ACTA CHEMICA SCANDINAVICA 14 (1960) 846–860

On the Oxidation of Cholesterol in Liver Mitochondrial Preparations

Bile Acids and Steroids 99

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The oxidation of 4-^{14}C- and 26-^{14}C-cholesterol has been studied in mitochondrial preparations of mouse and rat liver. The simultaneous occurrence of autoxidation and enzymatic oxidation of the added labeled cholesterol, indicated by experiments on the metabolism of cholesterol in different cellfractions of liver and in mitochondria of heart and kidney, has been ascertained by analyses of the labeled products formed under non-enzymatic and "enzymatic" conditions. Among autoxidatively formed products 3β, 5α, 6β-trihydroxycholestane, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol have been identified. Two major enzymically formed compounds both retaining the C_{27}-side-chain, have been isolated. One is less polar than 7α-hydroxycholesterol but more polar than 7-ketocholesterol, while the other shows chromatographic properties similar to a neutral trihydroxy-C_{27}-sterol. This latter compound is metabolized to chenodeoxycholic but not cholic acid in the bile-fistula rat.

Cholesterol is catabolized by the liver *in vivo* to bile acids, which represent the major end-products of cholesterol metabolism in the animal body. The individual steps in this chain of reactions, which entail elimination of the terminal *iso*propyl-group and modifications of the nucleus by saturation of the Δ^5-double bond, reversal of the configuration of the hydroxyl-group at C_3 and introduction of hydroxyls in C_7 and C_{12} or C_6, have not been fully established. The work of Bergström and Lindstedt [1-4] on the metabolism in the bile-fistula rat of possible intermediates between cholesterol and cholic acid has yielded important informations on the likely sequence in this process. Thus, all available evidence makes it probable that the nucleus is hydroxylated, first at C_7 and then, at least in this species, at C_{12} prior to the completion of the side-chain degradation, and that one of the early steps is the introduction of the 7α-hydroxyl-group.

Studies of the metabolism of cholesterol *in vitro* appear a promising approach to further investigations on the individual steps in the degradation

of cholesterol to bile acids. Such investigations have also been undertaken by several groups of workers. Meier *et al.*[5] reported in 1952 on the formation of labeled CO_2 from 26-^{14}C-cholesterol in rat liver slices. Anfinsen and Horning [6,7] demonstrated the same reaction in mouse liver mitochondria in presence of AMP *, DPN and boiled liver juice. Gurin and coworkers [8,9] have reported similar findings and also the formation of labeled 25-dehydrocholesterol, a labeled C_{27}-acid and aldehyde from 26-^{14}C-cholesterol in a solubilized rat liver mitochondrial system.

Fredrickson and Ono [10,11] analyzed the products formed from 4-^{14}-C-cholesterol in the mouse liver mitochondrial system of Anfinsen and Horning and found that of the 25 % of cholesterol metabolized 15 % was present as acidic compounds not identical with cholic or deoxycholic acid, 7 % as 25- and 26-hydroxycholesterol and the remainder as cholesterol esters. In a preliminary communication Danielsson and Horning [12] have reported on the formation of an unknown compound both from 4-^{14}C- and 26-^{14}C-cholesterol in essentially the same mouse liver mitochondrial system. This compound, which had chromatographic properties similar to a trihydroxylated neutral C_{27}-sterol, was metabolized in the bile-fistula rat to chenodeoxycholic acid and to two acids with chromatographic mobilities identical with those of the 6β-hydroxylated acids formed from chenodeoxycholic acid.

Such *in vitro* studies of the oxidation of cholesterol might conceivably be complicated by the well-known fact that cholesterol is easily autoxidized in colloidal solutions in the presence of air. The products of autoxidation are formed by introduction of oxygen functions in the 5,6,7-positions of the cholesterol molecule.

In a thourough study of the autoxidation of cholesterol in aqueous colloidal solutions Bergström and Wintersteiner[13–15] demonstrated the formation of 7-ketocholesterol and the two epimeric 7-hydroxycholesterols. The oxidation proceeded equally well at 37° as at 85°, although slower, reaching completion after 24 h at 37° compared to 3 h at 85°. After this period of time about 60 % of the cholesterol had been transformed into the 7-oxygenated compounds. Later, Mosbach *et al.*[16] could isolate in low yield 3β, 5a, 6β-trihydroxycholestane from samples of autoxidized cholesterol and Schwenk *et al.*[17] detected small amounts of 3β-, 5a-dihydroxy, 6-ketocholestane in samples of cholesterol-extracts from liver.

In the present work the products formed from labeled cholesterol in presence of rat and mouse liver mitochondria have been isolated and partly identified and the conditions under which the products are formed have been investigated.

