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INFLUENCE OF CLOFIBRATE ON LIVER MICROSOMAL HYDROXYLATION OF CHOLESTEROL AND ANDROSTENEDIONE

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Abstract—The liver microsomal metabolism of 4-[4-^{14}C]androstene-3,17-dione and [4-^{14}C]cholesterol was studied in control and clofibrate-treated rats.

In the control rat 25 per cent of androstenedione metabolites were hydroxylated at the 6β-position. Another 25 per cent were recovered as 16-oxygenated derivatives and minor amounts (5 per cent) were hydroxylated at the 6α- or a 7α-position. Clofibrate stimulated all the hydroxylation reactions of this compound. The 6β-hydroxylation was elevated by 100 per cent, the 7α-hydroxylation by 70 per cent, and the 6α- and 16α-hydroxylations by 50 per cent. Furthermore, following treatment with clofibrate, the ratio between 17β-hydroxy-4-androstene-3,16-dione and 16α-hydroxy-5-androstene-3,17-dione increased from 0·15 to 0·68. The activity of the 17β-hydroxysteroid oxidoreductase increased by 100 per cent, whereas the 3β-hydroxysteroid oxidoreductase and 5α-reductase activities were only slightly affected.

The 7α-hydroxylation of labelled cholesterol was uninfluenced by treatment with clofibrate.

It is suggested that clofibrate stimulates the activity of the enzyme system involved in the hydroxylation of drugs in the liver.

CHOLESTEROL is eliminated from the body to a large extent as bile acids. The rate-limiting step in the hepatic formation of bile acids is probably the 7α-hydroxylation of cholesterol effected by the liver microsomes.[1,2]

Clofibrate is a widely used hypolipidemic drug but its mechanism of action is, as yet, not fully known. Salvador *et al.*[3] reported that clofibrate stimulated the microsomal hydroxylation of testosterone in the rat liver. In view of the importance of the hepatic microsomes in the degradation of cholesterol and the inactivation of steroid hormones, it seemed important to consider the possible effect of clofibrate on such processes. The present study reports on the liver microsomal metabolism of 4-androstene-3,17-dione and cholesterol in control and clofibrate-treated rats.

MATERIALS AND METHODS

Reference steroids. Several of the reference compounds used in this investigation were generous gifts from colleagues: Dr. J. Babcock (4-androstene-3,17-dione and 17β-hydroxy-4-androsten-3-one), Dr. M. Ehrenstein (6β-hydroxy-4-androstene-3,17-dione), Dr. B. P. Lisboa (17β-hydroxy-4-androstene-3,16-dione), Dr. W. Staib (7α-hydroxy-4-androstene-3,17-dione), and Dr. J. Ufer (5α-androstane-3,17-dione and 3β-hydroxy-5α-androstan-17-one). 16α-Hydroxy-4-androstene-3,17-dione was obtained from USP Steroid Reference Substance (New York, U.S.A.). Clofibrate (Atromidin®) was obtained from Scanmeda.

Radioactive steroids. 4-[4-^{14}C]Androstene-3,17-dione (specific radioactivity, 1·2 μCi/mg and [4-^{14}C]cholesterol (specific radioactivity, 145 μCi/mg) were obtained from the Radiochemical Centre (Amersham, England). Prior to use, the labelled cholesterol was purified by chromatography on a column of aluminium oxide, grade III (Woelm, Eschwege, Germany).

Animals and preparation of homogenates. Two groups, of seven male white rats of the Sprague–Dawley strain, weighing about 200 g were fed crushed rat diet pellets for 3 weeks *ad libitum*. The food given to one of the groups was supplemented with 0·3% clofibrate. The drug was dissolved in ethanol, which was added to the crushed pellets and left to evaporate at room temperature. At this dose the drug is reported to have a maximum effect on the plasma cholesterol level without producing toxic manifestations resulting in decreased food consumption or loss of weight gain.[4]

