Biochemical Pharmacology, Vol. 20, pp. 3423–3430. Pergamon Press, 1971. Printed in Great Britain

EFFECTS OF THE CCl_4–CYCLOHEXIMIDE INTERACTION ON PROTEIN SYNTHESIS AND LIPID METABOLISM IN RAT LIVER

ENRICO GRAVELA, PAOLO PANI, ARNALDO FERRARI and CLORINDA MAZZARINO
Istituto di Patologia Generale dell'Universitá di Torino, Corso Raffaello 30, Turin, Italy

(*Received* 21 *May* 1971; *accepted* 7 *July* 1971)

Abstract—A protective effect against triglyceride accumulation in liver was observed in CCl_4-poisoned rats pretreated with cycloheximide. This protection is not related to the action of cycloheximide on protein synthesis and secretion of triglyceride from liver into plasma in CCl_4-treated rats. The metabolism of CCl_4 is not affected by cycloheximide. Reduced levels of plasma free fatty acids in cycloheximide treated rats could explain the phenomenon, in spite of the behaviour of protein synthesis and triglyceride secretion.

THE ADMINISTRATION of CCl_4 to rats rapidly produces a marked inhibition in hepatic protein synthesis, associated with a disaggregation of the polyribosomes.[1] We have previously demonstrated[2,3] that antioxidants almost totally prevent these CCl_4-induced changes in polysomal structure and function, results consistent with the well-documented protective effect of antioxidants on the CCl_4-induced fatty infiltration.[4,5] These data support the hypothesis of a causal relationship between CCl_4-induced lipid peroxidation, dissociation of liver polyribosomes, inhibition of protein synthesis and fat accumulation. We have also shown[2] that cycloheximide completely prevents the CCl_4-induced dissociation of polyribosomes; this suggested the possibility that cycloheximide could in turn mitigate the CCl_4 effect on protein synthesis and fat accumulation. In this report we show the effects of the interaction of CCl_4 and cycloheximide on the behaviour of protein synthesis and lipid metabolism in rat liver.

MATERIALS AND METHODS

Male rats of the Long–Evans strain weighing 180–230 g were fasted for 18 hr before treatment, water was given *ad lib.* Cycloheximide (Sigma Chemical Co., St. Louis, Missouri) as 0·1% solution in 0·9% NaCl, was injected intraperitoneally in the amount of 1 mg/kg body wt., 10 min prior to CCl_4 administration, unless otherwise indicated. CCl_4 (Merck, Darmstadt, Germany), dissolved in mineral oil (1 : 1 v/v) was administered by stomach tube in the amount of 250 μl/100 g body wt. Triton WR 1339 (Rohm and Haas, Philadelphia), as 20% (w/v) solution in 0·9% NaCl was injected in the saphenous vein under light ether anaesthesia, in the amount of 50 mg/100 g body wt. Controls were given equal volumes of saline, mineral oil and saline respectively, in place of cycloheximide, CCl_4 and Triton WR 1339. Five min before sacrifice, 5 mg of pentobarbital/100 g body wt. was injected intraperitoneally. Blood samples were withdrawn from the abdominal aorta and transferred into tubes containing ethylenediaminetetraacetate. For the experiments on the structure and activity of ribosomes the animals were killed by decapitation.

Polyribosomal sedimentation patterns and *in vitro* incorporation of amino acids into liver microsomal protein were carried out as previously described[3] with the exception that the incubation medium for amino acid incorporation contained 20 mg equiv. microsomes and 20 mg equiv. cell sap from the same animal, and 0·1 μc of [^{14}C]l-L-leucine (The Radiochemical Centre, Amersham, England) diluted with carrier leucine in order to achieve a final concentration of 5 μM.

