Eur. J. Biochem. 21 (1971) 68—79

Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Rat Liver Microsomes

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(Received December 10, 1970/February 8, 1971)

The metabolism of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and the formation of lipid peroxides from endogenous lipids were studied in rat liver microsomes fortified with NADPH. Under conditions of NADPH-dependent lipid peroxidation cholesterol was oxidized into several metabolites including 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol. When lipid peroxidation was inhibited, cholesterol was converted predominantly into 5-cholestene-3β,7α-diol. Incubation of cholesterol with soybean lipoxidase and linoleic acid yielded the same pattern of products as incubation of cholesterol with microsomal fraction under conditions of NADPH-dependent lipid peroxidation. 3β-Hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were metabolized by the microsomal fraction in essentially the same way whether or not lipid peroxidation was inhibited. The main metabolite of 3β-hydroxy-5-pregnen-20-one could not be identified. Gas-chromatographic and mass-spectrometric analyses indicated that it was a monohydroxy derivative of 3β-hydroxy-5-pregnen-20-one. Other hydroxylated metabolites of 3β-hydroxy-5-pregnen-20-one were 3β,7α-dihydroxy-5-pregnen-20-one, 3β,7β-dihydroxy-5-pregnen-20-one and 3β,16α-dihydroxy-5-pregnen-20-one. Major hydroxylated metabolites of 3β-hydroxy-5-androsten-17-one were 3β,7α-dihydroxy-5-androsten-17-one, 3β,7β-dihydroxy-5-androsten-17-one and 3β,16α-dihydroxy-5-androsten-17-one.

Biliary drainage resulted in an eight-fold stimulation of the 7α-hydroxylation of cholesterol but inhibited all hydroxylations of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one. Phenobarbital treatment had no significant effect on the 7α-hydroxylation of cholesterol or the formation of lipid peroxides but significantly stimulated the formation of 3β,16α-dihydroxy-5-pregnen-20-one, 3β,7α-dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one. All hydroxylations were inhibited by carbon monoxide. No significant inhibition of lipid peroxidation by carbon monoxide was observed.

The introduction of a 7α-hydroxyl group is the first and rate-limiting step in the biosynthesis of cholic acid from cholesterol [1]. This reaction as well as a large number of other hydroxylations are catalyzed by the microsomal fraction of liver fortified with NADPH and in the presence of oxygen [2—5]. An electron-transport chain including cytochrome P-450 as the terminal oxidase appears to be involved in many of these hydroxylations including the 7α-hydroxylation of cholesterol [4]. In a previous communication from this laboratory the enzymatic formation of 3β-hydroxy-5-cholesten-7-one from cholesterol in the presence of the microsomal fraction of rat liver homogenate fortified with NADPH was reported [6]. It was also found that the extent of conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one was much lower in the presence of the 20000×*g* supernatant fluid and that there appeared to be an inverse correlation between the extent of conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one and into 5-cholestene-3β,7α-diol. The formation of 5-cholestene-3β,7α-diol was more efficient in the presence of the 20000×*g* supernatant fluid than in the presence of the microsomal fraction [6]. The microsomal fraction of liver homogenate fortified with NADPH has been shown to catalyze the formation of lipid peroxides from some polyunsaturated fatty acids such as linoleic acid and linolenic acid, and it has been suggested that the processes of peroxidation and hydroxylation are closely related and may be a result of the operation of the same electron-transport chain [7—10]. Since lipid peroxidation resembles the conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one in being much more efficient in the presence of microsomal fraction fortified with NADPH than in the presence of microsomal fraction plus the supernatant fluid, it appeared possible that there is a connection between the formation of lipid peroxides from endogenous

Nomenclature. The systematic name of cholic acid is 3α,7α,12α-trihydroxy-5β-cholanoic acid.

fatty acids and the formation of 3β-hydroxy-5-cholesten-7-one [11]. Recently, Wills [9] reported that when the microsomal fraction was prepared in a sucrose medium containing EDTA, the formation of lipid peroxides was inhibited efficiently. To obtain information on the possible relation between the formation of lipid peroxides and the oxidation of cholesterol in rat liver microsomes, the metabolism of cholesterol has been studied in fortified microsomal fractions prepared in the presence and absence of EDTA. Since the microsomal fraction catalyzes oxidation in the 7-position of Δ^5-3β-hydroxysteroids of the C_{19} and C_{21} series, it appeared of interest to include such steroids in the study.

EXPERIMENTAL PROCEDURE

Materials

[4-^{14}C]Cholesterol (specific radioactivity, 145 μCi/mg), 3β-hydroxy-5-[4-^{14}C]pregnen-20-one (1.2 μCi/mg) and 3β-hydroxy-5-[4-^{14}C]androsten-17-one (0.5 μCi/mg) were obtained from the Radiochemical Centre (Amersham, England). In experiments with soybean lipoxidase [4-^{14}C]cholesterol with a specific radioactivity of 10 μCi/mg and 3β-hydroxy-5-[4-^{14}C]-pregnen-20-one with a specific radioactivity of 0.6 μCi/mg were used. Prior to use, [4-^{14}C]cholesterol was purified by chromatography on a column of neutral aluminium oxide, grade III (Woelm, Eschwege, Germany). The other labeled compounds were purified by chromatography on columns of hydroxyalkoxypropyl-Sephadex with methanol—water—1,2-dichloroethane (7:3:1, v/v/v) as solvent [12].

5-Cholestene-3β,7α-diol (m.p. 183—185 °C, reported [13] m.p. 185 °C), 5-cholestene-3β,7β-diol (m.p. 172—173 °C, reported [14] m.p. 178 °C), 3β-hydroxy-5-cholesten-7-one (m.p. 172 °C, reported [15] m.p. 170—172 °C), 5-cholestene-3β,7α,12α-triol (m.p. 194 to 195 °C, reported [16] m.p. 194—195 °C) and cholestane-3β,5α,6β-triol (m.p. 231—234 °C, reported [17] m.p. 237—239 °C) were synthesized according to methods described previously [16,18]. 3β,7α-Dihydroxy-5-pregnen-20-one (m.p. 169—170 °C, $[\alpha]_D^{23}$ −46° (*c*, 0.4 in chloroform); reported [19] m.p. 190 °C, $[\alpha]_D$ −28°), 3β,7β-dihydroxy-5-pregnen-20-one (m.p. 188—190 °C, $[\alpha]_D^{23}$ +45° (*c*, 0.4 in chloroform)), 3β,7α-dihydroxy-5-androsten-17-one (m.p. 175 to 176 °C, $[\alpha]_D^{23}$ −70° (*c*, 0.4 in chloroform); reported [20] m.p. 181.5—183.5 °C, $[\alpha]_D$ −70.7°) and 3β,7β-dihydroxy-5-androsten-17-one (m.p. 184—185 °C, $[\alpha]_D^{23}$ +59° (*c*, 0.4 in chloroform); reported [20] m.p. 215 to 216 °C, $[\alpha]_D$ +67.5°) were prepared according to procedures described by Stárka [21] and were purified by chromatography on columns of hydrophobic Hyflo Super-Cel with phase system F1 [22]. The crystallized compounds were analyzed by thin-layer chromatography and as trimethylsilyl ethers by combined gas chromatography—mass spectrometry with the LKB 9000 instrument equipped with a 1.5% SE-30 column. These analyses confirmed the purity and identity of the synthesized compounds. Although the samples had been carefully dried, it is possible that the differences between the melting points reported for 3β,7α-dihydroxy-5-pregnen-20-one and 3β,7β-dihydroxy-5-androsten-17-one and those found in the present investigation are due to the presence of solvent in the crystals. 3β-Hydroxy-5-pregnene-7,20-dione (m.p. 208—211 °C; reported [23] m.p. 209 to 210 °C) and 3β-hydroxy-5-androstene-7,17-dione (m.p. 238—240 °C; reported [20] m.p. 243—244.5 °C) were prepared from 3β-hydroxy-5-pregnen-20-one 3-acetate and 3β-hydroxy-5-androsten-17-one 3-acetate, respectively, by oxidation with *tert*-butyl chromate followed by hydrolysis with potassium carbonate [24,25]. 3β,16α-Dihydroxy-5-pregnen-20-one (m.p. 250—252 °C; reported [26] m.p. 252—255 °C) was a generous gift of Dr. J.-Å. Gustafsson. 3β,16α-Dihydroxy-5-androsten-17-one (m.p. 174—175 °C; reported [27] m.p. 177—181 °C) was obtained from Sigma Chemical Co. (St. Louis, Mo.) NADH, NADPH, *cis*-linoleic acid and soybean lipoxidase (83000 units/mg) were obtained from Sigma Chemical Co.

