Van Handel and Zilversmit (17), and plasma free fatty acids by the method of Dole (18). Protein content of microsomes and ribosomes was measured by the method of Lowry *et al.* (19).

RESULTS

Studies on isolated microsomes. By the careful selection of concentrations of microsomes and supernatant fraction, it was possible to achieve linear kinetics for lipid peroxidation *in vitro* as measured by the thiobarbituric acid reaction. Figure 1 shows that peroxidation of microsomal lipids proceeds in the absence of any added prooxidants. When lipid peroxidation was increased slightly by CCl_4, or markedly by ascorbic acid, no change in the incorporation of L-leucine-^{14}C into protein was noted. In addition, when peroxidation was

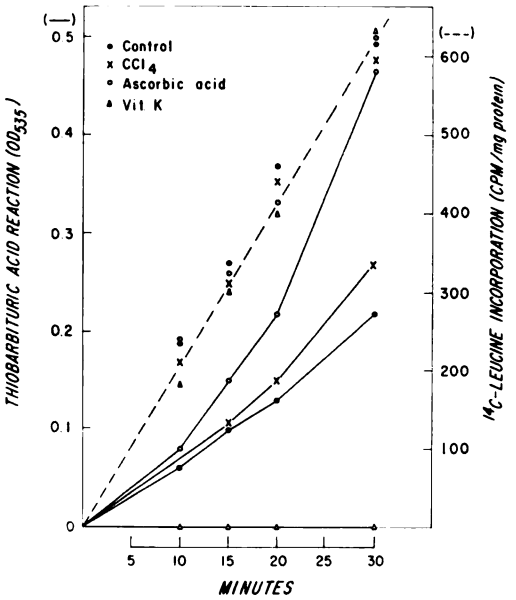

FIG. 1. *The effect of pro-oxidants and antioxidants on protein synthesis and lipid peroxidation in liver microsomes*

Microsomes were incubated as described in *Methods*. Ascorbic acid and menadione were added to a final concentration of 10^{-4} M and 3×10^{-5} M, respectively. The concentration of CCl_4 was 1 μl/ml of incubation medium. Protein synthesis and lipid peroxidation were assayed as described in *Methods*.

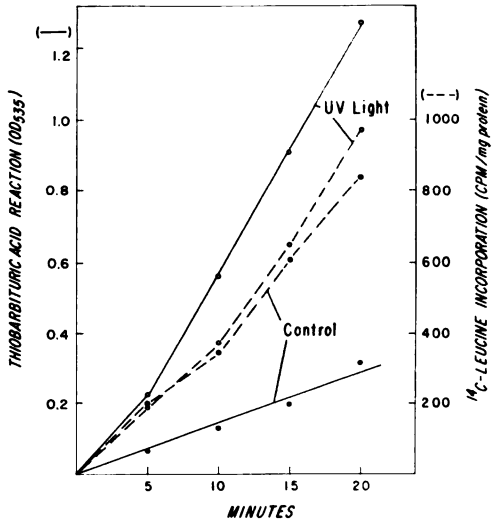

FIG. 2. *The effect of ultraviolet irradiation on protein synthesis and lipid peroxidation in liver microsomes*

Incubation and assays were performed as described in *Methods*.

completely prevented by the addition of vitamin K, leucine incorporation was unaffected (Fig. 1).

In an attempt to stimulate lipid peroxidation maximally, the microsomes were subjected to ultraviolet irradiation. Figure 2 shows that, despite a 4-fold increase in thiobarbituric acid-reacting material after ultraviolet irradiation, there was no change noted in incorporation of L-leucine-^{14}C into protein. The increase in lipid peroxidation was confirmed by extracting lipids and measuring difference in spectra at 232 mμ (20). These experiments show that marked changes in lipid peroxidation of microsomes *in vitro* do not alter the rate of protein synthesis.

