Activation of Fatty Acids in the Liver in Carbon Tetrachloride Poisoning

Recents experiments[1,2] have cast doubt upon the hypothesis that the increase in liver fat, which follows the oral administration of CCl_4, is the result of a lesion in the mitochondria.

Previous work in this laboratory[3] has shown a decrease of fatty acid activation in rat liver during the later stages of carbon tetrachloride intoxication. The present results seem to provide further insight into the mechanisms underlying CCl_4 hepatotoxicity in the initial stages.

Methods. Male rats weighing 180–200 g were used. All food was withdrawn 12 h before CCl_4 feeding. Carbon tetrachloride in a 1:1 mixture with olive oil was introduced into the stomach by intubation at a dose of 0.5 ml of the mixture/100 g of body weight. The animals were killed by cervical section and exsanguinated after 3, 4, 5, 7 h. The liver was homogenized with a teflon homogenizer of the Potter-Elvehjem type at 0°C in 0.25 *M* sucrose solution containing 0.03 *M* $MgCl_2$.

The activation of palmitic acid was tested by the hydroxamic acid method[4–8] without adding CoA, in order to evaluate mainly the initial step (palmityl-AMP) of activation[6,7].

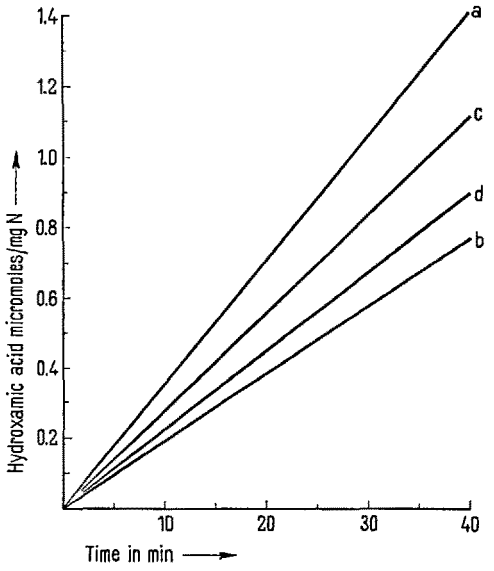
Total liver fat was examined by extraction in a Soxhlet apparatus for 24 h with alcohol-ether mixture (3:1).

The effect of CCl_4 *in vitro* was estimated as follows. For the activation of palmitic acid, the liver from normal rats was homogenized in one volume of 0.25 *M* cold sucrose and diluted to appropriate volumes in 0.25 *M* sucrose saturated or not with CCl_4 (100% saturation is about 0.005 *M*). The final suspension, corresponding to 2 mg of total liver nitrogen, contains 4,3 μM of CCl_4.

For the butyric acid activation, the enzyme was extracted from a liver acetone powder with 10 vol of 0.0128 *M* cold K-phosphate buffer pH 7.7[5,6,9] and diluted to appropiate volumes in the same buffer saturated or not with CCl_4. 1 ml of the extract corresponding to 0.9–1 mg of total nitrogen, contains 4 μM of CCl_4. In this case any formation of hydroxamic acid represents only the initial step of fatty acid activation (acyl-AMP), since the amount of CoA initially present in the extract cannot but be very low[6].

There are many evidences that our experimental conditions permit exclusively the measure of the hydroxamic acid produced by the reaction between *activated* fatty acids and NH_2OH. These evidences are the following:

(1) Lipman and Tuttle[8] showed that the amount of hydroxamic acid formed by liver lipase depends on the length of chain of the fatty acid: it has a maximal level for 8 C and is about zero for 16 C. This fact has been confirmed in our laboratories[4,5].



Influence of CCl_4 *in vitro* on the activation of palmitic and butyric acids. Conditions: see text and notes of the Table.
a = butyric acid μM 10. b = butyric acid μM 10 and CCl_4 μM 1.0. c = palmitic acid μM 2. d = palmitic acid μM 2 and CCl_4 μM 1.1.

	No. of rats	h post CCl_4	Liver fat mg/100 mg dry weight	τ	Significance	Hydroxamic acid μM/mg N	τ	Significance
Controls	6	—	19.8 ± 3.9	2.0	$P < 0.05$	1.03 ± 0.01	0.4	$0.6 < P < 0.7$
CCl_4 fed	6	3	23.8 ± 2.2			1.06 ± 0.05		
Controls	4	—	20.8 ± 2.5	2.4	$P < 0.05$	1,04 ± 0.06	1.6	$0.1 < P < 0.2$
CCl_4 fed	4	4	25.6 ± 2.5			0.96 ± 0.07		
Controls	5	—	19.4 ± 2.9	5.1	$P < 0.01$	1.04 ± 0.09	3.3	$P < 0.01$
CCl_4 fed	5	5	27.7 ± 1.7			0.85 ± 0.09		
Controls	7	—	19.1 ± 2.0	8.9	$P < 0.01$	1.10 ± 0.1	3.6	$P < 0.01$
CCl_4 fed	7	7	28.0 ± 1.5			0.84 ± 0.1		

Activation of palmitic acid in liver after CCl_4 feeding. *Conditions.* Liver homogenate corresponds to 0.5 mg of total nitrogen; time of incubation 40 min; temperature 38°C; pH 7.4. *Medium.* 10 μM of ATP; 30 μM of KF; 750 μM of $NH_2OH \cdot HCl$ (KOH to pH 7.4); 2 μM of palmitic acid (NH_4OH to pH 7.4). Blancs: without substrate and without ATP; Palmityl hydroxamic acid was determined by the method of Lipmann and Tuttle[8].