Methods

Animal Experiments. Male rats of the Sprague-Dawley strain weighing 150—200 g were used. In experiments with rats with a biliary fistula the animals were starved but had free access to drink; saline for the rats with a biliary fistula and tap water for the control rats. These experiments lasted for 60 h. Phenobarbital (100 mg/kg body weight) in 1 ml of saline was administered intraperitoneally daily for 5 days. The animals were killed 24 h after the last injection. In the phenobarbital experiments the animals had free access to drink and a commercial pellet diet.

Preparation of Rat Liver Microsomes. Liver homogenates (20%, w/v) were prepared in 0.25 M sucrose or in 0.25 M sucrose containing 0.001 M EDTA using a Potter-Elvehjem homogenizer equipped with a loosely fitting teflon pestle. The homogenate was centrifuged at 800×*g* for 10 min and at 20000×*g* for 10 min. The 20000×*g* supernatant fluid was centrifuged at 100000×*g* for 1 h. The resulting pellet was drained carefully and suspended in 0.1 M potassium phosphate, pH 7.0, containing 0.028 M nicotinamide. In some experiments 0.1 M Tris-Cl buffer, pH 7.0, was used. The microsomal fraction was suspended by homogenizing with a loosely fitting pestle in a volume corresponding to the original 20000×*g* supernatant. In experiments with variations of pH the microsomal fraction was suspended in 0.125 M potassium chloride. Boiled microsomal fraction was obtained by heating the microsomal suspension at 80 °C for 5 min followed by centrifugation at 800×*g* for 10 min.

Experiments with Rat Liver Microsomes

Incubations with [4-^{14}C]Cholesterol. Microsomal fraction, 3 ml; buffer, 2 ml; [4-^{14}C]cholesterol, 15 μg dissolved in 50 μl acetone; and NADPH, 5 μmoles; were incubated for 15 min at 37 °C. Incubation was terminated by the addition of 20 volumes chloroform—methanol (2:1, v/v). The precipitate was filtered off and 0.2 volumes of a 0.9% (w/v) sodium chloride solution were added. The residue of the chloroform phase together with appropriate reference compounds as internal standards was subjected to thin-layer chromatography with Kieselgel G (Merck, Darmstadt, Germany) as adsorbent and benzene—ethyl acetate (2:3, v/v), as solvent. The internal standards were visualized by iodine vapor. The iodine was evaporated at room temperature, the appropriate zones were scraped into test tubes and extracted with 5 ml methanol by vigorous stirring. The silica gel was allowed to settle by gravity and 1 ml of the methanol solution was evaporated in counting vials and assayed for radioactivity.

Incubations with 3β-Hydroxy-5-[4-^{14}C]pregnen-20-one. Microsomal fraction, 1 ml, and 3 μmoles NADPH were diluted with buffer to 3 ml. In experiments with phenobarbital-treated rats and the corresponding control rats, 0.5 ml of microsomal fraction was used. 3β-Hydroxy-5-[4-^{14}C]pregnen-20-one, 100 μg dissolved in 50 μl of acetone, was added and incubation was carried out for 10 min at 37 °C. The incubation was terminated, extracted and worked up as described above but with 2,2,4-trimethylpentane-isoamyl acetate—acetone (2:2:1, v/v/v), as solvent for thin-layer chromatography. The chromatoplates were run three times in the same solvent with drying of the plates between the runs.

Incubations with 3β-Hydroxy-5-[4-^{14}C]androsten-17-one. Microsomal fraction, 0.5 ml, and 3 μmoles of NADPH were diluted with buffer to 3 ml. 3β-Hydroxy-5-[4-^{14}C]androsten-17-one, 200 μg dissolved in 50 μl of acetone, was added. Incubation was carried out for 10 min at 37 °C. Analysis was performed as described above for incubations with 3β-hydroxy-5-[4-^{14}C]pregnen-20-one.

Assay of Lipid Peroxide Formation. To measure the rate of formation of lipid peroxides, samples of 0.5 ml were taken every fifth min from incubations with [4-^{14}C]cholesterol. The content of lipid peroxides in the samples was estimated by determining malonaldehyde with the thiobarbituric-acid method as described by Wilbur, Bernheim and Shapiro [28]. The molar absorption coefficient $\varepsilon_{530} = 1.56 \times 10^5\ M^{-1} \times cm^{-1}$ [29] was used.

Determination of Protein and Cholesterol. Protein was determined according to Lowry *et al.* [30]. The protein concentration of the microsomal fraction varied between 2 and 3.5 mg per ml. Cholesterol was determined as described by Hanel and Dam [31].

Experiments with Soybean Lipoxidase

[4-^{14}C]Cholesterol, 3β-hydroxy-5-[4-^{14}C]pregnen-20-one or 3β-hydroxy-5-[4-^{14}C]androsten-17-one, 200 μg dissolved in 50 μl of acetone, was suspended in 1.5 ml of 0.1 M Tris-Cl buffer, pH 7.5. Soybean lipoxidase, 100 μg (8300 units) dissolved in 0.1 ml of 0.1 M Tris-Cl buffer, pH 7.5, and/or 400 μg of linoleic acid were added and the mixture was shaken for 10 min at 30 °C. Prior to addition, the linoleic acid was converted into its ammonium salt by the addition of 0.25 ml 0.019 M NH_4OH/mg linoleic acid. Further analysis was carried out as described above for the different substrates.

Radioactivity Assay

Radioactivity was measured with a Packard scintillation spectrometer, model 4322, with a counting efficiency for ^{14}C of 73%. The scintillant consisted of a solution of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene.

Statistical Analysis

The Student *t*-test was used and the significance level was set at 0.01.