The effect of CCl_4 on lipid peroxidation and protein synthesis was next investigated. Although CCl_4 increased peroxidation in microsomal lipids *in vitro* only to a small and somewhat inconsistent extent (Fig. 1), when it was combined with ultraviolet irradiation a strong pro-oxidant effect was obtained. Table 1 reveals that the addition of CCl_4 alone, in increasing amounts, caused a slight increase in lipid peroxidation of isolated microsomes, but had no effect on incorporation of leucine into protein. Ultraviolet irradiation, in the presence of CCl_4, caused a larger increase in lipid peroxidation, more than twice that seen with ultraviolet light alone. However, the amount of peroxidation was dependent on the dose of CCl_4 added. In spite of the marked increase in peroxidation, the extent of protein synthesis by the microsomes remained unchanged (Table 1).

Since microsomes are composed of membranes and ribosomes, the fact that increased lipid peroxidation did not affect protein synthesis *in vitro* suggested that the ribosomes were preserved intact during peroxidation. It seemed possible, however, that increased lipid peroxidation might disrupt the bond between ribosomes and membrane, thus affecting the anatomical integrity of the microsomes. Microsomes were therefore incubated in the presence and absence of ultraviolet irradiation and subjected to discontinuous sucrose gradient centrifugation according to Henshaw *et al.* (21). Figure 3A

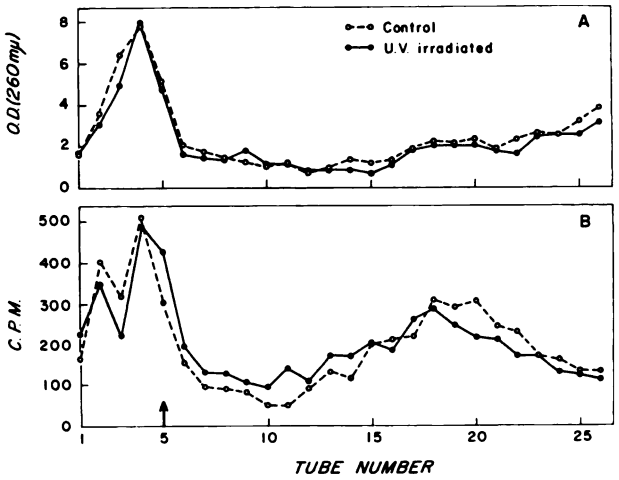

FIG. 3. *The effect of ultraviolet irradiation on sucrose density gradient patterns of liver microsomes*

Discontinuous sucrose gradients were prepared according to Henshaw *et al.* (21). One milligram of microsomal protein was added to the gradient in A and B, and both gradients were centrifuged in a Spinco SW 25-L rotor at 63,580 × *g* for 2 hr. The bottom of each tube was punctured and the effluent was collected. The tubes were analyzed at 260 mμ in A; 0.5 ml of each fraction from gradient B was used as the source of ribosomes for incorporation of L-leucine-^{14}C *in vitro* as described in *Methods*. After incubation for 30 min at 37°, the protein was precipitated with 5% trichloracetic acid. One milligram of carrier albumin was added, and the protein was isolated and assayed for radioactivity as previously described (8). The arrow at tube 5 indicates the interface between 50% and 30% sucrose.

shows the normal pattern of bound and free ribosomes on such a discontinuous sucrose gradient, with the membrane bound ribosomes at tube 5, the interface between 50 and 30% sucrose. Free ribosomes are present in very small amounts at tube 18. Ultraviolet irradiation did not cause any shift in optical density from membrane to free

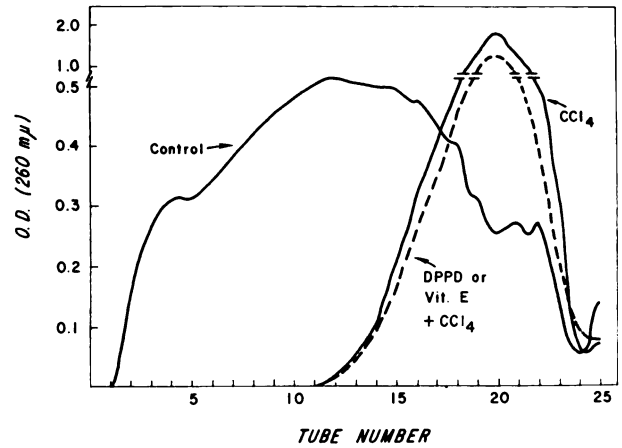

FIG. 4. *The effect of CCl_4 and antioxidants on sucrose density gradient patterns of liver polyribosomes*

