Biochemical Pharmacology, Vol. 19, pp. 1409–1417. Pergamon Press. 1970. Printed in Great Britain

A CRITICAL EXPERIMENTAL CONTRIBUTION CONCERNING THE VALUE OF CCl_4-INTOXICATED LIVER IN METABOLIC STUDIES*

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(*Received* 8 *August* 1969; *accepted* 18 *October* 1969)

Abstract—Experiments were performed with adrenalectomized, cortisol treated rats to examine the effect of CCl_4 intoxicated liver in metabolic studies, on the mechanism of hepatic steatosis. The results exclude any specificity of CCl_4 action. Using the model of liver tryptophan oxygenase induction after cortisol injection no reduction of protein biosynthesis and no disarrangement of endoplasmic reticulum could be seen without a concomitant decrease of the ATP/ADP ratio, glycogen storage and damage of mitochondrial structure, even if lowest doses were applied. On the other hand even lethal amounts of CCl_4 do not depress protein biosynthesis and ATP/ADP ratio below a relatively high minimal level. This may be caused by the fact that the dose dependent intoxication proceeds from the centre of the liver lobules to the periphery, as indicated by the histological study of stained glycogen granules. Thus CCl_4 intoxication appears to be an unspecific process, leading to the conclusion that CCl_4 poisoned liver should not be used as a model for studies on regulation of liver metabolism.

IT HAS been known for a long while that CCl_4, a strong liver poison, induces fatty liver[1, 2] by enhancing triglyceride deposition.[3, 4] In opposition to other suggestions[5–10] Recknagel and Lombardi[11] put forward the thesis that in the early state of intoxication, CCl_4 damages the endoplasmic reticulum while (up to 20 hr) mitochondrial functions remain unaffected. Since the endoplasmic reticulum was known to be involved in protein biosynthesis, they suggested the disturbance of protein synthesis to be the real cause of CCl_4-induced fatty liver. In fact, the inhibition of protein biosynthesis in the liver has been demonstrated by the reduction of (i) ^{14}C-glycin incorporation into plasma albumin and fibrinogen,[12] (ii) ^{14}C-glycin incorporation into protein fractions of liver microsomes, mitochondria and cytoplasm[13] and (iii) ^{14}C-leucin incorporation into total liver protein.[14] The connection between protein biosynthesis and liver triglyceride deposition becomes evident from the fact that liver triglycerides are secreted into the plasma as lipoproteins.[15–17] Thus in CCl_4-intoxicated rats the incorporation of ^{14}C-leucin into the protein moiety of lipoproteins,[14] the amount of lipoproteins in the blood[18] and the incorporation of ^{14}C-palmitate into serum lipoproteins[19] is diminished. Simultaneously the protein moiety of the diminished lipoproteins is overloaded with triglycerides.[18] The mechanism described has been ascertained in the isolated perfused rat liver.[20] Thus Recknagel's hypothesis has encouraged several

* The skilful technical assistance of Miss M. Seemann is gratefully acknowledged. This investigation was supported by a grant of the Deutsche Forschungsgemeinschaft.

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investigators to use CCl_4-induced fatty liver as a model to study the mechanisms of hepatic steatosis.

Following the same concept we originally intended to examine protein biosynthesis in CCl_4-intoxicated liver, using a more sensitive and specific system: the induction of liver tryptophan oxygenase by cortisol.[21, 22] ^{14}C-amino acid incorporation into liver protein fractions is not a very satisfactory method of protein biosynthesis measurement because of the high number of liver proteins with different turnover rates and because of its limited reproducibility. Therefore it cannot be used to indicate the real toxicity of CCl_4.

The unexpected results of our experiments demonstrated that even slight and early CCl_4 intoxication is a much more complex metabolic disturbance than postulated by Recknagel's hypothesis.