RESULTS

Oxidation of Cholesterol by Rat Liver Microsomes Prepared in Sucrose Medium

Fig. 1A shows a thin-layer chromatogram of the chloroform extract of an incubation of [4-^{14}C]cholesterol with microsomal fraction prepared in sucrose medium and fortified with NADPH. The major products were identified as 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol by crystallization to constant specific radioactivity together with the authentic compounds (for details concerning these results see Annex Table 1). As is evident from the thin-layer chromatogram shown in Fig. 1A several other products were formed. No attempt was made to identify these products. The results of the crystallizations also showed the presence of small amounts of other unknown compounds. The formation of all products required the addition of NADPH to the microsomal fraction (Fig. 1B) and was reduced by about 85% by boiling the microsomal fraction. Fig. 2 shows the effect of various factors on the conversion of cholesterol into 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol. The formation of lipid peroxides was measured simultaneously. Oxidation of cholesterol as well as formation of lipid peroxides were linear with time for at least 20 min (Fig. 2A). The rate of oxidation of cholesterol was constant between 0.5 and 1.5 mg of microsomal protein per ml. It should be pointed out

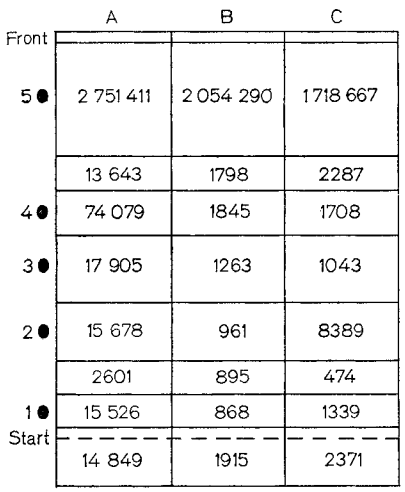

Fig. 1. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with microsomal fraction.* Three ml of microsomal fraction, prepared in sucrose (A and B) or in EDTA-sucrose (C) were diluted with buffer to 5 ml and incubated for 15 min with 15 μg of [4-^{14}C]cholesterol. In experiments shown in A and C, 5 μmoles of NADPH were added to the incubations. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v)

that the amount of [4-^{14}C]cholesterol added to the incubations is small in comparison with the amount of cholesterol present in the microsomal fraction and that estimation of cholesterol oxidation is based on radioactivity. The rate of formation of lipid peroxides decreased with increasing protein concentration (Fig. 2B). Between 0.5 and 1.5 mg of microsomal protein per ml, the total amount of lipid peroxides formed was constant and was thus not influenced by protein concentration. The effect of varying concentrations of NADPH on oxidation of cholesterol and formation of lipid peroxides is shown in Fig. 2C. The rate of reaction with NADH was less than 10% of that with NADPH. NADPH could be replaced by ascorbate but the rate of reaction was much slower (Fig. 2D). The pH-optimum for oxidation of cholesterol as well as formation of lipid peroxides was about 6.5 with NADPH as cofactor (Fig. 2E) and about 6.0 with ascorbate (Fig. 2F). With NADPH as cofactor cholesterol oxidation and lipid-peroxide formation were stimulated about four-fold by addition of Fe^{2+} at a concentration of 0.01 mM and about six-fold by Fe^{2+} at a concentration of 0.1 mM.

Oxidation of Cholesterol by Rat Liver Microsomes Prepared in EDTA-Sucrose Medium

Fig. 1C shows a thin-layer chromatogram of the chloroform extract of an incubation of [4-^{14}C]cholesterol with microsomal fraction prepared in EDTA-sucrose medium and fortified with NADPH. The predominant product was identified as 5-cholestene-3β,7α-diol (Annex, Table 2). Only small amounts of 3β-hydroxy-5-cholesten-7-one and 5-cholestene-3β,7β-diol were formed (Fig. 1C). The amounts of lipid peroxides formed were very small and were estimated to be less than 2% of those formed in incubations of microsomal fraction prepared without EDTA. The rate of conversion of cholesterol into 5-cholestene-3β,7α-diol was linear with time for at least 15 min (Fig. 3A). The rate of reaction was constant between 0.5 and 1.5 mg of microsomal protein per ml (Fig. 3B). The effect of varying concentrations of NADPH is shown in Fig. 3C. The rate of reaction with NADH was less than 10% of that with NADPH. The pH-optimum for the 7α-hydroxylation of cholesterol was about 7.0 (Fig. 3D). The effect on the reaction of various divalent metal ions is summarized in Table 1. All metal ions were added as chloride salts except Fe^{2+} which was added as sulfate. Sulfate as such had no effect on the reaction. In these experiments the microsomal fraction was suspended in Tris-Cl buffer instead of phosphate buffer. The rate of 7α-hydroxylation in Tris-Cl buffer was about half of that in phosphate buffer. The 7α-hydroxylation was stimulated by Fe^{2+} ions and inhibited by Cu^{2+} and Hg^{2+} ions. The stimulation of the 7α-hydroxylation by Fe^{2+} ions was accompanied by stimulation of the formation of 3β-hydroxy-5-cholesten-7-one and 5-cholestene-3β,7β-diol and of lipid peroxides. In fact, the oxidation of cholesterol and the formation of lipid peroxides in the presence of 0.1 mM Fe^{2+} ion proceeded as with microsomal fraction prepared without EDTA in the homogenizing medium. The effects of Zn^{2+} and Ni^{2+} ions were similar to those of Fe^{2+} ions but were less pronounced.

Oxidation of Cholesterol by Lipid Peroxides

[4-^{14}C]Cholesterol was incubated with soybean lipoxidase in the presence of linoleic acid. Fig. 4A shows a thin-layer chromatogram obtained from such an incubation. Several labeled products were formed including 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol (Annex, Table 3). No attempt was made to identify the other products. Without linoleic acid and/or lipoxidase very small amounts of oxidized products were formed (Fig. 4B—D).

Oxidation of 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Rat Liver Microsomes

Fig. 5 shows thin-layer chromatograms of chloroform extracts of incubations of 3β-hydroxy-5-[4-^{14}C]-pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one with the microsomal fraction prepared in EDTA-sucrose medium, with and without NADPH