N,N'-Diphenyl-*p*-phenylenediamine (DPPD), α-tocopherol, and CCl_4 were administered according to the schedule in Table 2. Animals were killed 1 hr after CCl_4 was given. Approximately 1 mg of ribosomal protein was added to a 15–30% linear sucrose gradient and centrifuged at 63,580 × *g* for 2 hr at 4°. Single ribosomes migrated to tube 21; double ribosomes, to tubes 18 and 19. The tubes were punctured, and the effluent passed through a continuous recording cell in a Gilford model 2000 spectrophotometer and analyzed at 260 mμ.

TABLE 1

Effect of CCl_4 on rat liver microsomes

Microsomes were isolated from rats fasted overnight as described in *Methods*. The CCl_4 was added to the medium and dispersed with vigorous shaking before addition of the microsomes. Lipid peroxidation was stimulated by ultraviolet light as described in *Methods*. All incubations were performed for 20 min. The figures are the mean of three separate flasks ± standard deviation.

Treatment	Amount of CCl_4 added	Thiobarbituric acid reaction	L-Leucine-^{14}C incorporation
	µl/mg protein	*OD_{535}/15 min*	*cpm/mg protein*
Carbon tetrachloride	0	0.116 ± 0.014	703 ± 34
	0.4	0.165 ± 0.013	722 ± 25
	1	0.168 ± 0.019	770 ± 47
	2	0.195 ± 0.007	701 ± 29
Carbon tetrachloride + ultraviolet irradiation	0	0.297 ± 0.016	712 ± 25
	0.4	0.475 ± 0.017	780 ± 14
	1	0.573 ± 0.017	771 ± 41
	2	0.706 ± 0.019	762 ± 16

ribosomes, suggesting that ribosomes remained attached to membrane during lipid peroxidation *in vitro*. When identical fractions from a duplicate gradient were used as the source of ribosomes for protein synthesis *in vitro*, no difference was noted in the distribution of labeled protein between control and ultraviolet-treated microsomes (Fig. 3B). In both preparations there was a large peak of protein synthesis associated with membrane-bound ribosomes, and no shift of this activity to the free ribosomes (tube 18) was noted as a result of ultraviolet light treatment. Thus, presumably the ribosomes remaining after ultraviolet irradiation are functionally unchanged, whether bound to membrane or not. No structural differences were observed when the treated and untreated microsomes were examined with the electron microscope. Thus, both the structure and function of the microsomes appeared to remain intact during extensive lipid peroxidation.

Use of antioxidants in vivo. In view of the lack of correlation *in vitro* between lipid peroxidation and protein synthesis, studies were performed *in vivo*, since much of the evidence in favor of lipid peroxidation as a mechanism for liver injury has been based on the use of antioxidants *in vivo*. Rats were treated with either α-tocopherol acetate or *N,N'*-diphenyl-*p*-phenylenediamine intraperitoneally for 48 hr prior to receiving CCl_4 (0.125 ml/100 g of body weight). Figure 4 shows that when rats were killed 1 hr after administration of CCl_4, ribosomes were found largely in the form of single and double ribosomes. This

TABLE 2

Effect of antioxidants in vivo on inhibition of protein synthesis 1 hr after CCl_4 administration

N,N'-Diphenyl-*p*-phenylenediamine (DPPD) suspended in corn oil (160 mg/ml) and α-tocopherol acetate dissolved in corn oil (25 mg/ml) were injected intraperitoneally 48 and 24 hr prior to death in doses of 60 mg and 10 mg, respectively, per 100 g of body weight. Control animals received corn oil alone. After overnight fasting, CCl_4 (0.125 ml/100 g of body weight) was given by stomach tube 1 hr before death in the groups indicated. Polyribosomes were isolated and tested for ability to incorporate leucine as described in *Methods*. Each treatment group consisted of three rats.

