The Metabolism of Steroids in the Fatty Liver Induced by Orotic Acid Feeding

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1. The metabolism of 4-[4-¹⁴C]androstene-3,17-dione, 4-[4-¹⁴C]pregnene-3,20-dione, 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol, [4-¹⁴C]cholesterol, 7 α -hydroxy-4-[6 β -³H]cholesten-3-one, 5 β -[7 β -³H]cholestane-3 α ,7 α -diol and [³H]lithocholic acid was studied in the microsomal fraction of livers from control and orotic acid-treated male rats. 2. As a result of the treatment the orotic acid-fed rats had fatty livers and subnormal concentrations of cholesterol and triglycerides in serum. 3. The 6 β - and 7 α -hydroxylation of 4-androstene-3,17-dione, the 2 α -, 2 β - and 18-hydroxylation of 5 α -androstane-3 α ,17 β -diol, and the 5 α -reduction of 4-androstene-3,17-dione and 4-pregnene-3,20-dione were decreased by 40–50% in orotic acid-fed rats. Other oxidative and reductive reactions of the steroid hormones were not significantly affected. 4. The 12 α -hydroxylation of 7 α -hydroxy-4-cholesten-3-one was decreased by about 50%, whereas the 7 α -hydroxylation of cholesterol and the 26-hydroxylation of 5 β -cholestane-3 α ,7 α -diol were not significantly decreased. The 6 β -hydroxylation of lithocholic acid was stimulated by 40%. 5. The results are discussed in relation to present knowledge of the hepatic drug-metabolizing enzymes and to the recent findings of an abnormal bile acid metabolism in liver disease.

The most common response of the injured liver is an abnormal accumulation of fat in the parenchymal cells. Indeed, various degrees of fatty infiltration have been found in association with many disease states in man, and there are many agents and means by which a fatty liver can be induced in experimental animals. Many agents seem to act via the same overall mechanism, which makes it difficult to differentiate between effects related to the specific agent and effects common to the group of agents (Farber, 1967; Witting, 1972). Ingestion of orotic acid in the rat is known to result in an experimental model characterized by a fatty liver and an almost complete inhibition of β -lipoprotein formation in and/or secretion from the liver (Standerfer & Handler, 1955; Handschumacher *et al.*, 1960; Windmueller, 1964; Novikoff *et al.*, 1966). The metabolic abnormalities associated with orotic acid feeding are not severe and animals have been maintained on such a diet for up to 250 days without major untoward effects (Creasey *et al.*, 1961).

Orotic acid treatment of male rats is reported to result in a decrease in the liver content of cytochrome P-450 (Holtzman & Gillette, 1969), but it has not been fully elucidated to what extent the various hydroxylase activities in the liver microsomal fraction are

influenced (Rubin, 1965; Holtzman & Gillette, 1969; Witting, 1972). We decided to find out whether the diminished amount of cytochrome P-450 in the fatty liver of orotic acid-treated rats would result in a common decrease in the activity of and/or in an altered substrate specificity of the microsomal hydroxylase systems. In the present paper we have studied the metabolism of some steroid hormones (androstene-dione, progesterone and 5 α -androstane-3 α ,17 β -diol), sterols (cholesterol, 7 α -hydroxy-4-cholesten-3-one and 5 β -cholestane-3 α ,7 α -diol) and one bile acid (lithocholic acid) in liver microsomal fractions from control and orotic acid-treated male rats.