For the experiments of amino acid incorporation *in vivo* into liver protein rats were intraperitoneally injected with 1 ml of [^{3}H]4,5-L-lysine solution containing 10 μc (specific activity 4·6 c/m-mole; The Radiochemical Centre, Amersham, England) per 100 g body wt. After 30 min, the livers were excised, weighed and homogenized in water (1 : 20, w/v); these procedures were carried out rapidly at 0–4°. Aliquots of 2 ml of homogenate were treated with 5% trichloroacetic acid (TCA). After centrifugation the pellets were washed four times with 5% TCA; after the second wash, they were left 15 min at 90° to eliminate nucleic acids. Water and lipids were then removed by washes in ethanol, ethanol and ether, and toluene. The pellets were then suspended in 3 ml of NCS (Nuclear Chicago Corp.) diluted with toluene (1 : 1); the solution of proteins was obtained after 2 hr in a water bath at 45°. This solution was employed for radioactivity and protein determinations. Aliquots of 1 ml were taken and added to 10 ml of a toluene–PPO–POPOP mixture (Nuclear Chicago Corp.) for radioactivity measurement with a liquid scintillation system (L.S. spectrometer, model mark-I, Nuclear Chicago Corp.). Proteins were determined according to Schmukler and Yiengst,[6] with a little modification: aliquots of 0·5 ml of the NCS solution were added to 2 ml of a biuret reagent containing per 100 ml: 225 mg $CuSO_4 . 5\ H_2O$, 49 ml of absolute methanol, 1 ml of ethylene glycol and 50 ml of a 0·1 N tetramethylammonium hydroxide solution in isopropanol–methanol (Merck, Darmstadt, Germany). The reagent was prepared fresh for each set of determinations. The reaction mixture was heated 15 min at 50° in a water bath and optical density was measured at 546 nm.

Plasma and liver total lipids were extracted and purified according to the method of Folch *et al.*,[7] dried at 40° under N_2 and then redissolved in a minimum amount of chloroform. The lipids were separated on thin layers of silicic acid (Silica Gel G, Merck, Darmstadt, Germany), using as solvent *n*-heptane–isopropyl ether–glacial acetic acid (60 : 40 : 2, by vol). The plates were air dried and the lipids were identified by exposure to I_2 vapors. The different fractions were scraped from the plates and quantitatively transferred to glass-stoppered tubes. Triglycerides were eluted with chloroform and free fatty acids with 0·22% formic acid in chloroform. Colorimetric analyses were performed for triglycerides according to Van Handel and Zilversmit[8] and for free fatty acids according to Duncombe.[9]

Diene conjugation of liver microsomal lipids was assayed 30 min after giving CCl_4, according to Rao and Recknagel.[10]

RESULTS AND DISCUSSION

Effects of cycloheximide and CCl_4 upon polyribosomes and amino acid incorporation. The protective effect of cycloheximide on liver polysomes, previously demonstrated 40 min after giving CCl_4,[2] is still present 2 hr after poisoning. In CCl_4-poisoned rats pretreated with cycloheximide, the amount of polysomes is only slightly decreased, with a corresponding small increase in the monomer peak (Fig. 1). The pattern of the

