The Metabolism of Cholesterol in the Presence of Liver Mitochondria from Normal and Thyroxine-Treated Rats

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1. [26-¹⁴C]- and [4-¹⁴C]-Cholesterol were incubated with liver mitochondria from normal and thyroxine-treated rats, and the radioactivity was measured in the carbon dioxide evolved during the incubation, in a butanol extract of the incubation mixture and in a volatile fraction containing substances of low molecular weight derived from the side chain of cholesterol. The butanol extract was separated by paper chromatography into three radioactive fractions, one of which contained the steroids more polar than cholesterol. 2. The butanol extract from incubations with [4-¹⁴C]cholesterol contained a radioactive substance moving with the same *R_F* as cholic acid on thin-layer chromatography. 3. After incubation with [26-¹⁴C]-cholesterol, 60–80% of the radioactivity extracted by steam-distillation of the incubation mixture at acid pH was recovered as [¹⁴C]propionic acid. 4. In the presence of [26-¹⁴C]cholesterol, mitochondria from thyroxine-treated rats produced more radioactivity in carbon dioxide and in the volatile fraction, and less radioactivity in the fraction containing the polar steroids, than did mitochondria from normal rats. In the presence of [4-¹⁴C]cholesterol, mitochondria from thyroxine-treated rats produced the same amount of radioactivity in the polar steroids as did normal mitochondria. 5. Thyroxine treatment had no effect on the capacity of the mitochondria to oxidize propionate to carbon dioxide. 6. These results are best explained by supposing that thyroxine stimulates a rate-limiting reaction leading to the cleavage of the side chain of cholesterol, but has little or no influence on the hydroxylations of the ring system or on the oxidation of the C₃ fragment removed from the side chain.

Bile acids are the main end products of the metabolism of the plasma cholesterol in rats (Siperstein & Chaikoff, 1955). Factors that modify the rate of conversion of cholesterol into bile acids may therefore help to regulate the plasma cholesterol concentration. Weiss & Marx (1955) have shown that thyroid hormone increases the excretion of radioactive acidic substances in the faeces of mice injected with [4-¹⁴C]cholesterol. Eriksson (1957) has shown that, in rats, thyroid hormone stimulates the excretion of bile acid through a cannula in the common bile duct, an effect that appears to be due entirely to an influence on the production of chenodeoxycholic acid. Strand (1963), using an indirect method to determine the rate of production of cholic acid and chenodeoxycholic acid in rats with intact enterohepatic circulation, found that in these more physiological conditions tri-iodothyronine also stimulates the formation of bile acids, and that the main effect is on the production of chenodeoxycholic acid.

Several groups of workers (Anfinson & Horning, 1953; Fredrickson, 1956; Whitehouse, Staple &

Gurin, 1959) have shown that washed liver mitochondria, suspended in a suitable medium, oxidize the end three carbon atoms of the side chain of cholesterol to carbon dioxide. Since the oxidation of cholesterol by mitochondria *in vitro* has been shown to result in the formation of at least one substance closely related to a naturally occurring bile acid (Fredrickson, 1956), the mitochondrial system affords an opportunity for studying the effect of thyroid hormone on the reactions involved in the conversion of cholesterol into bile acids.

In the present paper we show that the production of ¹⁴CO₂ from [26-¹⁴C]cholesterol incubated with rat-liver mitochondria is stimulated by treating the rats with thyroxine before removing their livers. Our observations suggest that this effect is due to stimulation of one or more of the reactions that lead to cleavage of the isopropyl unit from the side chain.

METHODS

Animals. The rats were adult males of a Wistar strain, inbred for more than 50 generations. They were given M.R.C. Diet no. 18 (Bruce & Parkes, 1946). In most cases

L-thyroxine (100 μ g./rat twice daily for 7 days) was given by subcutaneous injection of an aqueous solution at pH 8.5, the control animals receiving an injection of the same volume of the alkaline fluid used for dissolving the thyroxine. In some experiments, the solution of thyroxine was mixed with the food (2 mg. of thyroxine/100 g.) to form a paste that was rolled into pellets and dried at 37°; the food of the control animals was mixed with the solution used for dissolving the thyroxine and treated in the same way. All rats were kept under identical conditions and were allowed to eat *ad lib*. They were killed at about 11 a.m. on the day of the experiment.