Experimental

Materials

Radioactive steroids. 4-[4-¹⁴C]Androstene-3,17-dione (specific radioactivity 1.7 μ Ci/mg), 4-[4-¹⁴C]pregnene-3,20-dione (specific radioactivity 2 μ Ci/mg) and 4-[4-¹⁴C]cholesterol (specific radioactivity 145 μ Ci/mg) were from The Radiochemical Centre, Amersham, Bucks., U.K. Before use, [4-¹⁴C]cholesterol was purified by chromatography on a column (0.8 cm $\times$ 6.0 cm) of Al₂O₃, grade III (Woelm, Eschwege, Germany). 5 α -[4-¹⁴C]Androstane-3 α ,17 β -diol (specific radioactivity 3 μ Ci/mg) was synthesized as

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described by Berg & Gustafsson (1973). 7 α -Hydroxy-4-[6- ^{3} H]cholesten-3-one (specific radioactivity 6.7 μ Ci/mg) was prepared as described by Björkhem (1969). 5 β -[7- ^{3} H]Cholestane-3 α ,7 α -diol (specific radioactivity 16.7 μ Ci/mg) was synthesized as described by Björkhem & Gustafsson (1973). [^{3} H]-Lithocholic acid (specific radioactivity 10 μ Ci/mg) was from New England Nuclear Corp., Boston, MA, U.S.A. Before use, the radiochemical purity of the labelled compounds was tested.

Reference compounds. Several of the reference compounds used in this investigation to identify metabolites of 4-androstene-3,17-dione, 4-pregnene-3,20-dione and 5 α -androstane-3 α ,17 β -diol were generous gifts from colleagues: Dr. K. Babcock, Upjohn Co., Kalamazoo, MI, U.S.A. (4-androstene-3,17-dione, 4-pregnene-3,20-dione, 3 α - and 3 β -hydroxy-5 α -androstane-17-one, 7 α -hydroxy-4-androstene-3,17-dione, 20 α - and 20 β -hydroxy-4-pregnen-3-one and 6 β - and 16 α -hydroxy-4-pregnene-3,20-dione); Dr. W. Klyne, Medical Research Council Steroid Reference Collection, London NW3 7ST, U.K. (6 β - and 16 α -hydroxy-4-androstene-3,17-dione); Dr. M. Wolff, San Francisco, CA, U.S.A. (5 α -androstane-2 β ,3 α ,17 β -triol); Dr. P. N. Rao, San Antonio, TX, U.S.A. (2 α -hydroxy-4-pregnene-3,20-dione). 5 α -Androstane-3 α (and 3 β),17 β -diol and 17 β -hydroxy-5 α -androstane-3-one were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3 α -Hydroxy-5 α -pregnan-20-one and 3 β -hydroxy-5 α -pregnan-20-one were from Ikahparr (Ramat-Gan, P.O. Box 31, Israel).

Casein was manufactured by E. Merck, Darmstadt, Germany. Orotic acid (monosodium salt), NADP $^+$, NADPH, DL-isocitric acid (trisodium salt) and isocitrate dehydrogenase (type IV) were from Sigma. Vitamins (vitamin diet fortification mixture) and salts (Hawk Oser salt mixture no. 3) were from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.

Methods

Animals and preparation of homogenates. Adult male Sprague-Dawley rats weighing about 150 g were used. They were divided into two groups each of 12 animals. The first group received a semisynthetic diet containing (w/w): 70% glucose; 18% casein; 5% corn oil; 2% vitamins; 5% salts. The second group was fed on the same semisynthetic diet supplemented with 1% orotic acid. This food and water were given *ad libitum* for 10 days. After starvation overnight, six rats from each group were anaesthetized with diethyl ether. Blood drawn from the abdominal aorta was analysed for plasma cholesterol and triglycerides. EDTA (0.1 M, 25 μ l/ml of blood) was added as anticoagulant. The remaining six rats in each group were killed by a blow on the neck. The livers were excised, immediately chilled on ice, blotted, weighed and minced into small pieces. A

20% (w/v) homogenate was prepared in a modified Bucher medium (Bergström & Gloor, 1955) (0.1 M, pH 7.4), by using a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. A portion of this homogenate was refluxed at 80°C for 1 h with 5 vol. of chloroform/methanol (1:1, v/v), and the lipid extract was used for analysis of cholesterol and triglycerides. The remaining part of the homogenate was centrifuged for 15 min at 4°C and 20000g and the supernatant was spun at 4°C and 100000g for 70 min. The microsomal pellet was resuspended with Bucher medium to the same volume as before ultracentrifugation and gently rehomogenized.