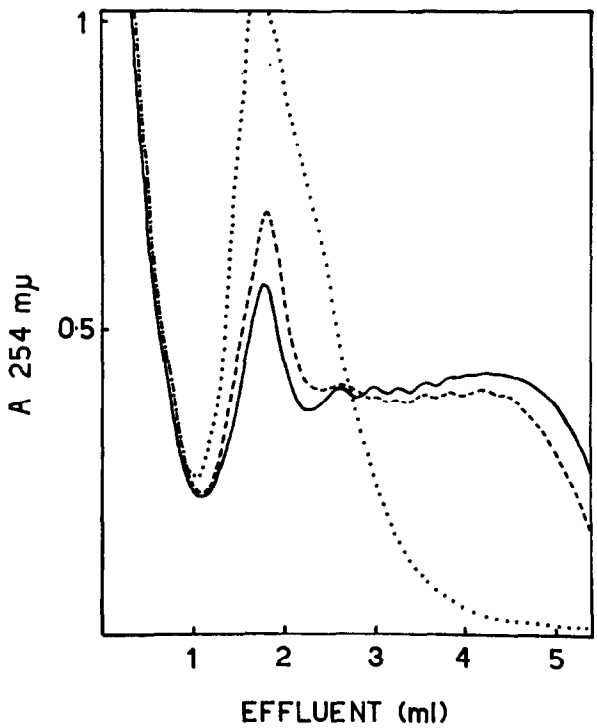

FIG. 1. Sedimentation patterns of liver ribosomes of control rats (solid line), CCl_4-poisoned rats (dotted line) and CCl_4-poisoned rats pretreated with cycloheximide (dashed line). Rats were killed 2 hr after CCl_4 poisoning. 0·2 ml of postmitochondrial supernatant, previously treated with sodium deoxycholate (1 %), were layered over 5·5 ml concave exponential gradients of 0·5–1·5 M sucrose, containing 50 mM tris-HCl buffer pH 7·8, 25 mM KCl and 5 mM $MgCl_2$, and centrifuged at 204,000 *g* for 40 min in the rotor SW 50 of the Beckman–Spinco, model HV, ultracentrifuge. The top of the gradient is to the left.

rats treated with CCl_4 alone show, on the contrary, a drastic shift from large polysomes to small polysomes and monomers. Furthermore, if the size distribution study of liver ribosomes of the CCl_4-poisoned rats is carried out at low Mg^{2+} concentration (Fig. 2), the monomer–dimer peak splits into three peaks, corresponding to the small and large ribosomal subunits and to residue monomers, in agreement with the results of Smuckler and Benditt.[1]

In vitro studies on the amino acid incorporating activity of the microsomes (Table 1) corroborated the sedimentation studies. Liver microsomes from CCl_4-poisoned rats previously treated with cycloheximide incorporate [^{14}C]leucine into protein at 80 per cent of the control value, compared to an incorporation of 40 per cent of the control by microsomes from unprotected CCl_4-treated rats. The incorporating activity of microsomes from liver of rats receiving cycloheximide alone is slightly higher than that of microsomes from untreated rats, in agreement with the results of Jondorf *et al.*[11] On the contrary, the amino acid incorporation *in vivo* into total liver protein (Table 1) appears to be similarly inhibited in rats receiving CCl_4 or cycloheximide or both the two drugs. Therefore, the protective effect of cycloheximide against the CCl_4-induced

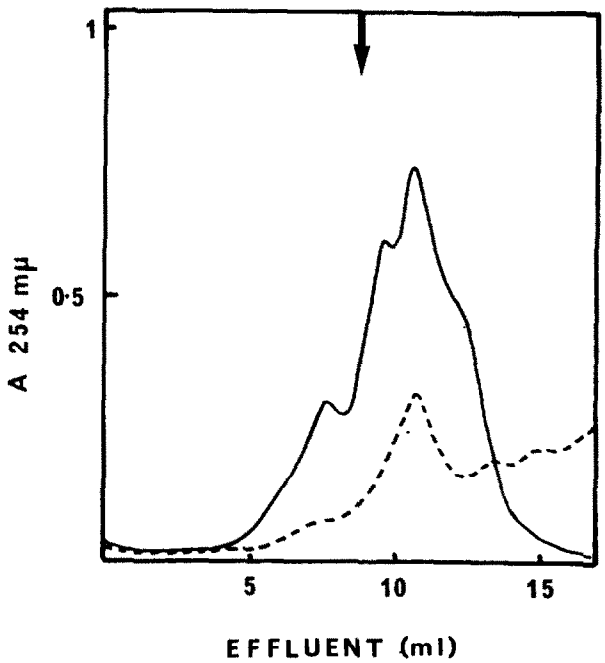

FIG. 2. Sedimentation patterns of liver ribosomes of control rats (dashed line) and CCl_4-poisoned rats (solid line). Rats were killed 2 hr after poisoning. Ribosomes were collected from postmitochondrial supernatant, previously treated with sodium deoxycholate (1%), by centrifuging it through 0·5 M sucrose, containing 50 mM tris-HCl buffer pH 7·8 and 25 mM KCl. Ribosomes were resuspended in water; samples corresponding to 8 E units were layered over 16·5 ml convex exponential gradients of 0·4–1·2 M sucrose, containing 50 mM tris-HCl buffer pH 7·8, 25 mM KCl and 0·5 mM $MgCl_2$, and centrifuged at 80,900 *g* for 13 hr in the rotor SW 27·1 of the Beckman–Spinco, model L2 65B ultracentrifuge. The arrow marks the position of 50 S *E.Coli* subunits.