The animals were killed by decapitation. The livers were removed as soon as possible and placed in cold buffer solution. Liver homogenates 20% (w/v) were prepared in a modified Bucher medium,[5] pH 7·4, using a Potter–Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 20,000 *g* for 15 min. The supernatant was diluted with Bucher medium to give a volume 1·5 times larger than that of the original homogenate. The microsomal fraction was obtained by centrifuging the supernatant at 100,000 *g* for 1 hr. The microsomal fraction was suspended in the homogenizing medium in a volume corresponding to that of the 20,000 *g* supernatant, and subsequently homogenized with a loosely fitting pestle. The protein concentrations of the 20,000 *g* supernatant fluid and the microsomal fraction were determined according to Lowry *et al.*[6] Cholesterol was analyzed according to Hanel and Dam.[7]

Incubations with labelled androstene-3,17-dione. 300 μg of 4-[4-^{14}C]androstene-3,17-dione, dissolved in 50 μl acetone, was added to a mixture of 0·5 ml of the microsomal fraction and 2·5 ml of Bucher medium fortified with 3 μmoles of NADPH. Incubations were carried out for 10 min at 37°. Under these conditions the conversion of substrate was linear with respect to time and enzyme concentration. The incubations were terminated by adding 20 vol. of chloroform–methanol (2:1, v/v). The precipitate was filtered off and 0·2 vol. of 0·9% sodium chloride were added. The chloroform phase was collected and the solvent was evaporated. The residue was dissolved in 0·5 ml of chloroform–methanol (2:1, v/v) and applied to precoated silica gel plates (250 μ, Merck, Darmstadt, Germany) subsequently developed in chloroform–ethylacetate, 4:1 (v/v) and subjected to autoradiography for 10 days. The radioactive zones on the thin-layer chromatograms were determined exactly from the X-ray film. The radioactive material was scraped off, eluted with methanol and measured for radioactivity.

Incubations with labelled cholesterol. [4-^{14}C]Cholesterol, 10 μg in 50 μl of acetone, was incubated for 20 min with 5 ml of 20,000 *g* supernatant fluid. The incubation was terminated by adding 20 vol. of chloroform–methanol (2:1, v/v). Further analysis of the chloroform–methanol extract was performed as described previously.[1]

RESULTS

Liver weight, cholesterol and protein

The liver weights of the clofibrate-treated rats were about 20 per cent higher than those of the control rats ($P < 0·005$). The concentrations of cholesterol and protein in

the 20,000 *g* supernatant fluid and the protein concentration of the liver microsomal fraction did not differ significantly between the two groups of animals (Table 1).

TABLE 1. INFLUENCE OF CLOFIBRATE TREATMENT ON LIVER WEIGHT, AND ON THE CONCENTRATIONS OF CHOLESTEROL AND PROTEIN IN THE LIVER

	CFT	Control
Liver weight (g)	15·8 ± 1·6	13·4 ± 1·0
Cholesterol concentration of the 20,000 *g* supernatant fraction (mg/ml)	0·112 ± 0·016	0·099 ± 0·016
Protein concentration of the 20,000 *g* supernatant fraction (mg/ml)	21·6 ± 2·0	19·2 ± 3·4
Protein concentration of the microsomal fraction (mg/ml)	1·19 ± 0·30	1·62 ± 0·32

CFT = clofibrate-treated rat.

Biochemical decomposition of 4-[4-^{14}*C*]*androstene*-3,17-*dione*

TLC of extracts obtained after incubation of labelled androstene-3,17-dione with the liver microsomal fraction of the control rats demonstrated that 20–25 per cent of the substrate was converted into other compounds. Autoradiography of the thin-layer chromatograms revealed 11 zones with radioactive material (Fig. 1), subsequently identified on the basis of the data summarized in Table 2.

No steroids were detected in zones 1 and 2. Zones 3, 4, 5 and 7 were found to contain the hydroxylated derivatives 7α-, 6α-, 6β- and 16α-hydroxy-4-androstene-3,17-dione, which in the control rats constituted 5, 5, 25 and 25 per cent, respectively, of the total metabolites formed.