The cholesterol concentrations of plasma, liver extracts and of the 20000g supernatant fluid were measured by the method of Hanel & Dam (1955). Plasma and liver triglycerides were determined as described by Laurell (1966). The protein concentrations of the 20000g supernatant fraction and of the microsomal fraction were assayed by a modification of Lowry's technique (Lowry *et al.*, 1951), with albumin as standard.

Incubations. 4-[4- ^{14} C]Androstene-3,17-dione (300 μ g), dissolved in 50 μ l of acetone, was added to a mixture of 0.3 ml of microsomal fraction and 2.7 ml of Bucher medium supplemented with 3 μ mol of NADPH. 4-[4- ^{14} C]Pregnene-3,20-dione (250 μ g), dissolved in 50 μ l of acetone, was mixed with 0.5 ml of microsomal fraction and 2.5 ml of Bucher medium supplemented with 3 μ mol of NADPH. 5 α -[4- ^{14} C]-Androstane-3 α ,17 β -diol (200 μ g), in 50 μ l of acetone, was added to 2.5 ml of the microsomal fraction, 1.5 ml of Bucher medium, 0.03 μ mol of MnCl $_2$, 3 μ mol of NADP $^+$, 12.5 μ mol of isocitrate and 10 μ l of isocitrate dehydrogenase solution (36 units/ml). Incubations were carried out for 10 min at 37°C and were terminated by the addition of 20 vol. of chloroform/methanol (2:1, v/v). The conditions under which the incubations were performed have been shown to give reactions that are linear with respect to enzyme concentration and time. The precipitate was filtered off, and 0.2 vol. of 0.9% NaCl was added. The chloroform phase was collected, and the solvent was evaporated. The residue was further analysed by t.l.c. (pre-coated silica-gel plates; 250 μ m thick; Merck, Darmstadt, Germany). The extracts from the incubations with 4-[4- ^{14} C]androstene-3,17-dione were developed in chloroform/ethyl acetate (4:1, v/v) and the extracts from the incubations with 4-[4- ^{14} C]-pregnene-3,20-dione in benzene/ethyl acetate (3:1, v/v). The organic material recovered after incubations with 5 α -[4- ^{14} C]androstane-3 α ,17 β -diol was developed in the solvent system ethyl acetate/cyclohexane (3:2, v/v). The t.l.c. plates were subjected to radioautography with an exposure time of about 10 days. The radioactivity zones on the t.l.c. plates traced from the X-ray films were scraped off, eluted with methanol and measured for radioactivity.

[4-¹⁴C]Cholesterol (13 µg), dissolved in 50 µl of acetone, was incubated for 20 min at 37°C with 3 ml of the 20000g supernatant fraction. The incubation was terminated by the addition of 20 vol. of chloroform/methanol (2:1, v/v). Analysis of the chloroform/methanol extract was performed as described previously (Danielsson *et al.*, 1967). 7α-Hydroxy-4-[6β-³H]cholesten-3-one (50 µg), dissolved in 50 µl of acetone, was added to a mixture of 2 ml of microsomal fraction and 1 ml of Bucher medium supplemented with 3 µmol of NADPH. The incubation was carried out for 12 min at 37°C. The incubation was terminated as described above and the organic extract was analysed as described by Björkhem & Einarsson (1970).

[³H]Lithocholic acid (50 µg), dissolved in 50 µl of aq. 95% (v/v) ethanol, was added to 1 ml of microsomal fraction and 2 ml of Bucher medium supplemented with 3 µmol of NADPH. The incubation was carried out for 20 min at 37°C and was terminated with an equal volume of 95% ethanol. Further analysis was performed as reported previously (Einarsson & Gustafsson, 1974).