Prior treatment of animals	CCl_4	L-Leucine-^{14}C incorporation	Inhibition
		cpm/mg protein	%
None	0	17,050	
	+	1,810	89
DPPD	0	21,000	
	+	2,430	88
α-Tocopherol	0	18,160	
	+	2,160	88

TABLE 3

Effect of antioxidants in vivo *on CCl_4-induced liver injury*

N,N'-Diphenyl-*p*-phenylenediamine (DPPD) was administered as in Table 2. Nembutal (32 mg/100 g of body weight) was given just before the experiment, and animals were exsanguinated via the aorta. This blood was centrifuged in a tube containing heparin, and the supernatant plasma was used for free fatty acid determinations. The liver was removed, and portions were saved for triglyceride determination. The rest of the liver was homogenized, and polyribosomes were isolated and treated as in Table 2. The number of animals in each treatment group is given in parentheses.

Time of death	Treatment	Liver triglycerides	Plasma free fatty acids	L-Leucine-^{14}C incorporation	Inhibition of leucine incorporation
				cpm/mg polyribosomal	
hr		*mg/g liver*	*μEq/l*	*protein*	*%*
4	None (8)	8.4	252 ± 26	17,800	
	CCl_4 (6)	13.7	235 ± 58	9,740	45
	DPPD (6)	7.7	236 ± 59	20,720	
	DPPD + CCl_4 (6)	8.0	205 ± 16	12,750	39
24	None (4)	11.5	302 ± 43	16,280	
	CCl_4 (4)	44.6	205 ± 25	11,940	27
	DPPD (4)	10.4	305 ± 34	22,350	
	DPPD + CCl_4 (4)	18.9	230 ± 18	16,930	24

pattern was not altered by prior treatment with antioxidants. Furthermore, the prior injection with antioxidants did not reverse the inhibition of protein synthesis noted in liver polyribosomes isolated after treatment of the rats with CCl_4. Table 2 shows that protein synthesis by isolated polyribosomes was decreased to about 10% of control levels by treatment with CCl_4, and this was not significantly altered by prior treatment with either *N,N'*-diphenyl-*p*-phenylenediamine or α-tocopherol.

When animals were killed 4 and 24 hr after CCl_4 administration, lesser degrees of inhibition (25–40%) of protein synthesis were noted (Table 3), as measured by leucine incorporation. However, even this smaller inhibition was not reversed by treatment with *N,N'*-diphenyl-*p*-phenylenediamine.

Similar results were obtained when protein synthesis was measured in liver slices from the same animals. Although *N,N'*-diphenyl-*p*-phenylenediamine did not reverse the effect of CCl_4 on protein synthesis, it prevented almost completely the formation of a fatty liver. Table 3 shows that liver triglycerides were nearly normal 4 hr after CCl_4 administration in animals that had first been treated with *N,N'*-diphenyl-p-phenylenediamine. The striking increase in liver triglycerides at 24 hr was diminished 75% by prior treatment with this antioxidant. No consistent change in plasma free fatty acids was noted, suggesting that the antioxidant did not act by changing plasma free fatty acid concentrations.

DISCUSSION

Di Luzio and Costales have recently presented evidence that antioxidants given intraperitoneally prevent the induction of fatty livers by CCl_4 (16). Our results confirm this finding. In addition, however, Crafton and Di Luzio have shown that antioxidants restore the ability of the liver to release triglycerides after CCl_4 administration, and on this basis suggested that lipoprotein synthesis is restored by the antioxidants (22). The data presented above show that *N,N'*-diphenyl-*p*-phenylenediamine does not protect against the CCl_4-induced inhibition of protein synthesis, yet almost completely prevents the formation of a fatty liver. Since lipo-

proteins constitute a very small percentage of total liver protein, it is possible that lipoprotein synthesis could have been selectively protected by this antioxidant, but the evidence presented above suggests no definite relationship *in vivo* between inhibition of protein synthesis and lipid peroxidation after CCl_4 administration.