Preparation of mitochondria. The rats were killed by a blow on the head and their livers removed immediately. The livers were minced in a stainless-steel mincer and homogenized with 4 vol. of 10% (w/v) sucrose solution in a glass tube with a Teflon plunger. Washed mitochondria and the 'soluble cofactor' were prepared from the homogenate by the method of Whitehouse *et al.* (1959). The washed mitochondria were suspended in 10% sucrose, 1 ml. containing the mitochondria from 1 g. wet wt. of liver. During these operations the vessels were immersed in ice-water and the centrifugations were carried out at 0°. The soluble cofactor was stored at -15°. Disrupted mitochondria were prepared by freezing the washed suspension in a mixture of acetone and solid CO₂ and allowing the frozen suspension to thaw at room temperature. This was repeated twice.

Incubations. Except in a few preliminary experiments to compare different conditions of incubation, all incubations were carried out in duplicate in Warburg flasks, silicone-treated before each experiment by coating the inside with dichlorodimethylsilane. As absorbent for CO₂, 0.2 ml. of a 1 M solution of hyamine base benzyl-{2-[2-(*p*-1,1,3,3-tetramethylbutylcresoxy)ethoxy]ethyl}dimethylammonium hydroxide in methanol was placed in a detachable cup fitted into the centre well of the flask. The side arm contained 0.3 ml. of 20% HCl. The flasks were shaken in a water bath at 37° with air as the gas phase.

The standard incubation mixture contained: tris-HCl buffer, pH 8.5 (0.1 M); ATP (2.3 mM); NAD⁺ (0.4 mM); GSH (3.0 mM); MgCl₂ (2.2 mM); sodium citrate (4.0 mM); sucrose (15 mM); 0.7 ml. of soluble cofactor solution (prepared from untreated rats); 0.5 ml. of mitochondrial suspension, containing 4–5 mg. of mitochondrial protein; 0.1 ml. of [¹⁴C]cholesterol emulsion (3.8 μ g., with 0.1–0.2 μ Ci); the final volume was 3.5 ml. The emulsion of [¹⁴C]cholesterol was prepared by evaporating a portion of the stock solution to dryness under N₂, dissolving the residue in 0.3 ml. of methanol and adding this to 5 ml. of a 1% (w/v) solution of bovine serum albumin in water. The methanol was removed as far as possible by blowing a stream of N₂ over the emulsion at 40°. Glass-distilled deionized water was used for making up all solutions.

In the experiments in which [¹⁴C]propionic acid was isolated from the steam-distillate by column chromatography (Fig. 5), 1.5 mg. of non-radioactive potassium propionate was added at the beginning of the incubation and a further 2 mg. was added at the end. In the experiments with [1-¹⁴C]propionate, the [¹⁴C]cholesterol in the incubation mixture was replaced by 1 mM-[1-¹⁴C]propionate. The experimental conditions were otherwise the same as those used for incubations with [¹⁴C]cholesterol.

Analysis of incubation mixture. At the end of the incubation,

the acid in the side arm was tipped into the main compartment and the flasks were allowed to stand at room temperature for a further 12 hr. The flasks were then opened and the hyamine cups removed. The outside of each cup was washed by dipping it into two lots of toluene to remove contamination from the incubation mixture. The incubation mixture was transferred to stoppered glass tubes, the inside of the flask was washed four or five times with a total of about 15 ml. of ethanol to ensure complete removal of all steroids, and the washings were added to the incubation mixture. The ethanol was evaporated at about 40° under reduced pressure. The aqueous residue was made up to 15 ml. with water and extracted three times with 2 vol. of water-saturated butan-1-ol. The combined butanol extracts were washed twice with 10 ml. of water and the washings added to the aqueous residue. The aqueous residue, plus washings, was evaporated to a small volume under reduced pressure and a known proportion taken for radioassay. The butanol was evaporated to dryness under a stream of N₂ at about 40° and the residue dissolved in 10 ml. of chloroform-methanol (2:1, v/v). A 0.5 ml. sample was taken for radioassay and the remainder submitted to reversed-phase paper chromatography by the method of Kodicek & Ashby (1954), with propan-1-ol-methanol-water (15:82:3, by vol.) as the mobile phase and liquid paraffin as the stationary phase.

For thin-layer chromatography, Kieselgel H was used as the solid phase. The residue from the butanol extract of the incubation mixture was first run with chloroform-methanol (47:3, v/v) as the solvent and the radioactivity located from the positions of cholesterol and bile acid markers run in parallel. The Kieselgel containing the radioactivity more polar than cholesterol was eluted with chloroform-methanol (2:1, v/v), and the eluate run on a fresh plate with chloroform-methanol (3:1, v/v) as solvent. The plates were stained by spraying with the ammonium molybdate-perchloric acid spray of Wagner, Hörhammer & Wolff (1961) and heating with a hair-drier for 10 min. After being stained, they were prepared for radioautography by spraying with a solution of Neotan.