The trimethylsilyl ether of the steroid accumulated in zone 6 yielded a molecular ion at *m/e* 374, indicating a monohydroxy-dioxo-androsten structure. Prominent peaks were also found at *m/e* 359 (M-5; base peak) and 129. By comparison with the authentic reference substance the compound in zone 6 was identified as 17β-hydroxy-4-androstene-3,16-dione (16-ketotestosterone).

Zones 8, 9 and 11 contained reduced derivatives of the parent compound 4-androstene-3,17-dione recovered in zone 10. In the control rats the radioactivity in zone 8 (17β-hydroxy-4-androsten-3-one, testosterone) and in zone 9 represented about 8 and 20 per cent, respectively, of the metabolites. The latter zone harboured two compounds, of which 5α-androstene-3,17-diol constituted 10 per cent and 3β-hydroxy-5α-androstan-17-one (epiandrosterone) 90 per cent of the material as judged by GLC. The steroid recovered in zone 11 corresponded to about 12 per cent of the metabolites and was identified as 5α-androstane-3,17-dione. The amounts of the various metabolites formed per mg microsomal protein/10 min are given in Table 3.

The administration of clofibrate to the rats had a stimulatory effect on the microsomal hydroxylation of 4-androstene-3,17-dione. In these animals, compared to the controls, the formation of 7α-hydroxy-4-androstene-3,17-dione was elevated by a

TABLE 2. TLC FRACTIONATION AND GLC-MASS SPECTROMETRIC ANALYSES OF RADIOACTIVE MATERIAL OBTAINED AFTER INCUBATION OF LABELED 4-ANDROSTENE-3,17-DIONE WITH LIVER MICROSOMES

TLC zone	Radioactivity recovered %		t_R (SE-30)	Characteristic mass spectrometric peaks (m/e)	Identification
	Untreated	Clofibrate-treated			
1	1·3	1·3	—	—	—
2	0·8	0·8	—	—	—
3	0·8	1·0	0·72	374 (M), 359 (M-15, base peak), 284 (M-90)	7α-hydroxy-4-androstene-3,17-dione (9)
4	1·0	1·1	0·85	374 (M), 359 (M-15, base peak), 318 (M-56)	6α-hydroxy-4-androstene-3,17-dione (10)
5	4·3	6·8	0·65	374 (M), 359 (M-15, base peak), 318 (M-56)	6β-hydroxy-4-androstene-3,17-dione (9)
6	0·7	2·0	0·99	374 (M), 359 (M-15, base peak), 129	17β-hydroxy-4-androstene-3,16-dione (16-ketotestosterone)
7	3·8	3·0	0·87	374 (M), 303 (M-71, base peak)	16α-hydroxy-4-androstene-3,17-dione (9)
8	1·3	1·2	0·67	360 (M), 129 (base peak)	17β-hydroxy-4-androsten-3-one (testosterone)
9	3·6	2·2	0·51	436 (M), 129 (base peak)	5α-androstane-3α,17β-diol (11)
			0·53	362 (M), 347 (M-15, base peak)	3β-hydroxy-5α-androstan-17-one (11) (epiandrosterone)
10	78·0	77·0	0·54	286 (M, base peak)	4-androstene-3,17-dione
11	1·9	1·5	0·54	288 (M, base peak)	5α-androstane-3,17-dione

t_R = relative retention time (5α-cholestane = 1·00); M = molecular ion.

TABLE 3. METABOLISM OF 4-[4-^{14}C]ANDROSTENE-3,17-DIONE BY MICROSOMES FROM CLOFIBRATE-TREATED (CFT) AND CONTROL RATS

Compound	nmoles of metabolite formed/mg protein/10 min	
	CFT	Control
7α-Hydroxy-4-androstene-3,17-dione	9·1 ± 2·5*	5·4 ± 0·5
6α-Hydroxy-4-androstene-3,17-dione	10·3 ± 3·5	6·9 ± 1·4
6β-Hydroxy-4-androstene-3,17-dione	63·7 ± 26·8*	28·4 ± 5·3
17β-Hydroxy-4-androstene-3,16-dione	19·0 ± 6·9*	4·8 ± 0·6
16α-Hydroxy-4-androstene-3,17-dione	27·8 ± 10·0	25·2 ± 4·7
17β-Hydroxy-4-androsten-3-one	11·1 ± 3·1	8·6 ± 1·3
3β-Hydroxy-5-androstan-17-one	20·2 ± 4·6	24·2 ± 5·8
5α-Androstane-3,17-dione	14·0 ± 3·0	13·2 ± 5·2

* Significally different from the control ($P < 0·01$).

