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CHANGES IN RABBIT LIVER STEROL PATTERNS AFTER ADMINISTRATION OF CARBON TETRACHLORIDE IN DOSES EFFECTIVE AGAINST *FASCIOLA HEPATICA*, THE LIVER FLUKE

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Abstract—Administration of carbon tetrachloride, CCl_4, to rabbits at a dose of 150 mg/kg i.m., which is fully effective against liver fluke infection in this laboratory animal, caused changes in the liver sterol pattern. Thirty hr after injection some methylsterols, not present in livers from untreated animals, were found in minute quantities; they were separated by means of chromatographic techniques and identified by mass spectrometry. As these sterols are possible intermediates in the biosynthesis of cholesterol, it is assumed that carbon tetrachloride blocks this synthesis between lanosterol and cholesterol by inhibition of demethylation reactions. A hypothesis for the mechanism of this action is given. Both liver homogenates and the sterol fractions from the CCl_4-treated rabbits demonstrated, in comparison with corresponding preparations from control animals, lethal effects on the liver fluke in an *in vitro* test. This lethal effect might be the mode of action of the anthelmintic carbon tetrachloride on the trematode parasite, *Fasciola hepatica*.

CARBON tetrachloride has been used for many years as an anthelmintic against *Fasciola hepatica*, the common liver fluke, and in spite of its rather high toxicity it still finds application.

The therapeutic dose in the rabbit, as determined in our laboratories, is 150 mg/kg i.m.

Studies[1] with $^{14}CCl_4$ in sheep showed that from an oral dose of 180 mg/kg an amount of labelled substance is recovered in the bile, corresponding to a concentration of at the most 65 ppm CCl_4; in the blood this concentration was 21 ppm.

Both levels are far from sufficient to give an explanation of the lethal effect on the parasite, since the effective dose *in vitro*[2] is about 1·500 ppm. It is therefore generally accepted now that CCl_4 exerts its anthelmintic action indirectly, either via one or more unknown metabolites or by inducing the formation of toxic substances in the liver.[1, 3] Concerning this second possibility, accumulation of a normal intermediate to relative high levels by disturbances of metabolic processes cannot be excluded.

The finding* that homogenates of livers from CCl_4-treated rabbits showed a greater *in vitro* activity on the liver fluke, as compared to control homogenates, led to the discovery, in the livers from treated animals, of methylsterols, which were not demonstrable in the livers of control animals.

* Experiments performed by De Zoeten and co-workers in our laboratories.

EXPERIMENTAL

Rabbits, 1·5–2·0 kg body wt., received intramuscular injections of 150 mg/kg carbon tetrachloride in a 50% (v/v) solution in arachis oil. Controls received an equal volume of the oil. The animals were sacrificed 30 hr later and the livers removed for further examination.

After removal of the gall bladders the livers were homogenized in a Waring Blendor and subjected to lipid extraction according to Folch *et al.*[4] with 3 ml of a mixture of chloroform and methanol (2:1, v/v) per gram of liver. After separation and evaporation, the residue of the chloroform layer was treated with 25 ml 15% (w/v) ethanolic KOH per gram of residue for 4 hr at the boiling temperature. Sterols were then extracted with petroleum ether (40–65°). All steps were followed by thin layer chromatography on silica[5] (Silica gel F 254, Merck, Darmstadt, W. Germ.), using as the solvent systems benzene, benzene–ethyl acetate (4:1, v/v), methylene chloride–benzene (2:1) + 10% (v/v) acetone, and the system of Skipsky *et al.*,[6] which gave a good separation of the different lipid classes. For detection[5] the Carr–Price reagent was used, as well as the less specific 10% sulphuric acid. Isolation of separate sterols from the petroleum ether extracts was performed by column chromatography. Satisfactory results were obtained by using a modification of the method of Frantz.[7] One g of the mixture, dissolved in 10 ml of the solvent system CH_2Cl_2–benzene (1:2, v/v), was separated on 30 g of silica (Merck, 0·05–0·2 mm) with an acetone gradient. Starting without acetone, the percentage of acetone was increased by 1 after each 10 fractions of 3 ml eluate.