EXPERIMENTAL

Labeled compounds. 4-^{14}C-cholesterol (60 μC/mg) was obtained from the Radiochemical Centre, Amersham, England. 26-^{14}C-cholesterol (250 μC/mg) was purchased from New England Nuclear Corp., Boston, Mass., USA. Prior to use the labeled cholesterol was checked for absence of autoxidation products by chromatography [18].

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* Abbreviations used are: AMP = adenosine-monophosphate: DPN = diphosphopyridine nucleotide; Tris = tris (hydroxymethyl)aminomethane: EDTA = ethylenediaminetetraacetic acid.

Reference compounds. *7-Ketocholesterol* (m.p. 169–170°; reported [15] 170°–172°) was prepared from cholesteryl acetate by oxidation with *t*-butyl chromate according to Heusler and Wettstein [19], followed by hydrolysis with potassium carbonate [20]. *7β-Hydroxycholesterol* (m.p. 172–173°, $[\alpha]_D^{25}$ +5°; reported [24] m.p. 178°, $[\alpha]_D$ +7°) was obtained by lithium aluminum hydride reduction of 7-ketocholesteryl acetate and purified by formation of the dibenzoate [21]. *7α-Hydroxycholesterol* was prepared according to Henbest and Jones [22,23]. Cholesteryl benzoate was treated with N-Br-succinimide in boiling carbon tetrachloride for 5–6 min. The resulting 7α-Br-cholesteryl benzoate was treated with sodium formate to yield 7α-formoxycholesteryl benzoate, purified by chromatography on aluminum oxide (Merck, Darmstadt, Germany) and the free sterol was then obtained by saponification. The resulting 7α-hydroxycholesterol had a m.p. of 184–85°, $[\alpha]_D^{25}$ –92°; reported [23,24] 185° $[\alpha]_D$ –86.6° and 91.6°. *3β, 5α, 6β-Trihydroxycholestane* (m.p. 228–231°; reported [25,26] 229–233° and 237–239°) was prepared according to Fieser *et al.*[26] by treatment of cholesterol with hydrogen peroxide in formic acid. 3β, 5α-Dihydroxy, 6-ketocholestane (m.p. 230–233°; reported [26] 231–232°) was obtained by treatment of cholesterol in methanol with N-Br-succinimide, as described by Fieser *et al.*[26].

Preparation of homogenates. White male mice of the Danish State Serum Institute strain weighing approx. 25 g and white male rats of the Sprague-Dawley strain weighing approx. 150 g were used. 10 or 33 % (liver wet weight/volume) homogenates were prepared in 0.25 M sucrose containing 3.6 mg nicotinamide/ml with a tight-fitting Teflon Potter-Elvehjem homogenizer. Mitochondria were isolated by centrifugation for 10 min at 10 000 × *g* and then washed once with the homogenizing medium. Microsomes were spun down in the Spinco preparative ultracentrifuge at 100 000 × *g*, 60 min. Boiled liver juice was prepared from the supernatant of the 10 000 × *g* centrifugation of mouse liver homogenates by heating for 10 min at 85–95° followed by centrifugation for 20 min at 20 000 × *g* to remove denatured protein. In some cases boiled liver juice was prepared according to Horning *et al.*[7] The boiled liver juice was kept at –15° for not more than 4–6 weeks prior to use.

Mitochondria and microsomes were resuspended in a few ml of the nicotinamide-sucrose medium by homogenization with a loose-fitting pestle 10–20 sec and then diluted to suitable volumes. The composition of the incubations, in which mitochondria, AMP, DPN (purchased from the Sigma Chemical Co., St. Louis, USA and Boehringer, Mannheim, W-Germany, respectively) and boiled liver juice were used simultaneously, was as follows:

Mitochondria from 15 g of liver in 10 ml nicotinamide-sucrose medium
Boiled liver juice 9 ml
0.01 M DPN 1.5 ml
0.04 M AMP 1.5 ml
0.05 M Tris buffer, pH 7.8, 5 ml
Serum albumin suspension of labeled cholesterol 1–2 ml

When less or more mitochondria were used the amounts were proportionally changed. When boiled liver juice was omitted no change in the concentrations of the other components was made. In incubations with microsomes, these were suspended in the amount of solution used for suspending mitochondria.

The labeled cholesterol was added to the incubation mixtures as an emulsion with serum albumin, prepared by dissolving the sterol in 0.1–0.2 ml ethanol and adding 0.9–1.8 ml of a 1 % solution of bovine serum albumin (Armour Lab., Kankakee, Ill. USA) in distilled water. In some instances the cholesterol emulsion was prepared with Tween 20 (Atlas Powder Co., Wilmington, Delaware, USA) as described by Meier *et al.*[5]

Incubations were conducted aerobically at 38° for 4 h with shaking in 50 ml Erlenmeyer-flasks containing each not more than 5–7 ml of the incubation mixture. The incubations were terminated by addition of 3–4 volumes of ethanol.