Gas-liquid chromatography-mass spectrometry analysis of metabolites of 4-[4-¹⁴C]androstene-3,17-dione, 4-[4-¹⁴C]pregnene-3,20-dione and 5α-[4-¹⁴C]-androstane-3α,17β-diol. The methanol extracts of radioactive zones pooled from the t.l.c. plates were evaporated to dryness, and all material left after radioactivity measurements was trimethylsilylated and analysed by g.l.c.-mass spectrometry (LKB 9000 instrument) with the use of a 1.5% SE-30 column (Shackleton & Gustafsson, 1971). Mass spectra were recorded on magnetic tape with the incremental mode of operation and were then processed in an IBM 1800 computer (Reimendal & Sjövall, 1972). A compound was considered identified only if it had the same mass spectrum and g.l.c. behaviour as the reference compound.

Radioactivity measurements. Radioactivity was assayed in a Packard liquid-scintillation spectrometer, model 3003. The scintillation fluid contained

3 g of 2,5-diphenyloxazole and 150 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene. All samples were counted for radioactivity for at least 5 min.

Recovery. The recovery of metabolites from the entire procedure of extraction and elution from t.l.c. plate was determined to be 85–90%.

Statistical analysis. Student's test was used.

Results

Body weight, liver weight, cholesterol, triglycerides and protein

Compared with the controls, the orotic acid-fed rats were less heavy, but their liver weights had increased by about 30% (Table 1). Orotic acid feeding also resulted in an increase in the liver triglycerides (5-fold) and cholesterol (2-fold). The two groups of animals did not differ with regard to the cholesterol concentration in the 20000g supernatant fluid or the amount of protein in the microsomal fraction, but the amount of protein in the 20000g supernatant was slightly decreased in the orotic acid-fed animals.

The concentrations of serum cholesterol and triglycerides in the control rats averaged 81 ± 6 (mean ± s.e.m.) and 53 ± 7 mg/100 ml respectively. The corresponding values for the orotic acid-fed rats were significantly lower ($P < 0.02$) and amounted to 61 ± 4 (cholesterol) and 20 ± 6 (triglyceride) mg/100 ml.

Incubations with 4-[4-¹⁴C]androstene-3,17-dione

The conversion of substrate averaged about 11% in the control rats. On the basis of their chromatographic and mass spectrographic characteristics (Einarsson *et al.*, 1974) the various metabolites were identified as 6β-, 7α- and 16α-hydroxy-4-androstene-3,17-dione, 17β-hydroxy-4-androsten-3-one, 3α- and 3β-hydroxy-5α-androstan-17-one and 5α-androstane-3,17-dione. Individual values were not determined for the 3α- and 3β-hydroxy steroid reduction products as the corresponding derivatives were not separated in the t.l.c.

Table 1. *Body weight, liver weight and concentrations of lipids and protein in the liver of control and orotic acid-treated rats*

The values listed are the means ± s.e.m. of experiments with six male rats. N.S., No significance ($P > 0.05$).

	Control	Orotic acid-treated	Significance of differences
Body weight (g)	190 ± 3	176 ± 3	$P < 0.01$
Liver weight (g/100 g of rat)	5.0 ± 0.2	6.5 ± 0.3	$P < 0.005$
Liver triglycerides (mg/g)	8.4 ± 0.9	44.3 ± 3.7	$P < 0.001$
Liver cholesterol (mg/g)	2.5 ± 0.2	5.0 ± 0.1	$P < 0.001$
Cholesterol concentration of the 20000g supernatant fraction (mg/ml)	0.16 ± 0.01	0.16 ± 0.02	N.S.
Protein concentration of the 20000g supernatant fraction (mg/ml)	15.2 ± 0.4	13.4 ± 0.2	$P < 0.005$
Protein concentration of the microsomal fraction (mg/ml)	2.4 ± 0.2	2.2 ± 0.04	N.S.