TABLE 1. AMINO ACID INCORPORATION *in vitro* AND *in vivo*

Treatment*	*in vitro* [^{14}C]leucine incorporation dis./min/mg of microsomal protein†	*in vivo* [^{3}H]lysine incorporation dis./min/mg of liver protein†
None	(5) 4602 ± 430	(6) 1705 ± 129
Cycloheximide	(6) 4838 ± 890	(5) 268 ± 157
CCl_4	(6) 1801 ± 805	(6) 342 ± 136
Cycloheximide + CCl_4	(6) 3548 ± 370	(5) 306 ± 112

* The animals were sacrificed 2 hr after treatment.
† Mean ± S.D. (The number of the animals is reported in parentheses.)

polysome breakdown does not correspond to an improvement of the *in vivo* protein synthesis, as measured by amino acid incorporation.

Effects of cycloheximide and CCl_4 on lipid metabolism. In rats pretreated with cycloheximide, the degree of the CCl_4-induced hepatic steatosis was much less than observed in rats receiving CCl_4 alone (Table 2). However, in the rats receiving both CCl_4 and cycloheximide, as well as in a control group receiving cycloheximide alone, a slight increase in hepatic triglyceride was found, as compared with untreated control.

TABLE 2. EFFECT OF CYCLOHEXIMIDE AND CCl_4 ON HEPATIC TRIGLYCERIDE (TG) AND PLASMA FREE FATTY ACID (FFA) LEVELS

Treatment*	Liver TG (mg/100 g body wt.)†	Plasma FFA (mVal/1000 ml plasma)†
(a) None	(8) 24·62 ± 9	(7) 0·977 ± 0·121
(b) Cycloheximide	(8) 40·25 ± 9·1	(8) 0·745 ± 0·126
(c) CCl_4	(8) 75·87 ± 16	(7) 1·275 ± 0·152
(d) Cycloheximide + CCl_4	(11) 43·27 ± 19·9	(11) 0·841 ± 0·083
(e) CCl_4 + Cycloheximide	(8) 70·81 ± 20·4	(8) 1·040 ± 0·126

* The animals were sacrificed 5 hr after treatment; (d) cycloheximide 10 min before CCl_4; (e) cycloheximide 50 min after CCl_4.

† Mean ± S.D. (The number of the animals is reported in parentheses.)

Statistical significance of the differences, P values by *t*-test: Liver TG: a–c, b–c, b–e, a–e: <0·001. a–b, c–d: <0·005. a–d, d–e: <0·025. Plasma FFA: b–c, c–d: <0·001. a–b, a–c, c–e: <0·01.

These data indicate that cycloheximide does induce a slight accumulation of triglycerides in liver, in agreement with data recently reported by Jazcilevich and Villa-Treviño.[12] However, in spite of this effect of cycloheximide, pretreatment of rats with this drug paradoxically appears to minimize the triglyceride accumulation usually induced by CCl_4 alone. In these experiments a fifth group of rats received cycloheximide 50 min after CCl_4, at a time when polyribosome breakdown induced by CCl_4 is complete.[2] Under these conditions cycloheximide does not protect against triglyceride accumulation caused by CCl_4, suggesting that the protective effect of cycloheximide against the CCl_4-induced fat infiltration in liver does not occur when liver polysomes are already dissociated by CCl_4.

The failure of cycloheximide to produce a great fatty infiltration is in contrast to the strong inhibition of protein synthesis. It has been reported that a secondary response of the rat to CCl_4 administration is a release of epinephrine[13] that could cause a mobilization of fatty acids from the adipose tissue. We considered, therefore, the possibility that this drug might prevent lipid accumulation in liver by decreasing the supply of fatty acids from adipose tissue via blood plasma. To test this hypothesis the levels of plasma free fatty acids were measured at the same time in which liver triglycerides were determined. As reported in Table 2 plasma free fatty acids levels were significantly affected by cycloheximide and CCl_4. In rats receiving cycloheximide alone plasma free fatty acids levels appeared decreased, as compared with untreated controls. In rats receiving cycloheximide prior or after CCl_4 plasma free fatty acids levels appeared decreased, as compared with controls receiving CCl_4 alone. Analysis of covariance in the groups of animals receiving CCl_4 and (or) cycloheximide indicates that the significance of the regression between plasma free fatty acids and liver triglycerides is very high ($P < 0{\cdot}02$).