MATERIALS AND METHODS

Male Wistar rats, 200–250 g, maintained on laboratory standard diet (Altromin-Company, Lage/Lippe, Germany) were used. Adrenalectomy was performed during ether anaesthesia 3 days before the experiments and drinking water was replaced by 0·9% NaCl solution. After 16 hr starvation a certain number of rats was injected i.p. with hydrocortisone acetate (Hoechst AG, Frankfurt/Main-Hoechst, Germany), 5 mg/150 g body weight. Controls received 0·9% NaCl solution instead of hormone. One hr later animals were slightly anaesthetized with ether and CCl_4, mixed with paraffinum liquid (1:1, v/v) was applied by stomach tube. The rats were decapitated after 4 hr.

Biochemical analysis

Tryptophan oxygenase and metabolites were measured as follows:

Tryptophan oxygenase: in the whole homogenate according to Civen and Knox.[23]

Glycogen: enzymatically as glucose, after decomposition in 4 N KOH for 20 min at 100°, followed by hydrolysis with 2 N HCl for 45 min at 100°, by the method of Slein.[24]

Lipids were extracted with chloroform–methanol (1:1, v/v) and purified according to Folch *et al.*[25]

Phosphatides: as inorganic phosphate after oxidation of an evaporated aliquot of the total lipid extract with a mixture of 65% HNO_3–70% $HClO_4$, 1:1 (v/v) by the method of Fiske and Subbarow.[26]

Triglycerides: enzymatically as glyceride glycerol according to Kreutz,[27] after silicic acid column chromatography and alkaline hydrolysis of another aliquot.

ATP and ADP: enzymatically according to Adam[28] from an acid extract of liver, suddenly frozen between the branches of a freeze stop clamp, which was precooled in liquid nitrogen.[29]

Blood sugar: enzymatically in whole blood, using the glucose oxidase method.[30]

Free fatty acids were extracted from the serum according to Trout *et al.*,[31] purified chromatographically,[32] esterified to the methylesters[33] and analyzed by quantitative gas chromatography.

Histology

For *light microscopy* liver material was fixed in Gendre's fluid,[34] dehydrated and embedded in paraffinum. Sections were cut 10 μ and stained with periodic acid Schiff

(PAS). For *electron microscopy* liver material was fixed in glutaraldehyde (2·5g%), followed by OsO_4 (1g%), buffered according to Dalton, embedded in VESTOPAL W or EPON 812 and stained with lead citrate according to Reynolds[35] or uranyl acetate.

RESULTS

Protein synthesis

In the liver of adrenalectomized rats injected with 5 mg cortisol/150 g body weight i.p. the amount of tryptophan oxygenase induction was enhanced 10-fold within 5 hr (Table 1). This rapid and excessive protein synthesis was inhibited by CCl_4. Even the smallest doses applied (0·125 ml/100 g), reduced the cortisol-induced tryptophan oxygenase augmentation by more than 60 per cent (Table 1). A further enhancement of the CCl_4 dose depressed the induction to a final value of about 25 per cent of the controls. This level cannot be reduced essentially by higher doses, even if the animals are pretreated with CCl_4 1 hr prior to glucocorticoid injection (Table 2). Our results demonstrate that in adrenalectomized, cortisol-substituted rats the protein synthesis is severely disturbed even by a dose very much lower than that usually recommended in the literature.

Triglycerides, phosphatides and free fatty acids

In our experimental conditions we found a liver triglyceride accumulation, dependent on the CCl_4 dose, from 4·46 μmoles to maximal 16·25 μmoles/g liver within 4 hr after CCl_4 application (Table 1). This rate of deposition is about half of that reported by Lombardi and Ugazio[18] for normal rats. Even in adrenalectomized, non-substituted animals liver fat was doubled by CCl_4 (Table 1). As described by others the level of liver phosphatides remained constant under all experimental conditions used (Table 1). Serum free fatty acid level was not significantly influenced by CCl_4 intoxication (Table 1).

Gluconeogenesis

Injection of 5 mg cortisol/150 g body weight was followed by forced gluconeogenesis apparent from glycogen deposition of 41·4 μmoles of glucosyl units/g liver as well as from blood sugar elevation up to 95mg% within 5 hr (Table 1). The rapid glycogen deposition was inhibited by even the smallest CCl_4 dose used (Table 1). But obviously CCl_4 did not impair gluconeogenesis. For even if glycogen deposition was greatly reduced blood sugar level continued to rise (Table 1).