The conversions were calculated from the amounts of radioactivity in the different zones of the thin layer chromatograms. The values listed are the means ± S.D. of experiments with six rats.

factor of 1·7 (Table 3). The corresponding values for the 6α- and the 6β-derivatives of the parent compound were 1·5 and 2·2, respectively. Oxygenation at the C 16 position (as evidenced by the formation of 17β-hydroxy-4-androstene-3,16-dione) increased 4-fold whereas the formation of 16α-hydroxy-4-androstene-3,17-dione was essentially unchanged. Some oxidoreductase activities were also influenced by the treatment of clofibrate. Thus the 17β-oxidoreductase (measured by the combined amount of 17β-hydroxy-4-androstene-3,16-dione and 17β-hydroxy-4-androsten-3-one) increased about twice ($P < 0·01$). On the other hand, the 3β-hydroxysteroid oxidoreductase and

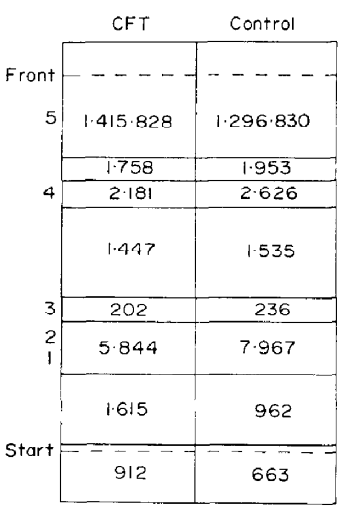

FIG. 2. TLC of radioactive material recovered after incubation of [4-^{14}C] cholesterol with the 20,000 *g* supernatant fraction of liver homogenates from clofibrate-treated (CFT) and control (control) rats. The figures on the chromatograms represent counts/min. Reference compounds were: (1) 7α,12α-dihydroxy-4-cholesten-3-one; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 7α-hydroxy-4-cholesten-3-one; (5) cholesterol.

the 5α-reductase were essentially unaffected by clofibrate as evidenced by the formation of 5α-androstane-3,17-dione and 3β-hydroxy-5α-androstan-17-one. The mean ratio between 17β-hydroxy-4-androstene-3,16-dione and 16α-hydroxy-4-androstene-3,16-dione and 16α-hydroxy-4-androstene increased from 0·15 to 0·68.

Biochemical decomposition of [4-^{14}C]*cholesterol.* The distribution of radioactivity upon TLC of extracts obtained after incubation of radioactive cholesterol with liver microsomes is shown in Fig. 2. In accordance with previous observations[12], 5-cholestene-3β, 7α-diol, 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one were the main cholesterol metabolites. The pattern of products formed was about the same in the clofibrate-treated and the control rats (Table 4).

TABLE 4. METABOLISM OF [4-^{14}C]CHOLESTEROL BY THE 20,000 *g* SUPERNATANT FRACTIONS FROM CLOFIBRATE-TREATED (CFT) AND CONTROL RATS

	CFT	Control
nmoles of 7α-hydroxylated metabolites formed per mg protein	0·080 ± 0·028	0·133 ± 0·083
nmoles of 7α-hydroxylated metabolites formed per total liver weight	135·5 ± 47·3	163·0 ± 80·1

The conversions were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The values listed are the means ± S.D. of experiments with seven rats.