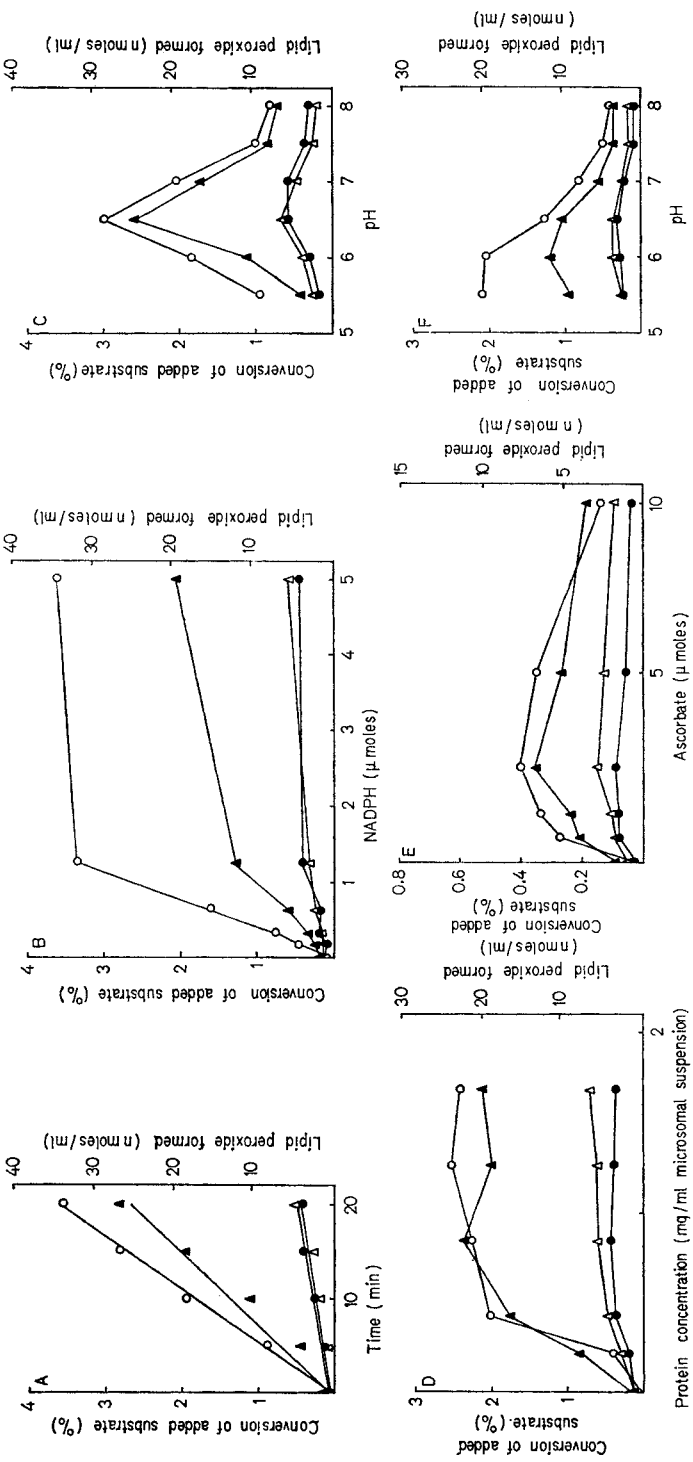

Fig. 2. *Effect of time (A), amount of microsomal fraction (D), NADPH (B), ascorbate (E) and pH (C, F) on oxidation of cholesterol and formation of lipid peroxides by microsomal fraction prepared in sucrose.* The standard incubation mixture consisted of 3 ml of microsomal fraction, 5 μmoles of NADPH and buffer, pH 7.0, to a final volume of 5 ml. This mixture was incubated for 15 min with 15 μg of [4-^{14}C]cholesterol. In D and F, NADPH was substituted with 2.5 μmoles of ascorbate. O, Formation of lipid peroxides, in nmoles of malonaldehyde formed per ml microsomal suspension; ▲, formation of 3β-hydroxy-5-cholesten-7-one; ●, formation of 5-cholestene-3β,7α-diol; △, formation of 5-cholestene-3β,7β-diol

added to the incubations. The main products isolated had the chromatographic properties of the corresponding 7-oxo, 7α-, 7β- and 16α-hydroxy derivatives. At least one other labeled compound was formed from both 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one. No attempt was made to identify these compounds. The identity of the 7-oxo, 7α-, 7β- and 16α-hydroxy compounds was established by crystallization to constant specific radioactivity together with the authentic compounds (Annex,

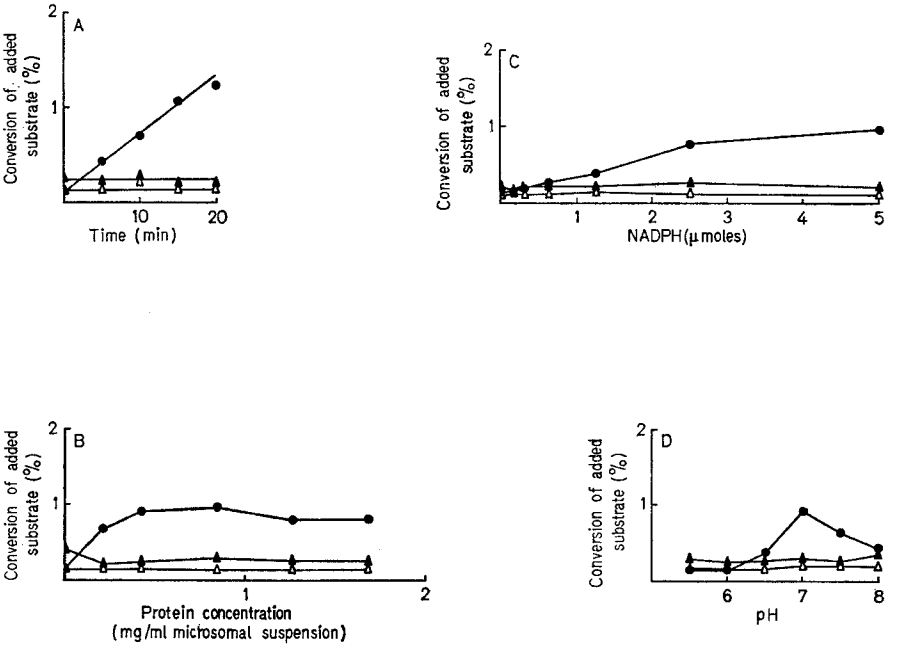

Fig. 3. *Effect of time (A), amount of microsomal fraction (B), NADPH (C) and pH (D) on oxidation of cholesterol by microsomal fraction prepared in EDTA-sucrose.* The incubation mixture and the incubation conditions were as those given in legend to Fig. 2. ▲, Formation of 3β-hydroxy-5-cholesten-7-one; ●, formation of 5-cholestene-3β,7α-diol; △, formation of 5-cholestene-3β,7β-diol

Table 1. *Effect of metal ions on oxidation of cholesterol and formation of lipid peroxides by microsomal fraction prepared in EDTA-sucrose*

Metal ion was added as chloride salt except Fe^{2+} which was added as sulfate. The concentration of metal ion in the incubation mixtures was in all instances 0.1 mM. The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The formation of lipid peroxides is expressed in the amount of malonaldehyde formed per ml of microsomal suspension (*cf.* Fig. 2B)

Metal ion added	Conversion of cholesterol into			Formation of lipid peroxides
	5-Cholestene-3β,7α-diol	5-Cholestene-3β,7β-diol	3β-Hydroxy-5-cholesten-7-one	
	% of added substrate			nmoles/ml
None	0.23	0.08	0.16	< 0.5
Mg^{2+}	0.22	0.09	0.14	< 0.5
Ca^{2+}	0.26	0.08	0.16	< 0.5
Mn^{2+}	0.18	0.08	0.10	< 0.5
Fe^{2+}	0.64	0.64	2.76	27.4
Co^{2+}	0.21	0.08	0.16	< 0.5
Ni^{2+}	0.23	0.12	0.31	0.7
Cu^{2+}	0.13	0.07	0.12	< 0.5
Zn^{2+}	0.22	0.20	0.86	7.7
Hg^{2+}	0.12	0.09	0.14	< 0.5

Tables 4 and 5). The crystallization data showed that only about 15% of the labeled material in the zone corresponding to 3β,7β-dihydroxy-5-pregnen-20-one was identical with this compound. Labeled material with the thin-layer chromatographic properties of 3β,7β-dihydroxy-5-pregnen-20-one was converted into the trimethylsilyl ether and analyzed by radio-gas chromatography and combined gas chromatography—mass spectrometry. Radio-gas chromatography (Fig. 6) showed that most of the radioactivity (peak II) appeared after 3β,7β-dihydroxy-5-pregnen-20-one (peak I). The mass spectrum of the unknown labeled compound indicated that the compound was a dihydroxy-5-pregnen-20-one. The base peak was at *m/e* 386 (M-90, loss of trimethylsilanol). Peaks were seen at *m/e* 461 (M-15), *m/e* 371 (M-(90 + 15)), *m/e* 343 (M-(90 + 43), loss of trimethylsilanol and side chain) and *m/e* 296 (M-2 × 90, loss of two molecules of trimethylsilanol). There was a small peak at *m/e* 476 (M). It appears reasonable to