Table 2. Metabolism of 4-[4-¹⁴C]androstene-3,17-dione in the hepatic microsomal fraction of control and orotic acid-treated rats

The conversions are calculated from the amounts of radioactivity in the different zones after t.l.c. and from peak-area determinations after g.l.c. The values listed are the means $\pm$ S.E.M. of experiments with six male rats. N.S., No significance ($P > 0.05$).

Reaction	Identified metabolites	Metabolism (nmol of metabolite formed/mg of protein)		Significance of differences
		Control	Orotic acid-treated	
6 β -Hydroxylation	6 β -Hydroxy-4-androstene-3,17-dione	21.2 $\pm$ 2.3	10.9 $\pm$ 0.5	$P < 0.01$
7 α -Hydroxylation	7 α -Hydroxy-4-androstene-3,17-dione	4.95 $\pm$ 0.81	2.76 $\pm$ 0.11	$P < 0.05$
16 α -Hydroxylation	16 α -Hydroxy-4-androstene-3,17-dione	21.4 $\pm$ 4.7	13.2 $\pm$ 1.2	N.S.
17 β -Hydroxy steroid reduction	17 β -Hydroxy-4-androsten-3-one	19.7 $\pm$ 1.7	20.9 $\pm$ 1.2	N.S.
3 α -+3 β -Hydroxy steroid reduction	3 α - and 3 β -Hydroxy-5 α -androstan-17-one	15.5 $\pm$ 1.0	7.35 $\pm$ 0.26	$P < 0.001$
5 α -Reduction	5 α -Androstane-3,17-dione, and 3 α - and 3 β -hydroxy-5 α -androstan-17-one	78.4 $\pm$ 6.3	40.6 $\pm$ 3.4	$P < 0.05$

system used. The identified metabolites constituted about 90% of the total conversion products formed.

Compared with the controls, the orotic acid-fed rats showed a 50% decrease in the 6 β - and 7 α -hydroxylation reactions as well as in 3-hydroxy steroid reduction and 5 α -reduction as evidenced by the combined formation of 5 α -androstane-3,17-dione and 3 α - and 3 β -hydroxy-5 α -androstan-17-one (Table 2). A similar tendency was also observed for the 16 α -hydroxylation reaction, whereas the 17 β -hydroxy steroid reduction was unaffected by orotic acid treatment.

Incubations with 4-[4-¹⁴C]pregnene-3,20-dione

The total conversion of this material when incubated with the microsomal liver fraction was 13–14% in the control rats. About 75% of the radioactivity not associated with progesterone was recovered in the various metabolites (Einarsson *et al.*, 1973) shown in Table 3. The parent compound had been subjected to 2 α -, 6 β - and 16 α -hydroxylation, 3 β - and 20-hydroxy steroid reduction, and 5 α -reduction. The 20-hydroxy-4-pregnen-3-one fraction was most probably made up of both the 20 α - and the 20 β -isomers. The 5 α -reduction (measured as the combined amount of 5 α -pregnane-3,20-dione, 3 β -hydroxy-5 α -pregnan-20-one and 2 α ,3 α -dihydroxy-5 α -pregnan-20-one) was the major reaction, contributing in the controls to more than 50% of the metabolites formed. The effect of orotic acid feeding was reflected in a 50% decrease in 5 α -reduction, whereas other reactions occurred at approximately the same rate in both groups of animals (Table 3).

Incubations with 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol

Approx. 12% of the incubated material in the controls was converted into various polar products (Table 4), which were all identified as described by Berg & Gustafsson (1973). The major one in both groups was 5 α -androstane-2 α ,3 α ,17 β -triol. Other metabolites were 2 β -, 7 α -, 7 β - and 18-hydroxylated derivatives of the parent compound. After treatment with orotic acid, the activities of the 2 α -, 2 β - and 18-hydroxylases decreased about 40%. The 7 α - and 7 β -hydroxylations were not significantly decreased (Table 4).