DISCUSSION

Clofibrate treatment of rats stimulates liver microsomal hydroxylation of 4-androstene-3,17-dione (as evidenced by the present study) and testosterone.[3] It has also been demonstrated that clofibrate treatment results in hepatomegaly,[13] induces proliferation of the smooth endoplasmic reticulum of rat liver[14] and increases the cytochrome P-450 concentration of rat liver microsomes.[3] These characteristics place clofibrate among the more than 200 drugs that are known to stimulate the activity of a variety of hepatic microsomal drug-metabolizing enzymes.[15,16]

Clofibrate also tended to stimulate the microsomal 17β-hydroxysteroid oxidoreduction of 4-androstene-3,17-dione. A similar effect has been noted recently in rats treated with the potent catatoxic steroid 16α-cyanopregnenolone, another inducer of microsomal hydroxylation reactions.[17]

Cholesterol is eliminated mainly as bile acids and neutral steroids in the feces. The formation of bile acids in the liver is regulated by homeostatic mechanisms whereby an interruption of the enterohepatic circulation results in a several-fold increase of the bile acid synthesis. The rate-limiting step in this process is the 7α-hydroxylation of cholesterol[1,2] and the activity of the enzyme system is enhanced upon stimulation of the bile acid synthesis. The present results provide no indication that treatment with clofibrate results in an increased 7α-hydroxylation of cholesterol and thus enhances the formation of bile acids in the rat liver. This finding is compatible with the observation that the elimination of cholesterol as bile acids in man is unchanged or decreased in hyperlipemic subjects receiving clofibrate in therapeutic doses.[18] It

appears then that the enzyme system inducing the 7α-hydroxylation of cholesterol and the systems involved in the hydroxylation of androstene-3,17-dione react differently in rats treated with clofibrate. A similar disassociation between the response of the 7α-hydroxylating enzyme system and that participating in the inactivation of drugs has been observed after administration of phenobarbital[19] and the catatoxic steroid 16α-cyanopregnenolone.[17]

This report presents evidence that clofibrate may influence the liver metabolism of steroid hormones. It also inhibits cholesterol biosynthesis in the liver.[20,21] As this drug is commonly used as a hypolipidemic agent it would be of interest to study its possible effects on steroid metabolism in man.

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REFERENCES

1. H. Danielsson, K. Einarsson and G. Johansson, *Eur. J. Biochem.* **2,** 44 (1967).
2. S. Shefer, S. Hauser, I. Bekersky and E. H. Mosbach, *J. Lipid Res.* **11,** 404 (1970).
3. R. A. Salvador, S. Haber, C. Atkins, B. W. Gommi and R. M. Wolch, *Life Sci.* **9,** 397 (1970).
4. J. M. Thorp and W. S. Waring, *Nature, Lond.* **194,** 948 (1962).
5. S. Bergström and U. Gloor, *Acta Chem. Scand.* **9,** 34 (1955).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
7. H. K. Hanel and H. Dam, *Acta Chem. Scand.* **9,** 677 (1955).
8. R. Reimendahl and J. Sjövall, *Anal. Chem.* **44,** 21 (1972).
9. J.-Å. Gustafsson and B. P. Lisboa, *Acta Endocr.* **65,** 84 (1970).
10. J.-Å. Gustafsson and B. P. Lisboa, *Eur. J. Biochem.* **12,** 369 (1970).
11. R. Vihko, *Acta Endocr.* Suppl. 109 (1966).
12. H. Danielsson and K. Einarsson, *J. biol. Chem.* **241,** 1449 (1966).
13. M. M. Best and C. H. Duncan, *J. Lab. Clin. Med.* **64,** 634 (1964).
14. R. Hess, W. Staubli and W. Riess, *Nature, Lond.* **208,** 856 (1965).
15. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
16. H. V. Gelboin, *Adv. Cancer Res.* **10,** 1 (1967).
17. K. Einarsson and J.-Å. Gustafsson, *Eur. J. Biochem.* **32,** 197 (1973).
18. K. Einarsson, K. Hellström and M. Kallner, *Eur. J. clin. Invest.* in press.
19. K. Einarsson and G. Johansson, *Eur. J. Biochem.* **6,** 293 (1968).
20. D. R. Avoy, E. A. Swyryd and R. G. Gould, *J. Lipid Res.* **6,** 369 (1965).
21. J. E. Fulton and S. L. Hsia, *J. Lipid Res.* **13,** 78 (1972).