Identification* of the sterols was achieved, with an AEI, MS 9 mass spectrometer, by means of micro-techniques with 1–10 μg aliquots obtained by elution of spots removed from thin layer plates.

The liver flukes were taken from the livers of freshly killed sheep and cows at the slaughterhouse and immediately immersed and stored at 37° in a medium composed of Dawes' saline medium[8] supplemented with calf serum, glucose and antibiotics: per litre: 700 ml Dawes' medium

300 ml calf serum
2 g glucose
25 × 10^4 Units penicillin-G
10 mg streptomycin
10 mg neomycin
} purchased from Mycofarm, Delft, The Netherlands.

The *in vitro* tests, duration 24–48 hr, were performed in this medium without the serum.

RESULTS

After the finding that liver homogenates from CCl_4-treated rabbits showed a significantly higher lethal activity against the liver fluke *in vitro* than corresponding control homogenates, our studies were aimed at the detection of the lethal factor(s) in the livers of the treated animals. Lipid extraction procedures showed that the activity was present in the neutral lipid fraction (see Table 1). Thin layer chromatographical data from some typical experiments are shown in Fig. 1. Here differences

* This part of the work was carried out at the department of Instrumental Analysis of Philips-Duphar Research Laboratories under the supervision of Dr. P. K. Korver.

TABLE 1. LETHAL ACTIVITY OF LIVER FRACTIONS FROM CCl_4-TREATED AND CONTROL ANIMALS ON *Fasciola hepatica*

Preparations	Control	CCl_4-treated single dose	CCl_4-treated during 4 days
Total liver homogenate	± 30·000	± 16·000	
After lipid extraction:			
methanol phase	> 10·000	> 10·000	
chloroform phase	± 5·000	2·000–3·000	± 5·000
$CHCl_3$-phase after digitonine treatment	> 5·000	> 5·000	
$CHCl_3$-phase after alkaline hydrolysis and petroleum ether extraction;			
dry petroleum ether extract (sterol fraction)	> 2·000	250–500	± 500
Combined fractions after removal of spot 1 (cholesterol) from the sterol fraction by means of column chromatography		< 50*	

In vitro tests: three flukes/5 ml medium in duplicate or triplicate. The fractions were solubilized in the medium using Tween 85. Values expressed in ppm (parts per million) corresponding to mg substance per liter medium.

Pure cholesterol: lethal activity 1·000–2·000 ppm.

* These results are liable to rather great variations and in accuracies as indicated in the text.

between the extracts of the control and the treated animals can be seen (I and II), e.g. the presence of spots 2 and 3, just above spot 1, attributed to cholesterol (IV). The finding that both the spots 2 and 3 as well as the lethal effect on the fluke disappeared after treatment of the extracts with digitonine, a reagent capable of precipitating 3β-hydroxy sterols, and the further fact that the lethal factor was resistant to alkaline hydrolysis, supplied evidence for the unsaponifiable sterol fraction being the lethal fraction. Alkaline hydrolysis (III), giving higher yields of sterol fractions, was used as a routine procedure in preparing the sterols from the liver extracts. It became clear that the maximum effect in the livers of treated animals appeared between 28 and 32 hr after injection. When carbon tetrachloride was administered to the rabbits during 4 successive days (150 mg/kg i.m., daily) and the livers were removed 30 hr after the last injection, the liver homogenates as well as the lipid extracts did show a *lower* lethal activity on the liver flukes than the corresponding preparations from animals receiving a single dose of CCl_4. After alkaline hydrolysis of the lipid extracts, the activity of the sterol fraction obtained, was comparable to that of sterol fraction from the single dose-treated animals (see Table 1). The chromatographical data indicated that esterification of the sterols could have taken place (VI and VII). These results indicate that by the action of carbon tetrachloride, some sterols, slightly less polar than cholesterol, are formed in rabbit liver. These compounds could not be demonstrated in normal livers. In the course of these studies they were also detected in the blood and in the bile of CCl_4-treated animals when these fluids were subjected to the same lipid extraction procedure as the liver.