ATP/ADP ratio

Similar to the tryptophan oxygenase activity and glycogen synthesis ATP, too, was sensible against increasing amounts of CCl_4. Even our smallest dose (0·125 ml/100 g) caused a significant decrease of the ATP/ADP ratio in the rat liver 4 hr later, reaching a minimal value of 2·3 after application of 0·5 ml/100 g (Fig. 1), an amount often chosen by investigators. These results indicate an early and severe disturbance of liver cell energy metabolism. They are completed by the concomitant changes of the mitochondrial structures, seen in the electron microscopic pictures.

Microscopy of glycogen storage

Using glycogen staining with periodic acid Schiff a decrease of liver glycogen

TABLE 1. DOSE-DEPENDENT EFFECT OF CCl_4 ON LIVER TRYPTOPHAN OXYGENASE INDUCTION AND SEVERAL LIVER AND SERUM METABOLITES IN ADRENALECTOMIZED, CORTISOL-SUBSTITUTED RATS

Cortisol (mg/150 g body weight)	CCl_4 (ml/100 g body weight)	Tryptophan oxygenase [U]*	Triglycerides (μmoles Glyc.Glycerol)	Phosphatides (μmoles PO_4)	Serum FFA (μmoles/ml)	Glycogen (μmoles glycosyl-units)	Blood sugar (mg/100 ml serum)
none	none	3·82 ± 0·63 (6)	7·93 ± 0·70 (12)	48·82 ± 1·76 (5)	0·28 ± 0·03 (7)	0·3 ± 0·1 (5)	69 ± 8 (5)
none	0·25	2·63 ± 0·68 (6)	14·10 ± 2·82 (4)	43·24 ± 1·88 (4)	0·24 ± 0·02 (10)	0·3 ± 0·1 (5)	54 ± 8 (5)
5·0	none	35·48 ± 3·73 (12)	4·46 ± 0·50 (19)	43·46 ± 2·35 (12)	0·29 ± 0·04 (8)	41·7 ± 8·5 (8)	95 ± 2 (13)
5·0	0·125	15·57 ± 2·32 (5)	12·99 ± 2·66 (5)	37·77 ± 3·17 (5)	0·23 ± 0·02 (5)	14·2 ± 3·1 (5)	104 ± 3 (10)
5·0	0·25	14·03 ± 2·29 (10)	16·25 ± 1·33 (12)	41·72 ± 2·88 (11)	0·30 ± 0·02 (10)	7·5 ± 0·8 (8)	108 ± 4 (10)
5·0	0·50	9·51 ± 1·80 (3)	15·39 ± 3·62 (5)	43·28 ± 1·20 (2)	0·33 ± 0·03 (6)	2·1 ± 0·2 (7)	113 ± 2 (9)
5·0	0·75	12·21 ± 1·92 (4)	11·44 ± 4·39 (5)	39·74 ± 2·45 (4)	0·29 ± 0·02 (7)	0·3 ± 0·2 (7)	107 ± 2 (12)
5·0	1·00	—	—	—	0·29 ± 0·01 (3)	1·3 ± 0·2 (3)	98 ± 3 (3)

Animals were injected with cortisol i.p. at zero time, received CCl_4/paraffin. liquid 1:1 (v/v) 1 hr later by stomach tube, and were sacrificed 4 hr later. The results are given as means ± S.D. per g liver wet weight for the numbers of the experiments in parentheses.
* μmoles kynurenin per hour at 37°.