For chromatography on KOH-treated silicic acid columns (Lewis & Myant, 1965) the butanol extract from the incubation mixture was evaporated under N₂ and the residue dissolved in 2 ml. of chloroform. The chloroform solution was put on to the column and eluted with 120 ml. of diethyl ether-hexane (7:3, v/v) and then with 100 ml. of 4% formic acid in methanol. Samples were taken from each eluate for radioassay and for reversed-phase paper chromatography.

Throughout all these procedures, precautions were taken to minimize exposure of the samples to light (Hais & Myant, 1965).

Propionic acid was extracted from the acidified incubation mixture by steam-distillation according to the method of Suld, Staple & Gurin (1962), and was separated by column chromatography on Celite columns according to the procedure of Swim & Utter (1957). Each 5 ml. fraction eluted from the column was titrated with 0.02 N-NaOH, with phenol red as indicator, and a portion of the aqueous phase taken for radioassay. The *S*-benzylisothiuronium salt of propionic acid was prepared according to the method of Vogel (1951) and recrystallized from hot water. Protein was estimated by the method of Cleland & Slater (1953). Endogenous cholesterol in the mitochondria was estimated

by extracting the mitochondrial suspension with chloroform-methanol (2:1, v/v) and measuring the cholesterol in the extract by the method of Carpenter, Gotsis & Hegsted (1957). The radioactivity in the samples was measured by methods described by Hais & Myant (1965). Radioactivity on the paper chromatograms was measured by cutting the strips into 1 cm. segments and laying each segment on the bottom of a counting vial containing the scintillation liquid. The radioactivity in each chromatographic fraction of the butanol extract was expressed as a proportion of the total radioactivity in the incubation mixture, by correcting for the fraction of the total radioactivity that was recovered in the butanol.

Materials. [26-¹⁴C]Cholesterol, [4-¹⁴C]cholesterol and sodium [1-¹⁴C]propionate were obtained from The Radiochemical Centre, Amersham, Bucks. The radioactive cholesterol was stored at 0° in the dark as a benzene solution; analysis of the samples by reversed-phase paper chromatography showed less than 1% of the total radioactivity in the non-polar zone and 2-4% in the polar zone. Other chemicals were obtained from the following sources: hyamine base from Nuclear Enterprises (G.B.) Ltd., Edinburgh; liquid paraffin from Hopkin and Williams Ltd., Chadwell Heath, Essex; Kieselgel H and Neatan from Camlab (Glass) Ltd., Cambridge; ATP and NAD⁺ from Sigma Chemical Co., St Louis, Mo., U.S.A.; CoA and GSH from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; cholic acid, deoxycholic acid, chenodeoxycholic acid and lithocholic acid from Steraloids Ltd., Croydon, Surrey. 7 α -Hydroxycholesterol was a gift from Dr O. Wintersteiner (The Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A.). All reagents were of A.R. grade.

RESULTS

Metabolism of [¹⁴C]cholesterol by mitochondria from normal rats

Experiments were first carried out with liver mitochondria from normal rats to find the best conditions for observing the breakdown of cholesterol. The agreement between values from paired flasks and between average values from different experiments was much improved when the Warburg flasks were treated with silicone, and when albumin, instead of Tween (Whitehouse *et al.* 1959), was used as emulsifying agent. Although the soluble cofactor of Whitehouse *et al.* (1959) was used in all the experiments described below, it had no effect on the production of ¹⁴CO₂ from [26-¹⁴C]cholesterol when the incubations were carried out in silicone-treated flasks, suggesting that the effect of the soluble cofactor is merely to stabilize the cholesterol emulsion.

Radioactivity recovered in carbon dioxide. When [26-¹⁴C]cholesterol was incubated for 6 hr. with mitochondria from untreated rats, the yield of ¹⁴CO₂ varied from about 0.3 to 2.0% of the added radioactivity. The rate of formation of ¹⁴CO₂ was constant between 4 and 8 hr. (Fig. 1); in two experiments in which the incubation was carried out for longer, ¹⁴CO₂ production continued at a

Fig. 1. Accumulation of radioactivity in CO₂ (○, control; ●, treated) and in the volatile fraction (□, control; ■, treated) during incubation of [26-¹⁴C]cholesterol with liver mitochondria from normal and thyroxine-treated rats. Experimental details are given in the Methods section. Radioactivity in the volatile fraction was calculated as the difference between the amount of radioactivity added and the amount recovered in the butanol extract and in CO₂.

constant rate until the 23rd hour. When the quantity of cholesterol added as carrier was increased, the amount of exogenous cholesterol oxidized to carbon dioxide during a 6 hr. incubation increased to a plateau at about 40 μ moles of cholesterol per flask (Fig. 2). With [4-¹⁴C]cholesterol, no radioactivity was recovered in carbon dioxide.