Incubations with [4-¹⁴C]cholesterol

The main products formed under the present conditions have previously been identified as 5-cholestone-3 β ,7 α -diol, 7 α -hydroxy-4-cholesten-3-one and 7 α ,12 α -dihydroxy-4-cholesten-3-one (Danielsson & Einarsson, 1966). The pattern of metabolites formed in the present experiments was the same in both groups of animals. The conversion of cholesterol into 7 α -hydroxylated products was about 0.3% (0.079 $\pm$ 0.008 nmol/mg of protein) in the control rats and almost the same in the orotic acid-fed animals (Table 5).

Incubations with 7 α -hydroxy-4-[6-³H]cholesten-3-one

The main metabolites formed from 7 α -hydroxy-4-cholesten-3-one in the presence of the microsomal liver fraction are 7 α ,12 α -dihydroxy-4-cholesten-3-one, 7 α -hydroxy-5 α -cholestan-3-one and 5 α -cholestone-3 β ,7 α -diol (Danielsson & Einarsson, 1966; Björkhem & Einarsson, 1970). In the control rats,

Table 3. *Metabolism of 4-[4-¹⁴C]pregnene-3,20-dione in the hepatic microsomal fraction of control and orotic acid-treated rats*

The conversions are calculated from the amounts of radioactivity in the different zones after t.l.c. and from peak-area determinations after g.l.c. The values listed are the means $\pm$ s.e.m. of experiments with six male rats. N.S., No significance ($P > 0.05$).

Reaction	Identified metabolites	Metabolism (nmol of metabolite formed/mg of protein)		Significance of differences
		Control	Orotic acid-treated	
2 α -Hydroxylation	2 α -Hydroxy-4-pregnene-3,20-dione and 2 α ,3 α -dihydroxy-5 α -pregnan-20-one	7.26 $\pm$ 1.50	4.78 $\pm$ 0.38	N.S.
6 β -Hydroxylation	6 β -Hydroxy-4-pregnene-3,20-dione	8.46 $\pm$ 0.85	6.22 $\pm$ 0.35	N.S.
16 α -Hydroxylation	16 α -Hydroxy-4-pregnene-3,20-dione	5.18 $\pm$ 0.64	4.60 $\pm$ 0.33	N.S.
20 α - + 20 β -Hydroxy steroid reduction	20 α - and 20 β -Hydroxy-4-pregnen-3-one	3.96 $\pm$ 0.28	3.56 $\pm$ 0.18	N.S.
3 β -Hydroxy steroid reduction	3 β -Hydroxy-5 α -pregnan-20-one	5.00 $\pm$ 0.30	6.18 $\pm$ 0.66	N.S.
5 α -Reduction	5 α -Pregnane-3,20-dione, 3 β -hydroxy-5 α - pregnan-20-one and 2 α ,3 α -dihydroxy- 5 α -pregnan-20-one	44.0 $\pm$ 2.6	20.9 $\pm$ 1.3	$P < 0.001$

Table 4. *Metabolism of 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol in the hepatic microsomal fraction of control and orotic acid-treated rats*

The conversions are calculated from the amounts of radioactivity in the different zones after t.l.c. and from peak-area determinations after g.l.c. The values listed are the means $\pm$ s.e.m. of experiments with six male rats. N.S., No significance ($P > 0.05$).

Reaction	Identified metabolites	Metabolism (nmol of metabolite formed/mg of protein)		Significance of differences
		Control	Orotic acid-treated	
2 α -Hydroxylation	5 α -Androstane-2 α ,3 α ,17 β -triol	6.76 $\pm$ 0.38	3.94 $\pm$ 0.61	$P < 0.05$
2 β -Hydroxylation	5 α -Androstane-2 β ,3 α ,17 β -triol	2.11 $\pm$ 0.19	1.31 $\pm$ 0.17	$P < 0.05$
7 α -Hydroxylation	5 α -Androstane-3 α ,7 α ,17 β -triol	2.50 $\pm$ 0.37	1.95 $\pm$ 0.31	N.S.
7 β -Hydroxylation	5 α -Androstane-3 α ,7 β ,17 β -triol	0.77 $\pm$ 0.08	0.62 $\pm$ 0.11	N.S.
18-Hydroxylation	5 α -Androstane-3 α ,17 β ,18-triol and 5 α -androstane-3 β ,17 β ,18-triol	2.30 $\pm$ 0.30	1.28 $\pm$ 0.18	$P < 0.05$