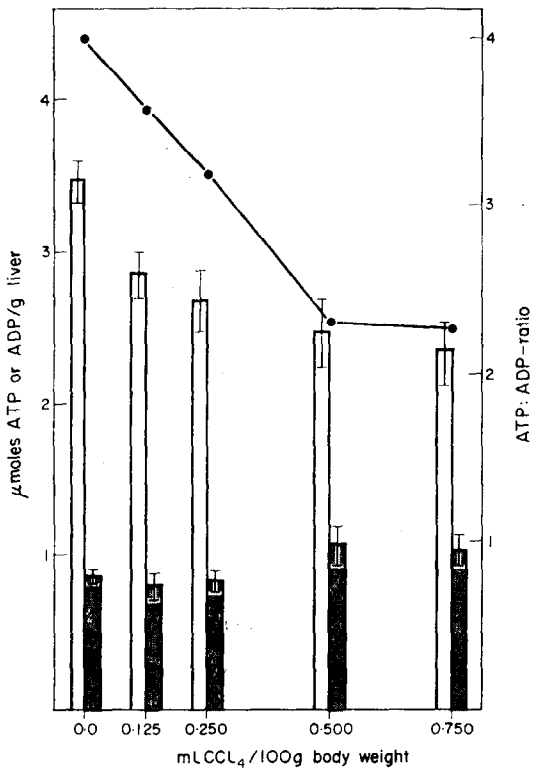

FIG. 1. Dose-dependent decrease of ATP and ATP/ADP ratio in CCl_4 intoxicated, adrenalectomized, cortisol-substituted rats. For experimental conditions see text of Table 1. Number of experiments per group: 7. □ = ATP, ■ = ADP.

content with increasing doses of CCl_4 (Fig. 2) was observed. This decrease was not the consequence of liver damage in the whole lobule. With our lowest dose glycogen depletion began in the center, whereas in the periphery polysaccharide content seemed to be unaffected. Increasing the doses glycogen depletion proceeded from the center to the periphery (Fig. 2b). Thus the analytical estimated glycogen content of the CCl_4-poisoned liver represents the sum of completely depleted and nearly unaffected areas of lobules.

Ultrastructure of the liver cells

Even a CCl_4 dose as low as 0·125 ml/100 g caused damage to the mitochondrial cristae, shrinking or lysis of mitochondria and an enlargement of ergastoplasm and its denuded membranes (Fig. 3a). A dose of 0·50 ml CCl_4/100 g increased the damage of all membrane systems, including that of the nucleus. On the other hand even after 0·75 ml CCl_4/100 g there were cells, in which ergastoplasm was unaffected (Fig. 3b).

DISCUSSION

At first sight our results seem to confirm the thesis of Recknagel and Lombardi[11] that early CCl_4 intoxication of the liver is caused by a damage of endoplasmatic reticulum, followed by impaired protein biosynthesis and, thereby, hepatic steatosis.

They demonstrate decreased tryptophan oxygenase induction even with lowest CCl_4 doses. From Fig. 4 the correlation between protein biosynthesis and hepatic steatosis is evident. This corresponds with the early disarrangement of endoplasmatic reticulum, which is obvious from the electron microscopic pictures.

Nevertheless we cannot agree to the thesis that CCl_4 intoxication, even in the early states, is a suitable model to study fat accumulation caused by diminished lipoprotein

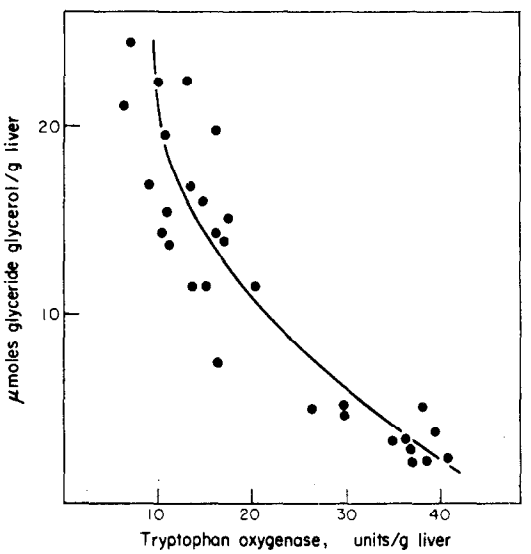

FIG. 4. Correlation between cortisol-induced tryptophan oxygenase induction and liver triglyceride accumulation. Each point represents one rat. For experimental conditions see text of Table 1.