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(2) Fluoride is known to be a powerful inhibitor of lipase[8,4]; for this reason our experiments have been carried out with fluoride 0.05 *M*.

(3) For short chain fatty acids (butyric), we have extracted the enzymes from a liver acetone powder by phosphate buffer 0.0128 *M* pH 7.7; it was demonstrated that fluoride completely inhibits any lipase activity in such conditions[5].

Apart from the above evidences, we usually have 2 controls in our determinations, i. e. without substrate and without ATP, in order to ascertain that hydroxamic acid formation we measure is merely due to presence of ATP.

Results. The force feeding of CCl_4 resulted in (a) an increase of total liver fat at 3–4 h, according to RECKNAGEL[1] and (b) a diminished activation of palmitic acid which appears to be almost contemporary to the fatty infiltration and in any case is already statistically significant 5 h after poisoning (Table).

CCl_4 added *in vitro* inhibited the activation of palmitic and butyric acid (Fig.).

Discussion. The experimental work reported here supports the view that the impairment in the ability to activate fatty acids is a direct result of CCl_4 hepatotoxicity. From other experiment[5], we have evidence that the activation takes place in the mitochondrial and microsomal fractions[10]. It is well known that an activation is the preliminary operation for fatty acid oxidation and for synthesis of phospholipids and triglycerides[11–14].

The fact that this activation is very precociously impaired shows that an enzymic lesion has set in just in the period when liver fat is rising. This lesion seems to represent one of the points of the pathological sequences correlated to the early increase in liver fat, which intervenes long before any demonstrable mitochondrial damage as defined[1,2] in terms of ATP ase transformation, loss of pyridine-nucleotide-dependant oxidative function, uncoupling of oxidative phosphorylation and impairment in the ability to reaccumulate potassium.

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Zusammenfassung

Bei CCl_4-Vergiftung zeigt die Leber eine vermehrte Aktivierung der Fettsäuren, die sich fast gleichzeitig mit der fettigen Infiltration kundgibt. Die Abnahme der Aktivierung äussert sich bereits 5 h nach der Vergiftung in statistisch signifikanter Weise. Zugabe von CCl_4 hemmt *in vitro* die Aktivierung von Palmitin- und Buttersäure.

The Histochemistry of the Dehydrogenase Systems in the Aortae of Rabbits with Experimental Atherosclerosis

In a previous work[1] we have studied alkaline and acid phosphatases, non-specific esterase and succinic dehydrogenase (the latter enzyme by means of blue tetrazolium and neotetrazolium) in the aortae of atherosclerotic rabbits and rats. Recently developed methods for succinic dehydrogenase[2], DPN-diaphorase[3], TPN-diaphorase[4], and the dehydrogenases linked to the latter based on the use of 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride (= nitro-BT) as the chromogenic electron acceptor gave the possibility of investigating the above-mentioned enzymes also in the vascular wall.

The present communication deals with results of a histochemical investigation of succinic dehydrogenase, DPN-diaphorase, TPN-diaphorase, lactic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, and glucoso-6-phosphate dehydrogenase in the aortae of 9 rabbits with cholesterol atheromatosis (produced by addition of 1 g cholesterol to the daily diet for 4 months) and 5 control animals. Directions given by PEARSE[5] have been adhered to. The tetrazolium used was nitro BT.

Activity of the enzymes mentioned in normal aortae can be detected chiefly in the muscle cells of media (in form of small granules-mitochondria) in decreasing order as follows: DPN-diaphorase, lactic and malic dehydrogenases, TPN-diaphorase, succinic dehydrogenase; the activity of glutamic and glucoso-6-phosphate dehydrogenases was negligible. The activity of the endothelium and cellular elements of the intima is weaker, and in some cases cannot be detected in the sections at all. In adventicial fibrocytes a more pronounced activity could be ascertained.

In rabbits with experimental atherosclerosis, enzyme activities in media and adventicia had mostly the same localisation and intensity as in the control samples.

An interesting pattern could be observed in the plaques. DPN-diaphorase (Fig. b), lactic dehydrogenase (Fig. c), malic dehydrogenase, and TPN diaphorase are here localised in the macrophages (lipophages), and in the fibrocytes ('Faserzellen' Benninghoff) in the form of small granules penetrating into all cellular processes. Preparations stained with Oil Red O showed that the localisation of enzymes activities were not always identical with the localisation of lipids. In the same elements succinic dehydrogenase (Fig. a) could be detected, but the corresponding activity was much slighter. A minute activity was shown by glucoso-6-phosphate (Fig. f) but only in some of the plaques. Similarly, glutamic dehydrogenase activity (Fig. e) which was the slightest of all the enzyme systems investigated, was weakly positive only in some cases in the surface parts of the plaques. It must be emphasized that the stain intensities of all the enzymes studied showed differences, which were most apparent upon comparing small plaques with large ones. In the small plaques with a small number of lipophages, reaction was very intense in nearly all cellular elements. In the large plaques, where substantial amounts of cholesterol could be detected, activity was only in the lipophages and fibrocytes at the surface and in depth close to the media. In the center of the plaques which showed the most intense reaction for cholesterol, enzyme reactions were negligible (Fig. b). Above the small plaques and in the neighbourhood of some larger plaques, reactions were rather pronounced also in the endothelia. Similar reactions are also shown by plaques on the semilunar valves (Fig. d).

The reported findings of high activity of the dehydrogenase systems in the small plaques present, as well as the previously reported activities of alkaline and acid phosphatases, and non-specific esterase, further evidence

Further work on the dehydrogenase systems in plaques in rabbit, rats, and humans is in progress and will be reported in detail elsewhere.

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