Radioactivity recovered in the butanol extract. When [4-¹⁴C]cholesterol was used, 100 $\pm$ 1% of the radioactivity added initially was recovered in the butanol extract. The butanol therefore contained all the unchanged cholesterol and all the steroids formed from cholesterol during the incubation. When either [26-¹⁴C]- or [4-¹⁴C]-cholesterol was used, radioautographs of the paper chromatograms made from the butanol extracts showed three zones of blackening: a small area near the origin due to non-polar steroids; a zone at about *R_f* 0.5 due to cholesterol; and a diffuse zone between cholesterol and the solvent front due to steroids more polar than cholesterol (Fig. 3). When the suspension of mitochondria was boiled before it was added to the incubation flask, radioactivity in the zone containing

Fig. 2. Yield of $^{14}\text{CO}_2$ from $[26\text{-}^{14}\text{C}]$ cholesterol after incubation for 6 hr. with mitochondria from normal rats in the presence of increasing concentrations of cholesterol. Experimental details are given in the Methods section.

the polar steroids was barely detectable (Fig. 3) and was usually no greater than that present in the polar zone on chromatograms of the stock solution of $[^{14}\text{C}]$ cholesterol.

Radioactivity was assayed in three zones on each paper strip: the area between cholesterol and the solvent front (polar zone); that between cholesterol and the origin (non-polar zone); the area containing cholesterol itself. After 6 hr. incubations, the radioactivity recovered in the polar zone accounted for 15–25% of the total radioactivity added initially, that in the cholesterol zone accounted for 60–70% and that in the non-polar zone for 5–8%.

With the solvent system used for the reversed-phase paper chromatography, mono-, di- and tri-hydroxycholelanic acid and 7α -hydroxycholesterol all ran with R_f values similar to the R_f of the radioactivity in the polar zone (Fig. 3). However, at least six radioactive components more polar than cholesterol could be distinguished on thin-layer chromatograms prepared from incubations with $[4\text{-}^{14}\text{C}]$ cholesterol. One of these components moved with the R_f of cholic acid.

Further evidence for the presence of acidic substances in the polar fraction was obtained from an analysis of the radioactivity in the butanol extract by chromatography on columns of potassium hydroxide-treated silicic acid, with a solvent system that gives almost complete separation between bile acids and neutral steroids (Lewis & Myant, 1965). In one of two experiments in which the butanol extract was made from an incubation with $[4\text{-}^{14}\text{C}]$ cholesterol, 88.9% of the radioactivity put on to the column was recovered in the neutral and acidic fractions. Of this, 8.9% was in the acidic fraction and 91.1% was in the neutral fraction.

Fig. 3. Radioautograph of reversed-phase paper chromatogram prepared from: (a) the butanol extract of an 8 hr. incubation of $[4\text{-}^{14}\text{C}]$ cholesterol with boiled (i) and normal (ii) liver mitochondria from untreated rats; (b) the acidic (iii) and neutral (iv) fractions in the butanol extract obtained by separation on a KOH-treated silicic acid column. Experimental details are given in the Methods section. A, Position occupied by samples of 7α -hydroxycholesterol, lithocholic acid, deoxycholic acid and cholic acid run in parallel on the same paper chromatogram; B, position occupied by cholesterol marker.

Analysis of the two eluates by reversed-phase paper chromatography showed that most of the radioactivity in the acidic fraction moved to the polar zone, whereas most of that in the neutral fraction moved to the position of cholesterol (Fig. 3). A sample of the eluate containing the acidic fraction was also submitted to thin-layer chromatography with chloroform-methanol (7:3, v/v) as solvent. Radioautographs of the chromatograms showed at least five bands of radioactivity (Fig. 4). One of these corresponded to the cholic acid marker. Another moved with R_f slightly greater than that of lithocholic acid. Similar results were obtained in the second experiment.