synthesis. This thesis postulates markedly different sensitivity of cell organelles in the sequence: microsomes–mitochondria–nuclei. But in our experiments we could never demonstrate, even after application of very small doses, any impairment of tryptophan oxygenase induction without concomitant lesions of mitochondria (Fig. 3). The electron microscopic pictures correspond with a continuous decrease of the ATP/ADP ratio Since now the reduction of ATP has not been observed as early as 4–15 hr after CCl_4-application to normal rats.[36-38] However, our results are in agreement with some other communications. For instance Artizzu and Dianzani[39] reported a decrease of the P/O ratio and a release of respiratory control in normal and adrenalectomized rats as early as 2 hr after feeding of 0·25–0·50 ml CCl_4/100 g. Early mitochondrial changes have also been noted using electron microscopy by Bassi[40] and Oberling and Rouillet,[41] who administered CCl_4 in a dose comparable to our smallest one. Furthermore it has been found that the liver concentration of Ca^{2+} which is concerned with mitochondrial swelling, was doubled as early as 2–4 hr after CCl_4 feeding.[42, 43] Finally, Kröner and Staib[36] have demonstrated, that as early as 2 hr after i.p. injection of the small amount of 0·10 ml CCl_4/100 g, malate dehydrogenase and glutamate dehydrogenase, both mitochondrial enzymes, have considerably increased in serum, indicating mitochondrial lesions.

A synopsis of our results with those reported so far by others favours the conclusion

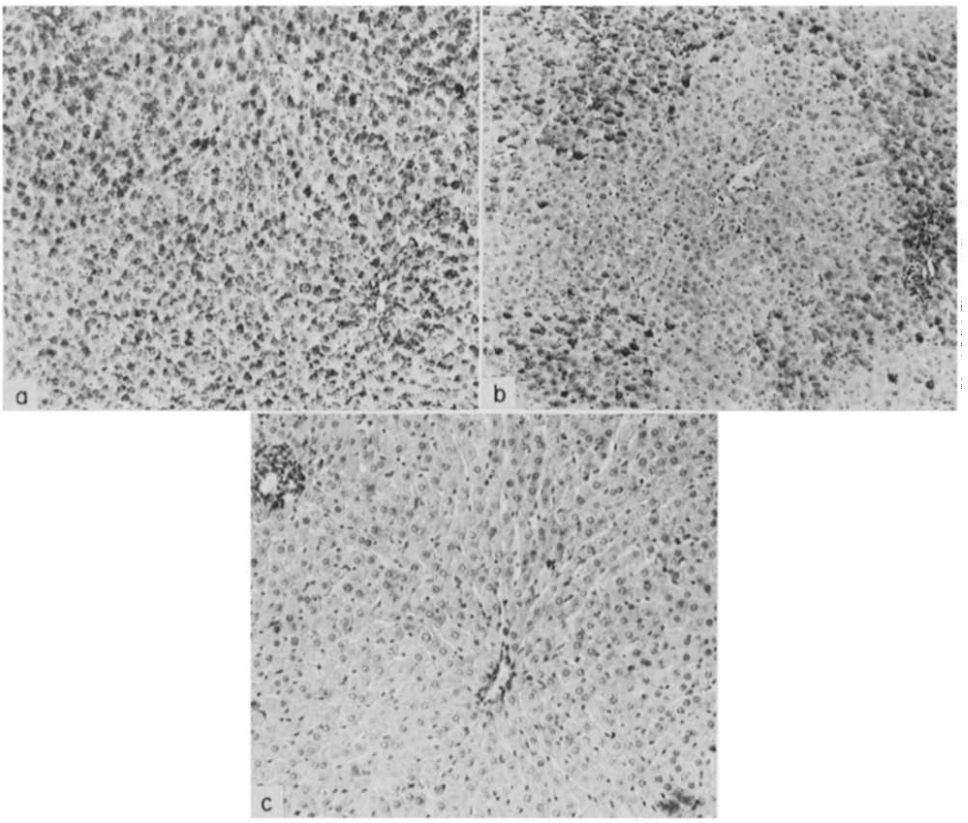

FIG. 2. Dose-dependent decrease of stained glycogen granules (dark spots) in liver lobules of CCl_4 intoxicated, adrenalectomized, cortisol-substituted rats. For experimental conditions see text of Table 1. (a) Normal liver lobule. (b) CCl_4 dose: 0·50 ml/100 g body weight. The center of the lobule is completely denuded of glycogen granules. (c) CCl_4 dose: 1·00 ml/100 g body weight. No glycogen granules at all.