Attempts to isolate and identify the individual sterols in the mixture encountered difficulties; repeated separations on silica columns with gradient elutions enabled us, however, to isolate minute quantities of chromatographically pure material. By scraping off thin layer plates, enough substance was collected to make possible an identification by means of mass spectrometry. Spot no. 1 on the chromatogram showed

good correspondence to a cholesterol reference. Mass analysis revealed a molecular weight of 386 with the breakdown pattern of a sterol; NMR analysis supplied further evidence that it did indeed represent cholesterol. In spot no. 2 two compounds were found, with molecular weights of respectively 400 and 398. Chemical hydrogenation (Adams catalyzer in ethyl acetate) resulted in a single compound, molecular weight

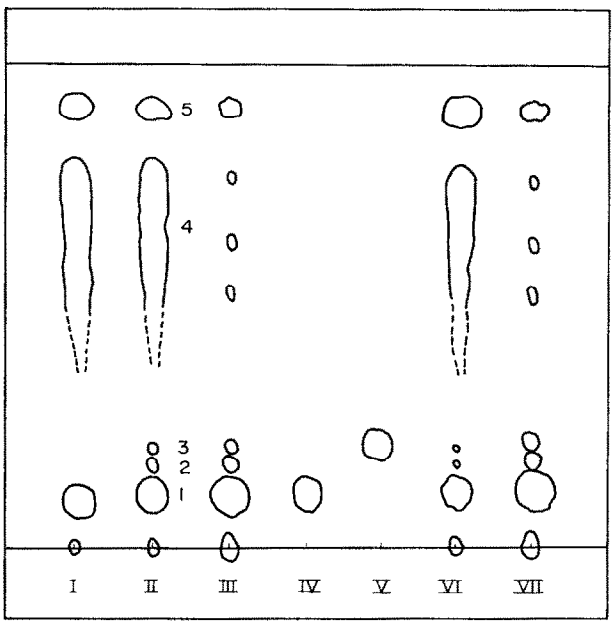

FIG. 1. Thin Layer Chromatography of neutral lipid fractions of rabbit livers on Silica gel F 254 (Merck).

Solvent : benzene
Detection : Carr–Price reagent

I. Liver extracts of control animals
II. Liver extract of carbon tetrachloride treated animals
III. Petroleum ether extract of fraction II after alkaline hydrolysis
IV. Reference: cholesterol
V. Reference: lanosterol
VI. Liver extract after administration of CCl_4 during 4 successive days (for details see text)
VII. Petroleum ether extract of VI after alkaline hydrolysis

Spots 1, 2 and 3 represent the sterol fraction. Spots 4 and 5 represent the triglyceride fraction and the sterol esters respectively.

In each case 100 μg of substance was applied to the plate; for the extracts I, II and VI this corresponds to about 1 mg of liver, for extracts III and VII: about 3 mg of liver.

400. The substance represented by spot no. 3, resembling lanosterol in chromatographical properties, consisted of two components with molecular weight 412 and 426. All four compounds showed in their mass spectra the characteristic breakdown pattern of sterols. From these data it was concluded that methyl sterols were involved, the differences between the molecular weights being 14 (CH_2-group).

In the last reaction sequences during biosynthesis of cholesterol three methyl-groups

from lanosterol (one at C-14 and two at C-4) are removed successively; the compounds corresponding to the given spots and molecular weights are therefore considered to be:

(1) 386 cholesterol
(2) 398 4α-methylcholest-7(or 8), 24-dien-3β-ol
    400 4α-methylcholest-7(or 8)en-3β-ol
(3) 412 4,4-dimethylcholesta-8,24-dien-3β-ol (14-desmethyllanosterol)
    426 lanosterol

As the isolated quantities of the separate methylsterols were sufficient only for good identification, we were not able to test the biological activity of each substance.

The whole methylsterol fraction (i.e. without cholesterol) and also pure lanosterol did show lethal effects *in vitro* on the liver fluke, but the tests gave rather erratic results. Effects in low doses as well as no lethal activity at all were observed. We assume that the results of the test system were confused by the condition of the liver flukes, obtained from the slaughterhouse; a factor that is not known and can hardly be controlled. Moreover the poor solubility of the sterols in water required solubilizing agents, such as Tweens, for the *in vitro* test. These surfactants are probably able to influence, with respect to the parasite, the uptake or penetration of the lethal agent.