The lack of correlation between the degree of lipid peroxidation and protein synthesis in isolated microsomes is in agreement with this conclusion. The use of the thiobarbituric acid method to measure the total extent of peroxidation *in vitro* seems justified, since it correlates well with other methods of measuring peroxidation (23). Isolated cell preparations were chosen because of the known difficulty of measuring peroxidation *in vivo* (24). The lack of correlation *in vitro* between increased lipid peroxidation caused by CCl_4 and the alterations in protein synthesis can be explained in at least three ways. (a) The products of lipid peroxidation might act on other subcellular organelles (e.g., lysosomes) not present in isolated microsomal preparations, and these might then affect the microsomes in the intact cell. Thus, an effect on protein synthesis would not be apparent in isolated cellular fractions. (b) The decrease in protein synthesis after CCl_4 treatment might not be mediated via lipid peroxidation. This possibility is suggested by the fact that the breakdown of polyribosomes, seen within the first hour after CCl_4 *in vivo,* is unaffected by administration of antioxidants (Fig. 4). Since CCl_4-induced polyribosome breakdown does not occur in isolated microsomes (6), it is not possible to investigate the effect of antioxidants *in vitro* on this parameter. (c) It is possible that the protein, once formed, would be altered by products of lipid peroxidation (25). Such an effect would not be detected by the types of assays used in this study. There would then be less functional lipoprotein available for secretion of triglycerides, and a fatty liver would ensue. However, this last possibility would not explain the breakdown of polyribosomes and decrease in protein synthesis seen after CCl_4 treatment (6).

In view of the fact that protein synthesis remains depressed after feeding of CCl_4 whereas fatty liver formation is prevented by antioxidants (Table 3), it is possible that antioxidants act at sites other than microsomes or polyribosomes. After the apoprotein portion of lipoproteins is formed, it must interact with lipid to form lipoproteins, which are then transported through the endoplasmic reticulum to the Golgi apparatus and released into the bloodstream. Antioxidants might act on any one of these steps and thus prevent a fatty liver, while not affecting protein synthesis per se. In addition, it is conceivable that these compounds might also exert their effect by a mechanism not related to their antioxidant property.

Further evidence supports the lack of correlation between protein synthesis and lipid peroxidation in the production of a fatty liver. Krone (26) found that α-tocopherol given orally, although able to correct a deficiency of this vitamin, gave no protection against CCl_4-mediated liver injury. Recently, McLean has confirmed that a single oral dose of α-tocopherol given to rats before CCl_4 does not protect against formation of fatty livers (27). He has suggested that the protection afforded by intraperitoneal injection of antioxidants may be unrelated to their antioxidant properties, since oral administration of α-tocopherol is ineffective. However, it is unclear whether α-tocopherol was in fact absorbed in these latter experiments. Antioxidants have been reported to prevent the production of fatty livers by ethanol (3), but ethanol has been shown *not* to affect protein synthesis (28), and the release of lipid from fatty livers after moderate doses of ethanol is normal. Furthermore, although the presence of CCl_4 in the intestine causes inhibition of protein synthesis and breakdown of polyribosomes, lipid peroxidation is not increased by such treatment (9).

Other experiments point out the lack of direct correlation between lipid peroxidation and other cellular functions. Gram and Fouts have shown that α-tocopherol decreases microsomal lipid peroxidation but has no effect on microsomal drug metabolism (29). These authors concluded that

peroxides are not responsible for the inactivation of drug-metabolizing enzymes. Rutin, which is a more potent antioxidant than α-tocopherol, was noted by Sharma and Murti to decrease the formation of peroxides in lysosomes *in vitro*, but not to prevent the release of acid phosphatase from the lysosomes (30).