Radioactivity recovered in the volatile fraction. When 6 hr. incubations were carried out with $[26\text{-}^{14}\text{C}]$ cholesterol, the radioactivity recovered in the butanol extract was usually less than 95% of the total added at the beginning of the incubation. Since recovery of steroids in the butanol extract was complete (see above), the radioactivity not recovered in the butanol in experiments with $[26\text{-}^{14}\text{C}]$ cholesterol may be assumed to be due to radioactive fragments removed from the end of the

Fig. 4. Stained thin-layer chromatograms of: (i) the acidic fraction of the butanol extract from a 6 hr. incubation with $[4-^{14}\text{C}]$ cholesterol, obtained by separation on a KOH-treated silicic acid column after adding non-radioactive cholesterol (1mg.) and cholic acid, deoxycholic acid, chenodeoxycholic acid and lithocholic acid (0.5 mg. of each) to the butanol as carriers; (ii) a mixture of cholesterol and the four bile acids run in parallel on the same thin-layer plate. Chloroform-methanol (7:3, v/v) was used as the mobile phase for the thin-layer chromatography. Other experimental details are given in the Methods section. *A*, Position occupied by cholesterol; *B*, position occupied by lithocholic acid; *C*, position occupied by deoxycholic acid and chenodeoxycholic acid; *D*, position occupied by cholic acid. A radioautograph of (i) is shown on the left.

side chain. These include carbon dioxide, and other substances of low molecular weight, which were removed during evaporation of the ethanol from the acidified incubation mixture or which remained in the aqueous phase after extraction with butanol. The yield of these radioactive products, other than $^{14}\text{CO}_2$, was calculated as the difference between the amount of radioactivity added initially to the incubation mixture and the amount recovered in the butanol, less that recovered in carbon dioxide. This fraction of the total radioactivity is referred to below as the volatile fraction. The rise in radioactivity in the volatile fraction was linear between the fourth and eighth hours of incubation (Fig. 1).

In a few experiments with $[26-^{14}\text{C}]$ cholesterol, the radioactive volatile compounds were isolated by steam-distillation of the acidified incubation mixture before evaporation of the ethanol and extraction with butanol. The steam-distillate was then

Fig. 5. Radioactivity (●) and titratable acid (○) in 5 ml. fractions eluted from a Celite column during chromatography of the steam-distillate from an incubation with $[26-^{14}\text{C}]$ cholesterol. Each flask contained the mitochondria from 2 g. of liver from a thyroxine-treated rat; CoA (1 mg./flask) was added at the beginning of the incubation. Other experimental details are given in the Methods section. In this experiment, 63% of the radioactivity in the distillate was recovered in the propionic acid peak.

Fig. 6. Effect of increasing the concentration of mitochondrial protein on the yield of radioactivity in CO_2 (○) and in the volatile (□) and polar (Δ) fractions during a 6 hr. incubation with $[26-^{14}\text{C}]$ cholesterol. The conditions of incubation were those described in the Methods section, except that the concentration of mitochondria in the sucrose suspension was adjusted to give the required final concentrations in the incubation flasks, and the amounts of added cofactors (ATP, NAD^+ , GSH and citrate) were adjusted to give the same ratio of cofactor concentration to concentration of mitochondria as that in the standard incubation mixture.

chromatographed on Celite columns. Up to 12% of the total radioactivity in the incubation mixture was recovered in the distillate, and between 60 and 80% of this was eluted from the column as a well-defined peak corresponding to the titration peak of propionic acid (Fig. 5). The *S*-benzylisothiuronium salt of propionic acid was prepared from the pooled fractions of the eluate corresponding to the propionic acid peak. A crystalline product was obtained (m.p. 151°) with a specific radioactivity of 20.1 counts/min./mg. After recrystallization from hot water the specific radioactivity of the crystalline material (m.p. 151°) was 19.4 counts/min./mg.

Effect of changing the quantity of mitochondria in the incubation mixture. With a given preparation of mitochondria from untreated rats, the yield of

$^{14}\text{CO}_2$ and the amount of radioactivity in the volatile fraction were both roughly proportional to the concentration of mitochondrial protein in the incubation flask (Fig. 6). The radioactivity recovered in the polar fraction tended to decrease with increasing concentrations of mitochondrial protein.

Production of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$ propionate. Propionate was readily oxidized to carbon dioxide by the mitochondrial preparation. When $[1\text{-}^{14}\text{C}]$ propionate was incubated at increasing concentrations with mitochondria from untreated rats, the amount oxidized to $^{14}\text{CO}_2$ in 6 hr. reached a maximum of about 250 μmoles (Fig. 7).

Effect of thyroxine treatment on the mitochondrial oxidation of $[^{14}\text{C}]$ cholesterol

To test the effect of thyroxine treatment, two or more rats to be treated were matched by weight with an equal number of control animals. At the end of the treatment, mitochondria were prepared from the pooled livers of each group of rats.