	A	B	C	D
Front				
5 ●	2 235 420	2 659 570	2 584 720	2 245 505
	21 618	4088	2892	2683
4 ●	29 131	3512	3706	3090
3 ●	11 160	1413	1421	1241
2 ●	10 054	1655	1518	858
	3 259	764	725	225
1 ●	8254	1681	1858	552
Start	4737	2691	4131	1451

Fig. 4. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with soybean lipoxidase and linoleic acid.* (A) [4-^{14}C]cholesterol, 200 µg, 100 µg of soybean lipoxidase and 400 µg of linoleic acid were added to 1.5 ml of 0.1 M Tris-Cl buffer, pH 7.5, and the mixture was incubated at 30 °C for 10 min; (B) same as in A with the omission of lipoxidase; (C) same as in A with the omission of linoleic acid; (D) same as in A with the omission of soybean lipoxidase and linoleic acid. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v)

	A	B	C	D
Front				
5 ●	150 038	127 697	10 ●	110 926	109 472
	1460	3626		851	3731
4 ●	302	4753	9 ●	406	2696
3 ●	0	4435		178	472
2 ●	106	13 272	8 ●	384	1247
1 ●	195	2342		129	664
	187	1328	7 ●	232	1533
Start	354	2544	6 ●	166	9584
				97	285
			Start	338	394

Fig. 5. *Thin-layer chromatograms of extracts of incubations of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C] androsten-17-one with microsomal fraction prepared in EDTA-sucrose.* (A) 3β-hydroxy-5-[4-^{14}C]pregnen-20-one, 100 µg, was incubated for 10 min with 1 ml of microsomal fraction in a final volume of 3 ml; (B) same as in A with the exception that 3 µmoles of NADPH were added; (C) 3β-hydroxy-5-[4-^{14}C]androsten-17-one, 200 µg, was incubated for 10 min with 0.5 ml of microsomal fraction in a final volume of 3 ml; (D) same as in C with the exception that 3 µmoles of NADPH were added. The numbers on the chromatograms represent counts/min. Reference compounds were: (1) 3β,7α-dihydroxy-5-pregnen-20-one; (2) 3β,7β-dihydroxy-5-pregnen-20-one; (3) 3β,16α-dihydroxy-5-pregnen-20-one; (4) 3β-hydroxy-5 pregnene-7,20-dione; (5) 3β-hydroxy-5-pregnen-20-one; (6) 3β,7α-dihydroxy-5-androsten-17-one; (7) 3β,7β-dihydroxy-5-androsten-17-one; (8) 3β-hydroxy-5-androstene-7,17-dione; (9) 3β,16α-dihydroxy-5-androsten-17-one; (10) 3β-hydroxy-5-androsten-17-one; Solvent, 2,2,4-trimethylpentane-iso-amyl acetate—acetone (2:2:1, v/v/v). The chromatoplates were developed three times in the same solvent

assume that one of the hydroxyl groups is located in the 3β-position. The location of the other hydroxyl group can not be completely deduced from the mass spectrum but position 18 is more probable than other positions. A very prominent base peak at M-90 occurs in mass spectra of trimethylsilyl ethers of Δ^5-3β-hydroxysteroids having a hydroxyl group in the C-7 or the C-18 position [31a]. The C-7 position is of course excluded by the chromatographic data (Fig. 5) as well as the crystallization data (Annex, Table 4).

Tables 2 and 3 summarize the effects of cofactors and addition of EDTA to the homogenizing medium on the oxidation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one by the microsomal fraction. The extent of oxidation was the same whether or not EDTA was added to the homogenizing medium. NADPH was several times more efficient as cofactor than NADH. In the presence of microsomal fraction alone or boiled microsomal fraction and NADPH, the extent of oxidation was very small.

Oxidation of 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one by Lipid Peroxides

3β-Hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one were incubated with soybean lipoxidase in the presence of linoleic acid. The pattern of products was essentially the same in both cases and similar to that observed with cholesterol. The predominant product was the corresponding 7-oxo derivative. The extent of formation of the 7-oxo derivative was in both cases about 2% of added substrate.

Effect of Biliary Drainage on Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one

Table 4 summarizes the effect of biliary drainage on the oxidation of [4-^{14}C]cholesterol, 3β-hydroxy-5-[4-^{14}C]pregnen-20-one and 3β-hydroxy-5-[4-^{14}C]androsten-17-one by rat liver microsomes. In the presence of microsomal fraction from an EDTA-sucrose homogenate of liver from rats with a biliary fistula, the 7α-hydroxylation of cholesterol was about eight times more efficient than was observed with control rats (Table 4, Fig. 7). Biliary drainage resulted

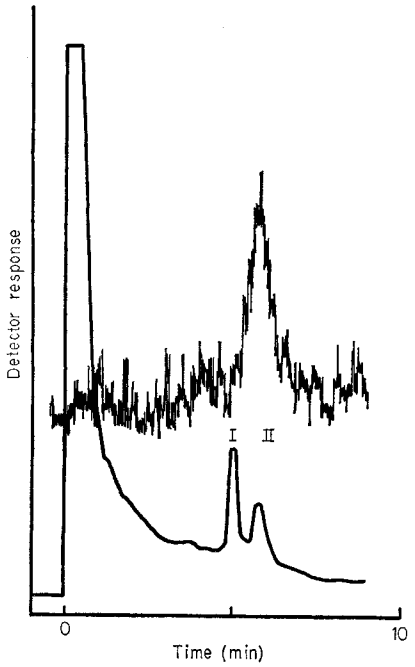

Fig. 6. *Radio-gas chromatogram of material in the zone corresponding to 3β,7β-dihydroxy-5-pregnen-20-one in the chromatogram shown in Fig. 5B.* Unlabeled 3β,7β-dihydroxy-5-pregnen-20-one was added to the labeled material and the mixture was converted to trimethylsilyl ether prior to chromatography. Peak I is the trimethylsilyl ether of 3β,7β-dihydroxy-5-pregnen-20-one. Upper curve, radioactivity tracing; lower curve, mass tracing. Column, 1% SE-30

in a marked increase also in the yield of labeled material with chromatographic properties similar to those of cholestane-3β,5α,6β-triol. Rechromatography of this material showed that the major part was 5-cholestene-3β,7α,12α-triol (Fig. 7C). The rates of 7α-, 7β- + X- and 16α-hydroxylation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one in the presence of microsomal fraction from rats with a biliary fistula were about 30% of the rates in the presence of microsomal fraction from control rats (Table 4). The rates of 7α-, 7β- and 16α-hydroxylation of 3β-hydroxy-5-[4-^{14}C]androsten-17-one in the presence of microsomal fraction from rats with a biliary fistula were 20—30% of the rates in control experiments.

When microsomal fraction was prepared from a sucrose medium without EDTA, there was no significant difference in rates of formation of 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol, and lipid peroxides between rats with a biliary fistula and control rats (Table 4).