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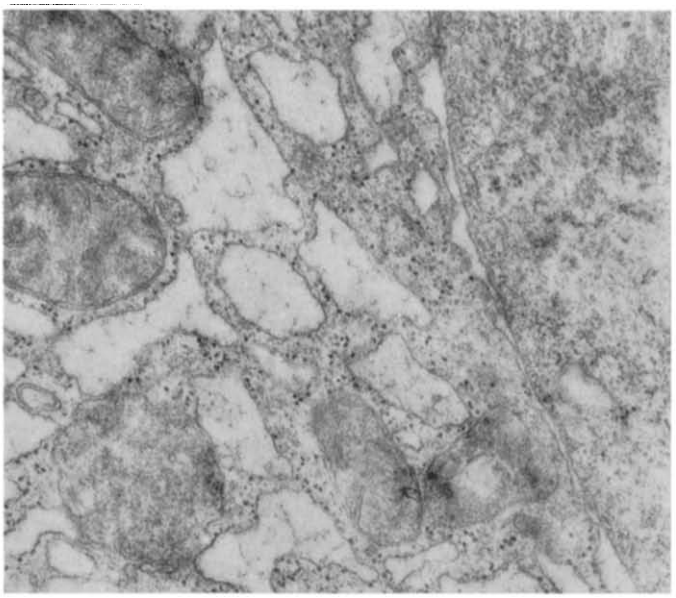

FIG. 3a

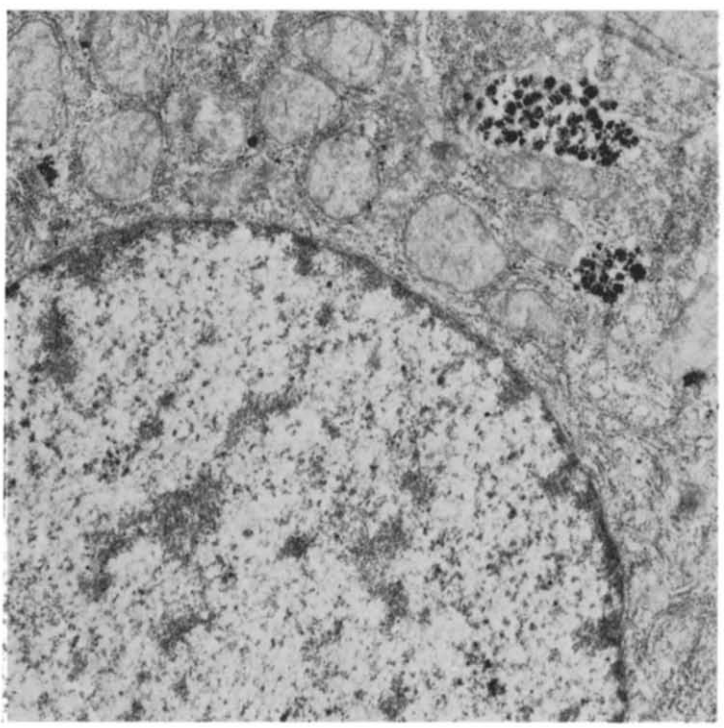

FIG. 3b

FIG. 3. Dose-dependent ultrastructural changes in liver cells of CCl_4-intoxicated, adrenalectomized, cortisol-substituted rats. For experimental conditions see text of Table 1. (a) CCl_4 dose: 0·125 ml/100 g body weight. Disarrangement of ergastoplasma, denuded membranes of endoplasmatic reticulum; shrinking and lysis of mitochondria. Enlargement: × 28,800. (b) CCl_4 doses: 0·75 ml/100 g body weight. Unaffected ergastoplasma. Enlargement: × 10,320.