Radioactivity recovered in carbon dioxide. The production of $^{14}\text{CO}_2$ from $[26\text{-}^{14}\text{C}]$ cholesterol was increased in mitochondrial preparations from thyroxine-treated rats (Fig. 1 and Table 1). In 18 comparisons between groups of treated and control rats, the ratio of the yield of $^{14}\text{CO}_2$ in the treated preparation to that in the control averaged 4.5 (range, 2.1–14.5). The stimulatory effect of thyroxine was not due to an increase in the permeability of the mitochondria to cholesterol, since it was equally marked when the mitochondria were disrupted by freezing and thawing before they were added to the incubation mixture (Table 2). Nor was it due to a decrease in the amount of endogenous cholesterol in the treated mitochondria ($27 \pm 6 \mu\text{g.}$ of cholesterol/mg. of mitochondrial protein) compared with that in the controls ($20 \pm 5 \mu\text{g./mg.}$).

Fig. 7. Yield of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]$ propionate after incubation for 6 hr. with mitochondria from normal rats in the presence of increasing concentrations of propionate. Experimental details are given in the Methods section.

Table 1. *Radioactivity in carbon dioxide, the volatile fraction and the three chromatographic fractions of the butanol extract from incubations of $[^{14}\text{C}]$ cholesterol for 6 hr. with mitochondria from control and thyroxine-treated rats*

Experimental details are given in the Methods section. Radioactivity in the volatile fraction was calculated as the difference between the amount of radioactivity added and the amount recovered in the butanol extract and in CO_2 . Results are expressed as percentages of the total radioactivity added at the beginning of the incubation.

		Percentage of added radioactivity in each fraction					
		CO_2	Volatile fraction	Polar fraction	Cholesterol fraction	Non-polar fraction	$\text{CO}_2/\text{volatile fraction radioactivity ratio}$
$[^{14}\text{C}]$ Cholesterol added	Control	0.56	3.9	23.0	67.4	5.1	0.14
	Treated	1.47	10.7	13.8	65.5	8.5	0.14
$[4\text{-}^{14}\text{C}]$ Cholesterol	Control	—	+0.2	18.9	74.6	6.3	—
	Treated	—	−0.9	20.0	72.8	8.1	—

Table 2. *Effect of disrupting mitochondria on $^{14}\text{CO}_2$ production from [26- ^{14}C]cholesterol incubated for 6 hr. in the presence of mitochondria from normal and thyroxine-treated rats*

Experimental details are given in the Methods section.

Conditions ...	Radioactivity in CO_2 (% of total added)	
	Control	Treated
Intact mitochondria	0.45	2.63
Disrupted mitochondria	0.39	2.88

Radioactivity recovered in the volatile fraction and in the butanol extract. Radioactivity in the volatile fraction from incubations with [26- ^{14}C]cholesterol was increased by thyroxine treatment (Fig. 1 and Table 1), and that in the polar fraction of the butanol extract was decreased. Thyroxine treatment also caused a slight, but reproducible, increase in the amount of radioactivity in the non-polar fraction of the butanol extract. In experiments with [4- ^{14}C]cholesterol, thyroxine treatment had no effect on the radioactivity recovered in the polar fraction. Table 1 shows the results of a typical experiment in which mitochondria from normal and thyroxine-treated rats were incubated for 6 hr. with [26- ^{14}C] and [4- ^{14}C]cholesterol. Radioactivity recovered in carbon dioxide, expressed as a proportion of the radioactivity in the volatile fraction, is also shown in Table 1 (see the Discussion section).

The effect of thyroxine treatment on the yield of radioactivity in carbon dioxide and in the volatile fraction was similar to that obtained by increasing the concentration of mitochondrial protein in incubation mixtures prepared from untreated rats (Fig. 6). However, thyroxine does not produce its effects by increasing the quantity of mitochondrial protein/g. of liver, because the values from the treated rats (8.5 ± 0.8 mg. of mitochondrial protein/g. of liver) were not significantly different from the control values (9.0 ± 0.3 mg./g.).

Production of $^{14}\text{CO}_2$ from [^{14}C]propionate. The production of $^{14}\text{CO}_2$ from [^{14}C]propionate was not significantly increased by thyroxine treatment. In six experiments in which mitochondria from control and thyroxine-treated rats were incubated for 6 hr. with [1- ^{14}C]propionate, $8.8 \pm 2.2\%$ was oxidized to $^{14}\text{CO}_2$ in the control preparations and $9.1 \pm 2.1\%$ in the treated preparations.