Effect of Phenobarbital Treatment and Carbon Monoxide on Oxidation of Cholesterol, 3β-Hydroxy-5-pregnen-20-one and 3β-Hydroxy-5-androsten-17-one

Treatment with phenobarbital had no significant effect on the 7α-hydroxylation of cholesterol by the microsomal fraction prepared in EDTA-sucrose (Table 5). Carbon monoxide inhibited the reaction (Table 6). In this case, microsomal fraction from rats with a biliary fistula was used. Phenobarbital had no significant effect on the 7α- and 7β- + X-hydroxylations of 3β-hydroxy-5-pregnen-20-one but significantly stimulated the 16α-hydroxylation (Table 5). The 7β- + X-hydroxylations as well as the 16α-hydroxylation were inhibited by carbon monoxide (Table 6). The 7α-hydroxylation tended to be lower in the presence of carbon monoxide but the inhibition was not significant statistically (Table 6). The 16α-hydroxylation of 3β-hydroxy-5-androsten-17-one was not influenced by phenobarbital, whereas the 7α- and 7β-hydroxylations were stimulated three- to four-fold (Table 5). All three hydroxylations were inhibited by carbon monoxide (Table 6).

Table 2. *Metabolism of 3β-hydroxy-5-pregnen-20-one*
The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms

Incubation	Homogenizing medium	Conversion of 3β-hydroxy-5-pregnen-20-one into			
		3β,7α-Dihydroxy-5-pregnen-20-one	3β,7β-Dihydroxy-5-pregnen-20-one + 3β,X-dihydroxy-5-pregnen-20-one [a]	3β,16α-Dihydroxy-5-pregnen-20-one	3β-Hydroxy-5-pregnene-7,20-dione
		% conversion of added substrate			
Microsomes	Sucrose	0.1	0.1	0.0	0.2
	EDTA-sucrose	0.4	0.2	0.1	0.6
Microsomes + 3 μmoles of NADPH	Sucrose	1.5	7.4	2.5	2.9
	EDTA-sucrose	2.4	9.6	3.5	1.8
Microsomes + 3 μmoles of NADH	Sucrose	0.5	1.6	0.5	0.4
	EDTA-sucrose	0.6	1.4	0.5	0.3
Boiled microsomes + 3 μmoles of NADPH	Sucrose	0.3	0.2	0.0	0.3
	EDTA-sucrose	0.2	0.2	0.1	0.3

[a] See text.

Table 3. *Metabolism of 3β-hydroxy-5-androsten-17-one*
The percentages were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms

Incubation	Homogenizing medium	Conversion of 3β-hydroxy-5-androsten-17-one into			
		3β,7α-Dihydroxy-5-androsten-17-one	3β,7β-Dihydroxy-5-androsten-17-one	3β,16α-Dihydroxy-5-androsten-17-one	3β-Hydroxy-5-androstene-7,17-dione
		% conversion of added substrate			
Microsomes	Sucrose	0.2	0.1	0.4	0.4
	EDTA-sucrose	0.2	0.2	0.4	0.4
Microsomes + 3 μmoles of NADPH	Sucrose	6.5	1.1	2.0	1.3
	EDTA-sucrose	7.6	1.2	2.0	1.0
Microsomes + 3 μmoles of NADH	Sucrose	0.5	0.1	0.4	0.4
	EDTA-sucrose	0.5	0.2	0.5	0.4
Boiled microsomes + 3 μmoles of NADPH	Sucrose	0.2	0.1	0.4	0.4
	EDTA-sucrose	0.2	0.1	0.4	0.3

Table 4. *Effect of biliary drainage on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*
The values listed are the means ± S.D. of experiments with four rats. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, control rats; bile fistula, rats with biliary drainage for 60 h

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Bile fistula	Control	Bile fistula
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	0.14 ± 0.03	1.30 ± 0.18[a]	0.98 ± 0.24	0.87 ± 0.59
	7β-Hydroxylation	0.08 ± 0.04	0.12 ± 0.02	1.32 ± 0.21	0.83 ± 0.67
	Formation of 3β-hydroxy-5-cholesten-7-one	0.13 ± 0.02	0.15 ± 0.03	5.21 ± 1.05	3.63 ± 3.30
				nmoles of malonaldehyde[b]	
Endogenous	Formation of lipid peroxides			40.1 ± 12.7	25.7 ± 12.2
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	1.8 ± 0.6	0.5 ± 0.1[a]		
	7β-Hydroxylation + X-hydroxylation[c]	5.9 ± 1.4	1.9 ± 0.3[a]		
	16α-Hydroxylation	3.0 ± 0.7	0.8 ± 0.2[a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	54.3 ± 4.3	13.0 ± 4.2[a]		
	7β-Hydroxylation	7.1 ± 1.0	2.4 ± 0.7[a]		
	16α-Hydroxylation	6.2 ± 1.6	2.3 ± 0.4[a]		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml of microsomal suspension (*cf.* Fig. 2B).
[c] See text.

The formation of 3β-hydroxy-5-cholesten-7-one, 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and lipid peroxides in the presence of microsomal fraction prepared in sucrose without EDTA was not influenced by phenobarbital treatment (Table 5) or carbon monoxide (Table 6).

DISCUSSION

The results of the present investigation show that there is a correlation between the formation of lipid peroxides and the oxidation of cholesterol by the microsomal fraction of rat liver homogenate. When peroxidation is inhibited, the major product of cholesterol is 5-cholestene-3β,7α-diol. On the other hand, under conditions of NADPH-dependent, enzymatic peroxidation of lipids, cholesterol is oxidized to several products including 3β-hydroxy-5-cholesten-7-one as the predominant product and 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and cholestane-3β,5α,6β-triol. The same situation prevails when peroxidation is achieved with microsomal fraction and ascorbate. The formation of 3β-hydroxy-5-cholesten-7-one and accompanying products may result from nonenzymatic oxidation of cholesterol by lipid radicals, generated by the lipid peroxidating system. This suggestion is based on the finding that cholesterol is oxidized to

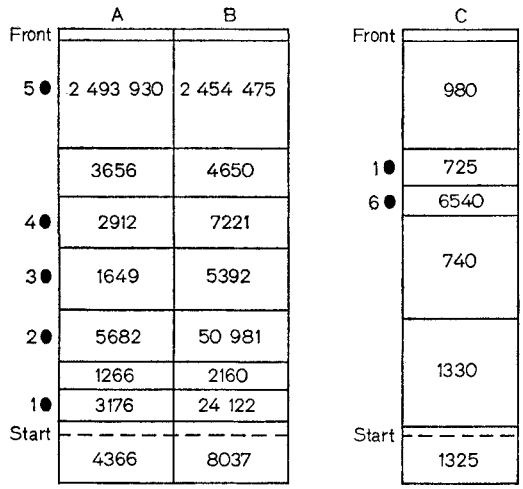

Fig. 7. *Thin-layer chromatograms of extracts of incubations of [4-^{14}C]cholesterol with microsomal fraction from control rat and rat with a biliary fistula.* (A) 3 ml of microsomal fraction from control rat, prepared in EDTA-sucrose, were diluted with buffer to 5 ml and incubated for 15 min with 5 μmoles of NADPH and 15 μg of [4-^{14}C]cholesterol. The numbers on the chromatogram represent counts/min. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (2) 5-cholestene-3β,7α-diol; (3) 5-cholestene-3β,7β-diol; (4) 3β-hydroxy-5-cholesten-7-one; (5) cholesterol. Solvent, benzene—ethyl acetate (2:3, v/v). (B) incubation with microsomal fraction, prepared in EDTA-sucrose, from rat that had had a biliary fistula for two days; incubation conditions, reference compounds and chromatographic conditions as in A. (C) chromatography of material in the zone corresponding to cholestane-3β,5α,6β-triol in chromatogram shown in B. Reference compounds were: (1) cholestane-3β,5α,6β-triol; (6) 5-cholestene-3β,7α, 12α-triol. Solvent, ethyl acetate. The chromatoplate was developed three times in the same solvent

the same products by a mixture of soybean lipoxidase and linoleic acid. The correlation between formation of lipid peroxides and oxidation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one by microsomal fraction was not as apparent as observed for oxidation of cholesterol. A possible explanation is that the enzymatic oxidation in the 7-position of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one is much more efficient than that of cholesterol and makes nonenzymatic oxidation difficult to detect. Both 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were oxidized in the 7-position by a mixture of soybean lipoxidase and linoleic acid. The pattern of 7-oxidized products formed was similar to that observed for cholesterol.