Carbon tetrachloride clearly has multiple sites of action within the cells: it inhibits the functions of microsomes (6), mitochondria (31), and lysosomes (8). The formation of a fatty liver begins after many of these cellular organelles have been affected. Thus, induction of fatty livers by CCl_4 is undoubtedly a very complex process. The results obtained by use of antioxidants *in vivo* suggest that lipid peroxidation may be one factor in producing a fatty liver. However, it is uncertain whether information obtained in whole animals, involving multiple processes, can be translated into a single biochemical lesion. Conversely, the lack of correlation in isolated cell fractions between lipid peroxidation and protein synthesis is not absolute evidence against some relationship *in vivo*.

ACKNOWLEDGMENT

We are indebted to Mrs. Katherine Porter for excellent technical assistance.

REFERENCES

1. E. L. Hove, *Arch. Biochem.* **17**, 467 (1948).
2. C. H. Gallagher, *Aust. J. Exp. Biol. Med. Sci.* **40**, 241 (1962).
3. G. H. Kalish and N. R. Di Luzio, *Science* **152**, 1390 (1966).
4. I. D. Desai, P. L. Sawant and A. L. Tappel, *Biochim. Biophys. Acta* **86**, 277 (1964).
5. P. E. Hoffsten, F. E. Hunter, Jr., J. M. Gebicki and J. Weinstein, *Biochem. Biophys. Res. Commun.* **7**, 276 (1962).
6. E. A. Smuckler and E. P. Benditt, *Biochemistry* **4**, 671 (1965).
7. A. K. Ghoshal and R. O. Recknagel, *Life Sci.* **4**, 2195 (1965).
8. D. H. Alpers and K. J. Isselbacher. *Biochim. Biophys. Acta* **137**, 33 (1967).
9. D. H. Alpers and K. J. Isselbacher, *Biochim. Biophys. Acta.* **158**, 414 (1968).
10. A. Ottolenghi and F. Bernheim, *Radiat. Res.* **12**, 37 (1960).
11. D. H. Alpers and K. J. Isselbacher, *Fed. Proc.* **26**, 326 (1967).
12. R. O. Wettstein, T. Staehelin and H. Noll, *Nature* **197**, 430 (1963).
13. R. J. Britten and R. B. Roberts, *Science* **131**, 32 (1960).
14. H. I. Kohn and M. Liversedge, *J. Pharmacol. Exp. Ther.* **82**, 292 (1944).
15. S. R. Fortney and W. S. Lynn, Jr., *Arch. Biochem. Biophys.* **104**, 241 (1964).
16. N. R. Di Luzio and F. Costales, *Exp. Mol. Pathol.* **4**, 141 (1965).
17. E. Van Handel and D. B. Zilversmit, *J. Lab. Clin. Med.* **50**, 152 (1957).
18. V. P. Dole, *J. Clin. Invest.* **35**, 150 (1956).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
20. R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15**, 132 (1966).
21. E. Henshaw, T. B. Bojarski and H. H. Hiatt, *J. Mol. Biol.* **7**, 122 (1963).
22. C. G. Crafton and N. R. Di Luzio, *Proc. Soc. Exp. Biol. Med.* **124**, 134 (1967).
23. J. G. Bieri, *Progr. Chem. Fats Other Lipids* **7**, 247 (1964).
24. R. O. Recknagel and A. K. Ghoshal, *Exp. Mol. Pathol.* **5**, 108 (1966).
25. A. L. Tappel, *Fed. Proc.* **24**, 73 (1965).
26. H. A. Krone, *Int. Z. Vitaminforsch.* **24**, 12 (1952).
27. A. E. M. McLean, *Brit. J. Exp. Pathol.* **48**, 632 (1967).
28. K. J. Isselbacher and N. J. Greenberger, *N. Engl. J. Med.* **270**, 402 (1964).
29. T. E. Gram and J. R. Fouts, *Arch. Biochem. Biophys.* **114**, 331 (1966).
30. S. K. Sharma and C. R. K. Murti, *Biochem. Pharmacol.* **15**, 2025 (1966).
31. G. S. Christie and J. D. Judah, *Proc. Roy. Soc. Ser. B, Biol. Sci.* **142**, 241 (1957).