DISCUSSION

Scheme 1 shows the probable sequence of changes leading from cholesterol to the two primary bile acids formed by the liver in rats (Bergström,

Danielsson & Samuelsson, 1960; Suld *et al.* 1962). According to Scheme 1, the ring system is first modified by saturation of the Δ^5 -double bond, removal of the 3β -hydroxyl group and the introduction of α -hydroxyl groups at positions 3 and 7 (II) or 3, 7 and 12 (IIa); the formation of 7α -hydroxycholesterol is probably an early intermediate in this sequence of changes. After the ring system has been modified, one of the end carbon atoms of the side chain is oxidized to give $3\alpha,7\alpha$ -dihydroxycoprostanic acid (III) or $3\alpha,7\alpha,12\alpha$ -trihydroxycoprostanic acid (IIIa). The di- and trihydroxycoprostanic acid are then converted into the corresponding C_{24} bile acids [chenodeoxycholic acid (IV) and cholic acid (IVa)] by removal of the end three carbon atoms of the side chain as a single propionyl-CoA unit. Propionyl-CoA is then oxidized to carbon dioxide by the mitochondria via succinyl-CoA (Friedberg, Adler & Lardy, 1956).

In Scheme 1, the branch between the di- and the tri-hydroxy series is assumed to lie before the oxidation of C-27 to a carboxyl group, in accordance with the finding of R. K. Bridgwater & S. Lindstedt (quoted by Bergström *et al.* 1960, p. 299) that $3\alpha,7\alpha$ -dihydroxycoprostanic acid cannot be converted into cholic acid in the intact rat, but that $3\alpha,7\alpha$ -dihydroxycoprostanic acid cannot. Steps between the main intermediates have been omitted. A possible pathway by which some chenodeoxycholic acid is formed via 26-hydroxycholesterol (Bergström & Danielsson, 1963) has also been omitted.

With the solvent system used for paper chromatography in the present work, 7α -hydroxycholesterol was well separated from cholesterol and ran with R_F similar to that of the polar fraction from the butanol extract. With the possible exception of 7α -hydroxycoprostan-3-one (B. Samuelsson, quoted by Bergström *et al.* 1960, p. 299), 7α -hydroxycholesterol is the least polar of the probable intermediates between cholesterol and bile acids. It is likely, therefore, that the polar fraction contained most, if not all, of the radioactive steroids in the incubation mixture that were more polar than cholesterol. In experiments with [26- ^{14}C]cholesterol, only the C_{27} compounds in the polar fraction from the butanol extract would be radioactive. With [4- ^{14}C]cholesterol, on the other hand, radioactivity would be present in all the steroids derived from cholesterol, including any C_{24} bile acids that may be formed *in vitro*.

Some of the polar steroids were acidic, as shown by their behaviour on potassium hydroxide-treated silicic acid columns. Moreover, thin-layer chromatography of the polar steroids showed that a radioactive substance with R_F similar to that of cholic acid was formed from [4- ^{14}C]cholesterol. This substance may have been cholic acid itself, or one of the radioactive acidic compounds, similar to

Scheme 1. Scheme, based on the suggestions of Bergström *et al.* (1960) and of Suld *et al.* (1962), for the formation of cholic acid and chenodeoxycholic acid from cholesterol. (I), 7 α -Hydroxycholesterol; (II), 3 α ,7 α -dihydroxycoprostanane; (IIa), 3 α ,7 α ,12 α -trihydroxycoprostanane; (III), 3 α ,7 α -dihydroxycoprostanic acid; (IIIa), 3 α ,7 α ,12 α -trihydroxycoprostanic acid; (IV), chenodeoxycholic acid; (IVa), cholic acid.

cholic acid, found by Fredrickson (1956) in ether extracts from incubations of [4- ^{14}C]cholesterol with mouse-liver mitochondria. This is in agreement with other evidence that isolated liver mitochondria catalyse the oxidation of cholesterol by a pathway similar to, if not identical with, that in the whole animal. Thus mitochondria have been shown to convert trihydroxycoprostanane (IIa) into trihydroxycoprostanic acid (IIIa) *in vitro* (Suld *et al.* 1962), and to convert the latter into cholic acid (Briggs, Whitehouse & Staple, 1961). Danielsson & Horning (1959) have also shown that cholesterol incubated with mouse-liver mitochondria gives rise to a trihydroxylated C_{27} compound, possibly an intermediate between compounds (II) and (III), which is converted into chenodeoxycholic acid in the whole rat.