It has been suggested that the NADPH-dependent, microsomal peroxidation of lipids involves the same electron carriers between NADPH and oxygen as those involved in a number of microsomal hydroxylations [7, 10]. The present results indicate, however, that cytochrome P-450 is not involved in lipid peroxidation since it was not inhibited by carbon monoxide nor stimulated by phenobarbital treatment.

Whether or not oxidation of cholesterol by lipid peroxides is of significance *in vivo* requires further studies. It should be mentioned that the major product of the reaction, 3β-hydroxy-5-cholesten-7-one, is not a presursor of the normally occurring bile acids [6].

The present findings explain the differences in results concerning oxidation of cholesterol by the microsomal fraction between a previous investigation

Table 5. *Effect of phenobarbital treatment on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one, and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*

The values listed are the means ± S.D. of experiments with four rats. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, control rats; phenobarbital, rats treated with daily injections of phenobarbital (100 mg/kg body weight) for 5 days

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Phenobarbital	Control	Phenobarbital
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	0.53 ± 0.19	0.29 ± 0.07	0.41 ± 0.14	0.31 ± 0.08
	7β-Hydroxylation	0.11 ± 0.04	0.06 ± 0.02	0.24 ± 0.13	0.14 ± 0.09
	Formation of 3β-hydroxy-5-cholesten-7-one	0.10 ± 0.02	0.10 ± 0.02	0.74 ± 0.45	0.49 ± 0.43
				nmoles of malonaldehyde [b]	
Endogenous	Formation of lipid peroxides			9.3 ± 2.7	15.3 ± 5.2
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	4.8 ± 0.8	7.1 ± 1.1		
	7β-Hydroxylation + X-hydroxylation [c]	19.2 ± 1.5	16.6 ± 2.9		
	16α-Hydroxylation	7.1 ± 0.5	13.8 ± 1.6 [a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	34.1 ± 2.2	120.3 ± 8.3 [a]		
	7β-Hydroxylation	6.4 ± 1.3	20.8 ± 2.2 [a]		
	16α-Hydroxylation	8.9 ± 0.9	6.1 ± 0.9		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml microsomal suspension (*cf.* Fig. 2B).
[c] See text.

Table 6. *Effect of carbon monoxide on oxidation of cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one and on formation of lipid peroxides*

The values listed are the means ± S.D. of four incubations. Incubations with cholesterol were carried out with microsomal fraction of liver from rats that had had a bilialy fistula for 48 h. Abbreviations: EDTA-sucrose, incubations with microsomal fraction prepared in EDTA-sucrose; sucrose, incubations with microsomal fraction prepared in sucrose; control, incubations in an atmosphere of 4% oxygen and 96% nitrogen; carbon monoxide, incubations in an atmosphere of 4% oxygen, 56% nitrogen and 40% carbon monoxide

Substrate	Reaction	EDTA-sucrose		Sucrose	
		Control	Carbon monoxide	Control	Carbon monoxide
		nmoles/mg protein		nmoles/mg protein	
Cholesterol	7α-Hydroxylation	2.89 ± 0.52	0.50 ± 0.15[a]	1.00 ± 0.10	1.07 ± 0.12
	7β-Hydroxylation	0.26 ± 0.04	0.11 ± 0.03	1.16 ± 0.28	1.29 ± 0.13
	Formation of 3β-hydroxy-5-cholesten-7one	0.20 ± 0.05	0.18 ± 0.05	3.46 ± 1.00	3.70 ± 0.50
				nmoles of malonaldehyde[b]	
Endogenous	Formation of lipid peroxides			22.5 ± 3.0	29.4 ± 2.3
3β-Hydroxy-5-pregnen-20-one	7α-Hydroxylation	2.9 ± 0.9	1.5 ± 0.2		
	7β-Hydroxylation + X-hydroxylation[c]	14.2 ± 1.6	4.8 ± 0.8[a]		
	16α-Hydroxylation	4.4 ± 0.3	2.5 ± 0.2[a]		
3β-Hydroxy-5-androsten-17-one	7α-Hydroxylation	40.9 ± 3.9	11.4 ± 0.8[a]		
	7β-Hydroxylation	5.7 ± 0.6	2.1 ± 0.8[a]		
	16α-Hydroxylation	11.8 ± 1.4	4.3 ± 0.4[a]		

[a] $P < 0.01$ as compared to control.
[b] The figures represent amount formed per ml microsomal suspension (*cf.* Fig. 2B).
[c] See text.

from this laboratory [6] and investigations by Shefer, Hauser and Mosbach [2] and Boyd, Scholan and Mitton [3]. In the investigation from this laboratory 3β-hydroxy-5-cholesten-7-one was found to be a major product and 5-cholestene-3β,7α-diol a minor product of cholesterol in the presence of microsomal fraction whereas Shefer, Hauser and Mosbach [2] and Boyd, Scholan and Mitton [3] found that 5-cholestene-3β,7α-diol was the predominant product. Shefer, Hauser and Mosbach [2] suggested that the difference in results was due to differences in mode of addition of cholesterol to the incubation mixtures. As is evident from the results of the present investigation, the difference is most probably due to presence or absence of lipid peroxidation in the system. Thus, Shefer, Hauser and Mosbach [2] added EDTA to the homogenizing medium, thereby inhibiting microsomal lipid peroxidation. Boyd, Scholan and Mitton [3] added β-mercaptoethylamine and some sulfhydryl compounds are known to inhibit lipid peroxidation [8, 32]. It might be mentioned in this connection that in short-time incubations with microsomal fraction cholesterol is subjected to true autoxidation only to a very limited extent.

The present investigation also aimed at a further study of the properties of the 7α-hydroxylase system catalyzing the conversion of cholesterol into 5-cholestene-3β,7α-diol. Previous results [33, 34] indicating that this 7α-hydroxylase differs in several respects from other microsomal hydroxylases have been confirmed and expanded. Special interest was paid to a comparison between 7α-hydroxylation of cholesterol and 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one [35, 36]. Marked differences in the efficiency of the 7α-hydroxylation of the three substrates were observed. The most efficient 7α-hydroxylation was obtained with 3β-hydroxy-5-androsten-17-one and the least efficient with cholesterol. Biliary drainage resulted in a several-fold increase in the 7α-hydroxylation of cholesterol, as has been previously found [2—4, 33, 37]. In contrast, the 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one was inhibited by biliary drainage. Phenobarbital treatment did not significantly affect 7α-hydroxylation of cholesterol and 3β-hydroxy-5-pregnen-20-one, whereas 7α-hydroxylation of 3β-hydroxy-5-androsten-17-one was stimulated. Carbon monoxide inhibited 7α-hydroxylation of cholesterol and 3β-hydroxy-5-androsten-17-one, indicating the participation of a cytochrome P-450 in these hydroxylations. Cytochrome P-450 may also participate in the 7α-hydroxylation of 3β-hydroxy-5-pregnen-20-one. Further investigations including studies on light reversibility of the inhibition by carbon monoxide are required to establish definitely the participation of cytochrome P-450 in

the 7α-hydroxylations. The apparent explanation for the differences between the three substrates with respect to 7α-hydroxylation is the existence of a specific 7α-hydroxylase for cholesterol and another 7α-hydroxylase(s) for 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one.