We considered two additional hypotheses: (a) cycloheximide inhibits the metabolism of CCl_4; (b) cycloheximide increases triglyceride secretion from liver into plasma in CCl_4-poisoned rats, in spite of the inhibition of protein synthesis.

Figure 3 shows the diene conjugation of microsomal lipids in the liver of rats treated with CCl_4, reported as one of the first changes induced by this poison.[10] The peroxidative alteration of microsomal lipids is evidenced by the u.v. difference spectrum

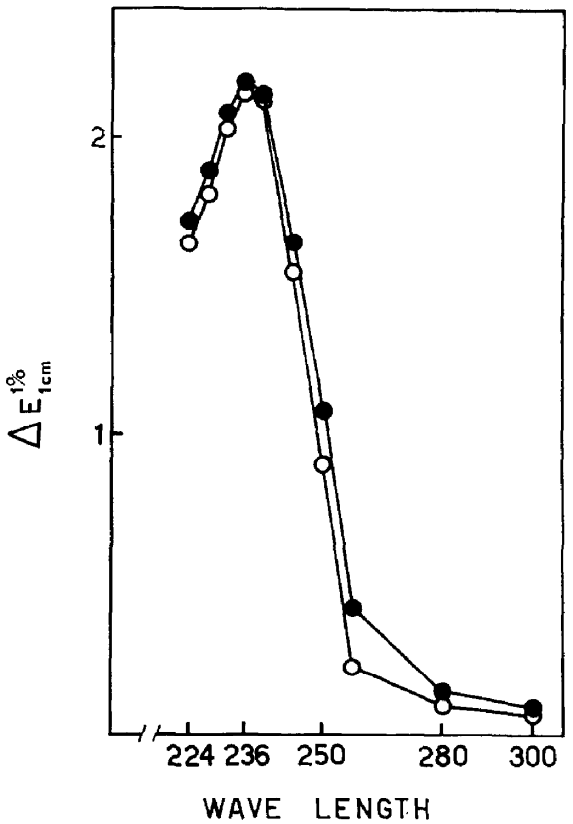

FIG. 3. Diene conjugation absorption in rat liver microsomal lipids. —○— CCl_4-poisoned rats, —●— CCl_4-poisoned rats pretreated with cycloheximide. Difference spectrum between treated and untreated animals (mean of five for group).

between intoxicated rats and control rats. Cycloheximide, administered prior to CCl_4-dosing, does not alter the extent of double bond shifting in unsaturated lipids. This finding is consistent with the previous data on the behaviour of CCl_4-stimulated production of malonyldialdehyde by liver homogenates, which is not affected by the presence of cycloheximide,[2] and seems to rule out the first hypothesis (a).

In order to study the ability of the liver to secrete triglycerides into the plasma, Triton WR 1339 was given to the animals. This nonionic detergent is known to cause a block in the exit of lipids from the plasma compartment,[14,15] due to physicochemical alterations of plasma lipoproteins.[16] From the levels of Triton hyperlipidemia the rate of liver secretion can be assessed. In some experimental models of fatty liver, tritonized rats showed a marked decrease in hypertriglyceridemia,[17–19] indicating an inhibition in triglyceride secretion from liver into the plasma. As reported in Table 3, we found a significant decrease in plasma triglyceride concentration in tritonized rats treated with cycloheximide. Moreover, tritonized rats treated with CCl_4 and cycloheximide showed no increase in the levels of plasma triglycerides, but it appears that the interaction of the two drugs have depressed even further these levels. When we compared the two drugs separately, we saw that cycloheximide alone inhibited triglyceride secretion even more than CCl_4.