6–7% (1.78 $\pm$ 0.12 nmol/mg of protein) of the 7 α -hydroxy-4-cholesten-3-one was converted into 7 α ,12 α -dihydroxy-4-cholesten-3-one and 8–9% of the incubated radioactivity was recovered as 7 α -hydroxy-5 α -cholestan-3-one and 5 α -cholestane-3 β ,7 α -diol. The 12 α -hydroxylation and the 5 α -reduction were both decreased by about 50% in the orotic acid-fed rats (Table 5).

Incubations with 5 β -[7-³H]cholestane-3 α ,7 α -diol

The metabolites produced in this system are 5 β -cholestane-3 α ,7 α ,12 α -triol and 5 β -cholestane-3 α ,7 α ,26-triol (Björkhem & Gustafsson, 1973). The conversion of the substrate into the former metabolite

was small in both groups of rats and averaged about 0.6%. The amount of 5 β -cholestane-3 α ,7 α -diol subjected to 26-hydroxylation amounted to 2.3 $\pm$ 0.2 (1.3%) and 1.8 $\pm$ 0.3 nmol/mg of protein in the control and orotic acid-fed rats respectively (Table 5).

Incubations with [³H]lithocholic acid

The main metabolite formed from lithocholic acid upon incubation with the microsomal fraction of rat liver homogenates is 3 α ,6 β -dihydroxy-5 β -cholanoic acid (Einarsson & Johansson, 1969; Einarsson & Gustafsson, 1974). The extent of 6 β -hydroxylation in the present study was about 11% (6.1 $\pm$ 0.7 nmol/mg of protein) in the control rats and about 15% in the

Table 5. *Metabolism of [4-¹⁴C]cholesterol, 7 α -hydroxy-4-[6 β -³H]cholesten-3-one, 5 β -[7 β -³H]cholestane-3 α ,7 α -diol and [³H]lithocholic acid in control and orotic acid-treated rats*

The conversions are calculated from the amounts of radioactivity in the different zones after t.l.c. N.S., No significance ($P > 0.05$).

Substrate	Reaction	Metabolism (nmol of metabolite formed/mg of protein)		Significance of differences
		Control	Orotic acid-treated	
Cholesterol	7 α -Hydroxylation	0.079 $\pm$ 0.008	0.066 $\pm$ 0.009	N.S.
7 α -Hydroxy-4-cholesten-3-one	12 α -Hydroxylation	1.78 $\pm$ 0.12	0.86 $\pm$ 0.07	$P < 0.001$
	5 α -Reduction	2.27 $\pm$ 0.24	1.26 $\pm$ 0.16	$P < 0.02$
5 β -Cholestane-3 α ,7 α -diol	26-Hydroxylation	2.3 $\pm$ 0.2	1.8 $\pm$ 0.3	N.S.
Lithocholic acid	6 β -Hydroxylation	6.1 $\pm$ 0.7	8.5 $\pm$ 0.4	$P < 0.05$

animals fed with orotic acid and this difference was significant ($P < 0.05$) (Table 5).

Discussion

The present findings that the ingestion of a semi-synthetic diet containing 1% orotic acid results in an accumulation of triglycerides and cholesterol in the liver and a lower concentration of both lipids in serum are in agreement with previous reports (Standerfer & Handler, 1955; Handschumacher *et al.*, 1960; Windmueller, 1964; Novikoff *et al.*, 1966). Perfusion studies in rats have demonstrated that the lower concentrations of serum lipids are due to inability of the liver of orotic acid-fed rats to secrete and/or produce more than a fraction of the low-density lipoproteins excreted by normal livers (Windmueller & Levy, 1967). This defect does not seem to reflect an impaired synthesis of the protein constituents of the lipoproteins, since both low-density-lipoprotein and very-low-density-lipoprotein antigens have been isolated from the liver cells of orotic acid-treated animals (Pottenger & Getz, 1971).