that CCl_4, even in the smallest amounts, not only disarranges the endoplasmic reticulum but also damages mitochondria and thus the energy metabolism of the liver cell. Therefore it is unlikely that the damage to the endoplasmic reticulum is the mere cause of protein synthesis inhibition in CCl_4-intoxicated liver. It is highly probable that a decrease of the ATP/ADP ratio down to about 50 per cent of control values may impair protein synthesis, too, independent from other lesions. On the other hand it is worth noticing that even under severe intoxication there are cells in which the endoplasmic reticulum seems to be unaffected: This is indicated by the fact that even the highest doses of CCl_4 do not depress the tryptophan oxygenase induction to zero (Tables 1 and 2) and corresponds with the electron microscopic picture (Fig. 3b).

TABLE 2. ACTION OF CCl_4 ON LIVER TRYPTOPHAN OXYGENASE INDUCTION IN ADRENALECTOMIZED, CORTISOL-SUBSTITUTED RATS

Treatment	Time of drug action (hr)	− CCl_4	+ CCl_4	
		+ cortisol	− cortisol	+ cortisol
± CCl_4, ± Cortisol,	4 5	35·48* ± 3·73 (12)	2·63* ± 0·68 (6)	14·03* ± 2·29 (10)
± CCl_4, ± Cortisol,	5 4	29·99* ± 2·41 (5)	2·25* ± 0·33 (5)	11·18* ± 0·78 (4)

The increased level of enzyme activity cannot be reduced further, even if the animals are pretreated with CCl_4 1 hr prior to glucocorticoid injection. Doses: cortisol, 5 mg/150 g, CCl_4: 0·25 ml/100 g body weight.

* Tryptophan oxygenase activity: μmoles Kynurenin/hr per g liver wet weight.

It has been demonstrated in several laboratories that administration of CCl_4 is rapidly followed by increased serum free fatty acid level,[44, 45] sufficient to produce a triglyceride deposition in the liver by itself (see refs. 46, 47, for review: see Steinberg[48]). Part of this free fatty acid increase may be ascribed to CCl_4 intoxication of the adrenal glands,[49] followed by enhanced secretion of catecholamines,[50, 51] which are known to stimulate free fatty acid release from adipose tissue. Using adrenalectomized, cortisol-substituted rats, we did not observe any marked alteration of free fatty acid level in the blood after CCl_4 intoxication (Table 1).

Furthermore, Stern *et al.*[44] pointed out that adrenalectomy prevents the induction of fatty liver by CCl_4. We cannot confirm this statement either. In our experiments enhanced triglyceride deposition following CCl_4 application to adrenalectomized rats was significant (Table 1). Our results are consistent with those of Artizzu and Dianzani,[39] who also were unable to notice any difference in CCl_4-induced steatosis between normal and adrenalectomized rats.

It is worth noticing that the inhibition of glycogen synthesis by CCl_4, indicated by the number of stained granules in the cells, is not homogeneously distributed in the liver lobules, but proceeds from the center vein to the periphery (Fig. 2). This fact clearly demonstrates that biochemical analysis, which is unable to differentiate between intact and poisoned cells, does not represent the true picture of the intoxication state. Histology, in connection with electron microscopy, favours the view that CCl_4, exceeding critical concentration in the liver cell, disturbs a lot of its vital functions in

all compartments: microsomes, mitochondria, nuclei and cytoplasm. Increasing amounts of CCl_4 do not only impair all liver cells slightly more, but result in a larger number of vitally impaired liver cells. Thus selectivity of CCl_4 action on these compartments is less pronounced than is generally accepted.

Apart from all contradictions in the literature on biochemical problems concerning the mechanism of CCl_4 intoxication, one fact seems to be apparent: CCl_4-induced fatty liver is not a suitable model for regulation-studies of fat metabolism in the liver.

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