The differences between the metabolism of cholesterol and 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were not limited to the 7α-hydroxylation. Whereas cholesterol was converted practically only to 5-cholestene-3β,7α-diol, which in turn was 12α-hydroxylated to some extent [16], 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one were converted into the corresponding 7-oxo, 7β-, and 16α-hydroxy derivatives [36,38]. 3β-Hydroxy-5-pregnen-20-one was also converted into another, as yet unidentified hydroxylated derivative. It should be mentioned that Hampl and Stárka [39] have reported recently that the microsomal fraction of rat liver homogenate catalyzes the interconversion of 3β,7α-dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one. The contribution of this reaction to the formation of the two 7-hydroxy derivatives observed in the present investigation cannot be assessed. However, there can be no doubt that there is predominantly an initial 7α-hydroxylation. The amount of 7α-hydroxy derivative formed was several times greater than that of the 7β-hydroxy derivative. Hampl and Stárka [39] found that the equilibrium was towards formation of the 7β-hydroxy derivative and equilibrium was reached not later than after an incubation time of 30 min.

The skilful technical assistance of Miss Yvonne Jacob and Mrs. Britt-Marie Johansson is gratefully acknowledged. This work is part of investigations supported by the Swedish Medical Research Council (Project 13X-218) and has also been supported by a grant from Karolinska Institutet.

ANNEXES

The following documents have been deposited at the *Archives Originales du Centre de Documentation du C.N.R.S.* (15 Quai Anatole France, F-75 Paris 7, France) where they may be ordered as microfiche or photocopies. Reference No: A. O.—494.

Annex, Table 1. Crystallization to constant specific radioactivity of products formed in incubations of [4-^{14}C]cholesterol with microsomal fraction prepared in sucrose.

Annex, Table 2. Crystallization to constant specific radioactivity of 5-cholestene-3β,7α-diol formed in incubation of [4-^{14}C]cholesterol with microsomal fraction prepared in EDTA-sucrose.

Annex, Table 3. Crystallization to constant specific radioactivity of products formed in incubation of [4-^{14}C]cholesterol with linoleic acid and soybean lipoxidase.

Annex, Table 4. Crystallization to constant specific radioactivity of products formed in incubation of 3β-hydroxy-5-[4-^{14}C]pregnen-20-one with microsomal fraction prepared in EDTA-sucrose.

Annex, Table 5. Crystallization to constant specific radioactivity of products formed in incubation of 3β-hydroxy-5-[4-^{14}C]androsten-17-one with microsomal fraction prepared in EDTA-sucrose.

REFERENCES

1. Danielsson, H., in *The Bile Acids: Chemistry, Physiology and Metabolism* (edited by P. P. Nair and D. Kritchevsky), Plenum Press, New York, in the press.
2. Shefer, S., Hauser, S., and Mosbach, E. H., *J. Lipid Res.* 9 (1968) 328.
3. Boyd, G. S., Scholan, N. A., and Mitton, J. R., *Advan. Exp. Med. Biol.* 4 (1969) 443.
4. Wada, F., Hirata, K., Nakao, K., and Sakamoto, Y., *J. Biochem. (Tokyo)*, 66 (1969) 699.
5. Hayaishi, O., *Ann. Rev. Biochem.* 38 (1969) 21.
6. Björkhem, I., Einarsson, K., and Johansson, G., *Acta Chem. Scand.* 22 (1968) 1595.
7. Orrenius, S., Dallner, G., and Ernster, L., *Biochem. Biophys. Res. Commun.* 14 (1964) 329.
8. Wills, E. D., *Biochem. J.* 113 (1969) 315.
9. Wills, E. D., *Biochem. J.* 113 (1969) 325.
10. Wills, E. D., *Biochem. J.* 113 (1969) 333.
11. Johansson, G., unpublished observation.
12. Ellingboe, J., Nyström, E., and Sjövall, J., *Biochim. Biophys. Acta*, 152 (1968) 803.
13. Henbest, H. B., and Jones, E. R. H., *J. Chem. Soc. (London)* (1948) 1792.
14. Wintersteiner, O., and Ruigh, W. L., *J. Amer. Chem. Soc.* 64 (1942) 2453.
15. Bergström, S., *Arkiv Kemi, Geol. Mineral.* 16A (1942) No. 10.
16. Berséus, O., Danielsson, H., and Einarsson, K., *J. Biol. Chem.* 242 (1967) 1211.
17. Fieser, L. F., and Rajagopalan, S., *J. Amer. Chem. Soc.* 71 (1949) 3938.
18. Danielsson, H., *Acta Chem. Scand.* 14 (1960) 846.
19. Schenck, G. O., Neumüller, O.-A., and Eisfeld, W., *Liebigs Ann. Chem.* 618 (1958) 202.
20. Dodson, R. M., Nicholson, R. T., and Muir, R. D., *J. Amer. Chem. Soc.* 81 (1959) 6295.
21. Stárka, L., *Collect. Czech. Chem. Commun.* 26 (1961) 2452.
22. Norman, A., and Sjövall, J., *J. Biol. Chem.* 233 (1958) 872.
23. Marshall, C. W., Ray, R. E., Laos, I., and Riegel, B., *J. Amer. Chem. Soc.* 79 (1957) 6308.
24. Heusler, K., and Wettstein, A., *Helv. Chim. Acta* 35 (1952) 284.
25. Barnett, J., Ryman, B. E., and Smith, F., *J. Chem. Soc. (London)*, (1946) 526.
26. Bernstein, S., Heller, M., and Stolar, S. M., *J. Amer. Chem. Soc.* 76 (1954) 5674.
27. Fotherby, K., Colás, A., Atherden, S. M., and Marrian, G. F., *Biochem. J.* 66 (1957) 664.
28. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., *Arch. Biochem. Biophys.* 24 (1949) 305.
29. Sinnhuber, R. O., Yu, T. C., and Yu, T. C., *Food Res.* 23 (1958) 626.
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* 193 (1951) 265.
31. Hanel, H. K., and Dam, H., *Acta Chem. Scand.* 9 (1955) 677.
31a. Gustafsson, J.-Å., personal communication.
32. Christophersen, B. O., *Biochem. J.* 106 (1968) 515.
33. Einarsson, K., and Johansson, G., *Eur. J. Biochem.* 6 (1968) 293.
34. Johansson, G., *Eur. J. Biochem.* 17 (1970) 292.
35. Stárka, L., Šulcová, J., Dahm, K., Döllefeld, E., and Breuer, H., *Biochim. Biophys. Acta*, 115 (1966) 228.
36. Šulcová, J., and Stárka, L., *Steroids*, 12 (1968) 113.
37. Danielsson, H., Einarsson, K., and Johansson, G., *Eur. J. Biochem.* 2 (1967) 44.
38. Colás, A., *Biochem. J.* 82 (1962) 390.
39. Hampl, R., and Stárka, L., *J. Steroid Chem.* 1 (1969) 47.

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