DISCUSSION

In the normal mammalian liver the biosynthesis of cholesterol takes place rapidly, so that many intermediates cannot be demonstrated directly. The reaction sequence from lanosterol to cholesterol, involving demethylations (C-14 and C-4), hydrogenation (Δ24,25), oxidation (to 3-keto intermediates), and a shift of the double bond (Δ8,9→Δ5,6) have now largely been determined.[9] Gaylor *et al.*[10] have shown with rat liver microsomes that isomerization (Δ8,9→Δ7,8) and demethylation can take place at the same time, although the isomerization velocity becomes significant only at later stages of demethylation. The hydrogenation of the side chain (Δ24,25) prior to completion of the demethylation seems possible, too. Our results with the livers of CCl_4-treated rabbits indicate a blockage in the cholesterol biosynthesis, in particular at the demethylation points.

Since the removal of the methyl groups is performed via three oxidative steps and a decarboxylation ($RCH_3 \rightarrow RCH_2OH \rightarrow RHC = 0 \rightarrow RCOOH \rightarrow RH + CO_2$), and because most of these steps of the cholesterol biosynthesis can be achieved *in vitro* in the presence of liver microsomes, NADPH and molecular oxygen,[11] it is assumed that carbon tetrachloride exerts the above-mentioned action on the cholesterol biosynthesis by influencing the microsomal electron transport chain.

It might be possible that the CO-sensitive cytochrome P-450, an essential factor in this chain, is involved in the action of carbon tetrachloride.[12–16]

Insufficient quantitative data are available to explain fully the *in vivo* anthelmintic action of CCl_4 from the *in vitro* activity of the sterol fraction on the liver fluke. We cannot indicate at present which sterols are involved or what the mechanism of action on the liver fluke is. The involvement of the formation of the above-mentioned methylsterols in the elimination of the trematode parasites by carbon tetrachloride treatment does not seem unlikely. Speculations concerning the mechanism of action imply that the parasite, in all probability incapable of *de novo* biosynthesis of sterols[17]

and therefore fully dependent on the host animal in this respect, may suffer serious consequences from a defect in the cholesterol biosynthesis of that host.

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REFERENCES

1. A. C. KONDOS and G. L. MCCLYMONT, *Nature, Lond.* **206**, 846 (1965).
2. W. STEPHENSON, *Parasitology* **38**, 116 (1947).
3. A. KHALDI and S. A. ZAKI, *Br. J. Pharmac.* **36**, 253 (1969).
4. J. FOLCH, I. ASCOLI, M. LEES, J. A. MEATH and F. N. LE BARON, *J. biol. Chem.* **191**, 833 (1951
5. E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin (1962).
6. V. P. SKIPSKY, A. F. SMOLOWE, R. C. SULLIVAN and M. BARCLAY, *Biochim. biophys. Acta* **106**, 386 (1965).
7. I. D. FRANTZ, JR., *J. lipid Res.* **4**, 176 (1963).
8. B. DAWES, *Nature, Lond.* **174**, 614 (1954).
9. I. D. FRANTZ, JR. and G. J. SCHROEPFER, JR., *Ann. Rev. Biochem.* **36** (pt. 2), 691 (1967).
10. J. L. GAYLOR, C. V. DELWICHE and A. C. SWINDELL, *Steroids* **8**, 353 (1966).
11. J. R. GILLETTE, in *Advances in Pharmacology* **4**, 229 (1966).
12. F. WADA, K. HIRATA and Y. SAKAMOTO, *Jap. J. Biochem.* **65**, 171 (1969).
13. J. L. GAYLOR and H. S. MASON, *J. biol. Chem.* **243**, 4966 (1968).
14. H. A. SASAME, J. A. CASTRO and J. R. GILLETTE, *Biochem. Pharmac.* **17**, 1759 (1968).
15. S. ORRENIUS and L. ERNSTER, *Biochem. J.* **103**, 3P (1967).
16. E. A. SMUCKLER, E. ARRHENIUS and T. MULTIN, *Am. J. Pathol.* **50**, 52a (1967).
17. TH. VON BRAND, *Biochemistry of Parasites*, Academic Press, New York (1966).