TABLE 3. EFFECT OF CYCLOHEXIMIDE AND CCl_4 ON THE TRITON-INDUCED HYPERTRIGLYCERIDEMIA

Treatment*	Plasma TG (mg/100 ml plasma)†
(a) Triton	(4) 164·57 ± 27·75
(b) Triton + cycloheximide	(4) 64·45 ± 12·88
(c) Triton + CCl_4	(4) 98·32 ± 18·49
(d) Triton + cycloheximide + CCl_4	(4) 51·28 ± 6·55

* With all treatments blood was withdrawn 90 min after Triton; (b) cycloheximide 30 min before Triton; (c) CCl_4 30 min before Triton; (d) cycloheximide 30 min before Triton and CCl_4 10 min after cycloheximide.

† Mean ± S.D. (The number of the animals is reported in parentheses.) Statistical significance of the differences, P values by *t*-test: a–b, a–d: <0·001. c–d: <0·005. a–c: <0·01. b–c: <0·05.

Cycloheximide, blocking protein synthesis, reduces available lipoproteins to secrete triglycerides. However, an extensive lipid accumulation was not seen in liver, at least as compared with the values observed in CCl_4 intoxication. The decreased levels of plasma free fatty acids in cycloheximide-treated rats could account for this phenomenon. In contrast, in CCl_4 intoxication there is an increased supply of fatty acids from the adipose tissue, which cannot be secreted from the liver because of the marked inhibition in protein synthesis.

Our conclusions are that the apparent ameliorating effect of cycloheximide upon the CCl_4-induced fatty liver is not due to a modification of the direct mechanism of CCl_4 action in the cell, but rather to a modification of some of the secondary responses of the animal to this poisoning injury; in this case a depression of the CCl_4-induced rise in plasma free fatty acids. The theoretical possibility exists, however, that cycloheximide can also act at other levels, i.e. on fatty acids penetration and transport in the cell and on triglyceride synthesis.

Acknowledgements—This work was aided by a grant from the Consiglio Nazionale delle Ricerche, Roma. We thank the technical assistance of the students M. T. Caselle and G. Pastore.

REFERENCES

1. E. A. SMUCKLER and E. P. BENDITT, *Biochemistry* **4**, 671 (1965).
2. E. GRAVELA and M. U. DIANZANI, *FEBS Letters* **9**, 93 (1970).
3. E. GRAVELA, L. GABRIEL and G. UGAZIO, *Biochem. Pharmac.* **20**, 2065 (1971).
4. G. UGAZIO and M. V. TORRIELLI, *Biochem. Pharmac.* **18**, 2271 (1969).
5. M. U. DIANZANI and G. UGAZIO, Commun. 2nd Intl. Symposium of Biochemical Pathology, Oxford, 13 July, 1969.
6. M. SCHMUKLER and M. J. YIENGST, *Analyt. Biochem.* **25**, 406 (1968).
7. J. FOLCH, M. LESS and G. H. SLOANE STANLEY, *J. biol. Chem.* **226**, 497 (1957).
8. E. VAN HANDEL and D. B. ZILVERSMIT, *J. lab. clin. Med.* **50**, 152 (1957).
9. W. G. DUNCOMBE, *Clin. chim. Acta* **9**, 122 (1964).
10. K. S. RAO and R. O. RECKNAGEL, *Exp. Mol. Path.* **9**, 271 (1968).
11. W. R. JONDORF, D. C. SIMON and M. AVNIMELECH, *Biochem. biophys. Res. Commun.* **22**, 644 (1966).
12. S. JAZCILEVICH and S. VILLA TREVIÑO, *Lab. Invest.* **23**, 590 (1970).
13. D. RUBINSTEIN, *Am. J. Physiol.* **203**, 1033 (1962).
14. A. KELLNER, *Am. J. Pathol.* **26**, 732 (1950).

15. M. FRIEDMAN and S. O. BYERS, *J. exp. Med.* **97**, 117 (1953).
16. A. SCANU and P. ORIENTE, *J. exp. Med.* **113**, 735 (1961).
17. R. O. RECKNAGEL, B. LOMBARDI and M. C. SCHOTZ, *Proc. Soc. exp. Biol. Med.* **104**, 608 (1960).
18. F. F. SCHLUNK and B. LOMBARDI, *Lab. Invest.* **17**, 299 (1967).
19. B. LOMBARDI, P. PANI and F. F. SCHLUNK, *J. Lipid. Res.* **9**, 437 (1968).