Analyses of the incubation mixtures. The precipitate formed by the addition of ethanol was removed through filtration and the ethanol was evaporated. The aqueous residue was acidified with hydrochloric acid and then extracted twice with water-saturated *n*-butanol. The combined butanol-extracts were washed with water until neutral and

then taken to dryness. The residue was chromatographed in the reversed phase partition systems earlier described. Phase systems I and III [17] were employed for separation of neutral steroids and systems C1 and F1 [27] for acidic steroids. Hyflo Supercel or Hostalene, prepared as earlier described [18,28], were used as supporting material for the stationary phase.

Phase systems:

	Moving phase	ml	Stationary phase	ml
I	*Iso*propanol:water	165:135	chloroform:heptane	10:40
III	» »	150:150	» »	15:60
C1	Methanol:water	150:150	chloroform:*iso*octanol	15:15
F1	» »	165:135	chloroform:heptane	45:5

Chromatograms were run at constant temperature of 23°. Radioactivity was determined in infinitely thin layers with an automatic end-window counter or in a window-less methane gas-flow counter (Frieseke and Hoepfner, Erlangen, Germany, FH 49 and FH 51, respectively). $^{14}CO_2$ was trapped in 2 N KOH, precipitated with 1.5 M $BaCl_2$ and counted in an infinitely thick layer of $BaCO_3$.

RESULTS

Fractionation of homogenates and optimal conditions for oxidation of 4-^{14}C-cholesterol

In this section experiments are described where the conversion of cholesterol to more polar products was analyzed by chromatography first on phase system I to separate cholesterol from the more polar products (Fig. 1) and then on phase system III (Fig. 2) to separate the more polar products into three fractions, one ''acidic'' (A Fig. 2), one ''trioxy neutral'' (B Fig. 2) and one ''dioxy neutral'' (C Fig. 2).

Fractionation of liver homogenates, which were prepared either as 10 or 33 % homogenates, by differential centrifugation in the usual manner, demonstrated (Table 1) that the oxidation of cholesterol to more polar products

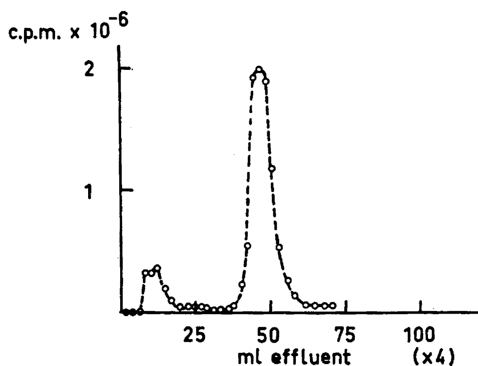

Fig. 1. Chromatogram of the butanol-extract from an incubation of 4-^{14}C-cholesterol with mouse liver mitochondria + boiled juice, AMP and DPN, 18 g Hostalene. Phase system I.

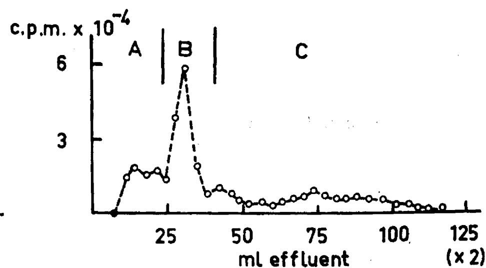

Fig. 2. Rechromatography of first peak in the chromatogram shown in Fig. 1. A = ''acidic'' fraction; B = ''trioxy neutral'' fraction; C = ''dioxy neutral'' fraction. 9 g Hostalene. Phase system III.

Table 1. Oxidation of cholesterol in different cellfractions of mouse and rat liver. 33 % homogenate.

	% conversion to more polar products	
	Rat	Mouse
Mitochondria	3	4.7
Microsomes	0.7	2.5
Supernatant	0.7	2
Mitochondria + supernatant	5	10
Mitochondria + microsomes + supernatant		5.5
Microsomes + supernatant	0.5	3.3

occurred most easily in presence of mitochondria isolated from mouse as well as rat liver. The addition of 100 000 $\times$ g supernatant (hereafter called supernatant) to mitochondria doubled the conversion. The oxidation was considerably slower in presence of microsomes and the addition of microsomes to mitochondria plus supernatant depressed the oxidation. Supernatant alone was capable of a small transformation of added cholesterol to more polar products. Use of boiled liver juice (prepared in either of the two ways described in the experimental part) instead of fresh supernatant in combination with mouse liver mitochondria resulted in the highest conversion of cholesterol to more polar products (10—15 %) and it was found that this magnitude of conversion could easily be reproduced from experiment to experiment. The extent of conversion was examined at various intervals during a 4 h incubation with this system (Fig. 3) and the oxidation was found to proceed in two phases, one rapid with leveling off after 1 h of incubation and afterwards one linear phase.