The almost complete absence of radioactivity in the polar zone on paper chromatograms from incubations with boiled mitochondria shows that most of the polar-steroid fraction in normal incubations was formed enzymically, and was not due to autoxi-

dation (Bergström & Wintersteiner, 1942) or photolysis (Hais & Myant, 1965) of the added cholesterol. To this extent, our findings do not agree with those of Danielsson (1960), who found that, in butanol extracts from emulsions of [4- ^{14}C]cholesterol incubated with boiled tissue extracts, 4–5% of the total radioactivity was due to compounds more polar than cholesterol. On the other hand, radioactivity in the non-polar fraction of the butanol extract may have been due partly to non-enzymic autoxidation or photolysis, since some radioactivity was present in this fraction from incubations with boiled mitochondria (Fig. 3). However, if all the non-polar radioactivity were due to this, it is difficult to see why it was increased by thyroxine treatment.

Most of the radioactivity in the volatile fraction from incubations with [26- ^{14}C]cholesterol was in a form that gave rise to propionic acid on steam-distillation at acid pH. This is consistent with the suggestion of Suld *et al.* (1962) that the end of the cholesterol side chain is removed as propionyl-CoA,

a suggestion based on their recovery of [^{14}C]-propionyl-CoA from incubations of trihydroxy-[26- ^{14}C]coprostane with liver mitochondria. Our finding is also consistent with Danielsson's (1963) conclusion that, in the formation of bile acids from cholesterol, a reaction in which acetone is the primary product of the cleavage of the side chain (Whitehouse, Rabinowitz, Staple & Gurin, 1960) is probably not of major importance. If the scheme of Suld *et al.* (1962) is accepted, it is reasonable to assume that the $^{14}\text{CO}_2$ formed during incubations with [26- ^{14}C]cholesterol was derived from the volatile fraction, and that the radioactive propionyl-CoA in the volatile fraction, recovered as [^{14}C]propionate in the steam-distillate, was derived from a radioactive C_{27} compound in the polar fraction of the butanol extract.

Treatment of the whole animal with thyroxine is known to stimulate the mitochondrial oxidation of several substrates of low molecular weight, including succinate (Lardy & Maley, 1954). This might suggest that the stimulatory effect of thyroxine on the production of carbon dioxide from cholesterol was due merely to an effect on the oxidation of propionyl-CoA. However, several of our findings argue against this. In the first place, the oxidation of the C_3 fragment is probably not rate-limiting for the oxidation of the side chain of cholesterol to carbon dioxide, since our preparation had a greater capacity for oxidizing propionate than for oxidizing C-26 of cholesterol to carbon dioxide. Thus, during a 6 hr. incubation, C-26 from a maximum of about 0.52 μmole of added cholesterol was oxidized to carbon dioxide (Fig. 2) (or 60 μmoles if the endogenous cholesterol in the mitochondria is assumed to be exchangeable with the added cholesterol), compared with 250 μmoles of added propionate oxidized during a similar period (Fig. 7). Secondly, if the effect of thyroxine is due to stimulation of the oxidation of propionate, there should be a decrease, and not an increase, in the net accumulation of radioactivity in the volatile fraction from the treated preparations. Thirdly, if thyroxine acts on cholesterol oxidation by stimulating propionate oxidation, the production of $^{14}\text{CO}_2$ per unit of radioactivity in the volatile fraction should be greater in the treated than in the control preparations, but this was not found to be so (Table 1). Finally, we were unable to demonstrate a significant stimulatory effect of thyroxine on the oxidation of exogenous [1- ^{14}C]propionate.

It seems more likely that thyroxine acts at a step earlier than the oxidation of the side-chain fragment. The combination of effects shown in Table 1 is most simply explained by supposing that thyroxine stimulates a rate-limiting reaction at one of the later steps leading to the cleavage of the side chain, and has little or no effect on the hydroxylations of

the ring system. In experiments with [26- ^{14}C]cholesterol this would lead to a decrease in the net accumulation of activity in the fraction containing the polar steroids and an increase in radioactivity in the volatile fraction and in carbon dioxide. With [4- ^{14}C]cholesterol, on the other hand, there would be no decrease in radioactivity in the polar fraction, since the radioactive carbon atoms would remain in this fraction after removal of the end three carbon atoms of the side chain.

Our observations provide no evidence as to the particular step that is sensitive to thyroid hormone. However, there is one possible site of action that would explain why thyroxine stimulates the production of chenodeoxycholic acid in the whole animal, but does not stimulate cholic acid production. If bile acids are formed according to Scheme 1, there might be a single enzyme system catalysing the oxidation of the side chain of both compounds (II) and (IIa). If it were rate-limiting, stimulation of this step would increase the formation of chenodeoxycholic acid by increasing the rate of formation of compound (III) and, in doing so, would diminish the supply of compound (IIa) for the formation of cholic acid.

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