As evidenced by the present study orotic acid feeding influenced the male rat liver metabolism of steroids in different and somewhat unpredictable ways. It led to a decreased activity of several hydroxylases involved in the hepatic metabolism of 4-androstene-3,17-dione (6 β - and 7 α -) and 5 α -androstane-3 α ,17 β -diol (2 α -, 2 β - and 18-) and of the 12 α -hydroxylase involved in cholic acid synthesis. On the other hand it did not significantly affect the hydroxylations of 4-pregnene-3,20-dione, the 16 α -hydroxylation of 4-androstene-3,17-dione and the 7 α - and 7 β -hydroxylation of 5 α -androstane-3 α ,17 β -diol. Neither were the 7 α - and 26-hydroxylases, involved in bile acid biosynthesis, influenced by orotic acid treatment. The oxidative metabolism of steroid hormones in the liver is catalysed by a microsomal enzyme system, which also participates in the inactivation of drugs. Lu *et al.* (1972, 1973) have resolved this enzyme

system and isolated a CO-binding haemoprotein (cytochrome *P*-450), and NADPH-dependent reductase and phosphatidylcholine as indispensable components. Using the reconstituted hydroxylation system from liver microsomal fraction, they also demonstrated that enzyme systems prepared from rats pretreated with a drug (phenobarbital) or a chemical agent (pregnenolone-16 α -carbonitrile) showed different substrate specificities and further that these specificities reside in the cytochrome fraction. Among the bile acid hydroxylases, the 6 β -hydroxylase seems to be similar to the drug-metabolizing enzymes. Although the hydroxylases involved in the conversion of cholesterol into bile acids seem to be more specific, they are all dependent on the activity of the cytochrome *P*-450 fraction (Björkhem & Danielsson, 1974). Since orotic acid feeding appears to result in a decrease in cytochrome *P*-450 in the male rat liver (Holtzman & Gillette 1969), it is possible that part of the enzymic changes recorded in the present study may be due to a diminished amount of the cytochrome. However, several hydroxylases were not affected by orotic acid treatment and the 6 β -hydroxylase active on lithocholic acid was stimulated by about 40%, which indicates that not only had the bulk of cytochrome *P*-450 been diminished, but the properties of the cytochrome had also been altered. 5 α -Reduction of 4-androstene-3,17-dione, 4-pregnene-3,20-dione and 7 α -hydroxy-4-cholesten-3-one was decreased by about 50% in the orotic acid-fed rats. These enzymic changes cannot be ascribed to a smaller amount of cytochrome *P*-450, but may suggest a more general effect on the liver system.

The finding that the 12 α -hydroxylase was more affected by liver damage than the 7 α -hydroxylase (i.e. the enzyme conducting the rate-limiting step in the conversion of cholesterol into bile acids) and the 26-hydroxylase may have a bearing on the following observations in human subjects with portal liver cirrhosis. In patients with mild to moderate liver damage the total bile acid formation remains within

normal limits. However, probably because of a defective 12 α -hydroxylase, the formation of cholic acid is subnormal, whereas the synthesis of chenodeoxycholic acid tends to be correspondingly increased (Einarsson *et al.*, 1975). With increasing degree of liver cirrhosis, the formation of cholic acid decreases gradually (Vlahcevic *et al.*, 1972; McCormic *et al.*, 1973).

As the liver concentration of cytochrome P-450 is decreased by about 50% in patients with severe hepatitis and cirrhosis (Schoene *et al.*, 1972) and further, the demethylation rate of aminopyrine and p-nitroanisole is similarly decreased, the hepatic metabolism of steroid hormones might be expected to undergo changes in the damaged human liver.

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