This system, mouse liver mitochondria plus boiled liver juice, is the same as the one described for achieving maximal $^{14}CO_2$-production from 26-^{14}C-cholesterol except that in the latter case there is a requirement for DPN and AMP. Using the amount of more polar products formed from 4-^{14}C-cholesterol as index of oxidation, as has been done in this investigation, the addition of DPN and/or AMP to the system had no influence on the magnitude of the oxidation nor could any effect be observed, when these cofactors, alone or in combination, were added to mitochondria alone (Table 2).

Incubation with boiled liver juice only resulted in 2—3 % oxidation of the added 4-^{14}C-cholesterol, easily reproduced in a number of experiments.

Table 2. The effect of cofactors on the oxidation of cholesterol in mouse liver mitochondria. 33 % homogenate.

	% conversion to more polar products
Mitochondria	6
» + DPN	5
» + AMP	5
+ DPN and AMP	6.5
+ boiled juice	11
» + boiled juice and DPN	11.5
» + boiled juice + AMP	9
» + boiled juice + DPN + AMP	11

Table 3. Oxidation of cholesterol in mitochondria isolated from different organs. 33 % homogenate.

	% conversion to more polar products	
	Rat	Mouse
Mitochondria (liver)	6.5	5.5
» + boiled juice	8.6	10
Boiled juice	3.3	3
Mitochondria (kidney)	0.7	1
» + boiled juice	2	
Mitochondria (heart)	2	1

Oxidation of cholesterol in presence of a deproteinized liver extract must be attributed to autoxidation. This finding is not entirely surprising in view of the long recognized sensitivity of cholesterol to autoxidation in colloidal solutions. Considering the appreciable autoxidation occurring in presence of boiled liver juice it was conceivable that at least part of the oxidation taking place in the optimal system (mouse liver mitochondria plus boiled liver juice) or even in mitochondria alone was due to autoxidation. One possible way of establishing if autoxidation occurred in presence of mitochondria alone was thought to be a study of the oxidation, if any, of cholesterol in mitochondria isolated from other organs, *e.g.* heart or kidney, which in the present state of knowledge can be regarded as unlikely sites of cholesterol catabolism *in vivo*. The extent of oxidation in heart or kidney mitochondria isolated from rat or mouse varied from 0.7 to 2 % (Table 3). Judging from the chromatographic behaviour of the polar products formed, these consisted of autoxidation products. It appears likely, therefore, that a certain degree of autoxidation of cholesterol occurs in mitochondrial systems.

Using the analytical procedure outlined at the beginning of this section, a consistent difference in the ratio "trioxy neutral" to "dioxy neutral" fraction was obtained when experiments using the complete system were compared with experiments using boiled liver juice only. Thus, under "enzymatic" conditions, *i.e.* using the full system, this ratio in the isolated products was 1.5—1.8, while non-enzymatic incubations, *i.e.* using boiled liver juice, yielded much less "trioxy neutral" steroids, resulting in a ratio of about 0.5—0.7. After incubations with mouse liver mitochondria alone roughly equal amounts of "trioxy neutral" and "dioxy neutral" steroids were isolated, and with rat liver mitochondria somewhat more of the "dioxy neutral" fraction was formed.

As it seemed apparent that part of the more polar products was formed by autoxidation of the added labeled cholesterol, to be described in more detail in the following section, it was conceivable that the mode of suspending the cholesterol could be a factor to be taken into account. Accordingly several experiments were performed using Tween 20 emulsions, a method that has been widely used for dispersing water-insoluble steroids in aqueous media. However, it was found, that the general pattern of polar products formed in the full system from Tween-emulsified cholesterol did not differ from that obtained when albumin-suspensions of cholesterol were used.

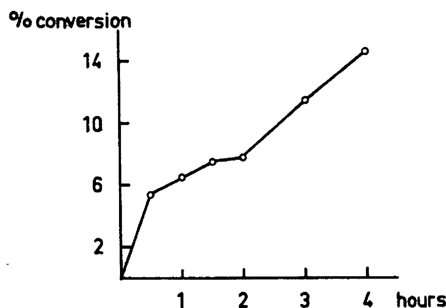

Fig. 3. Time curve of oxidation of cholesterol in mouse liver mitochondria + cofactors.

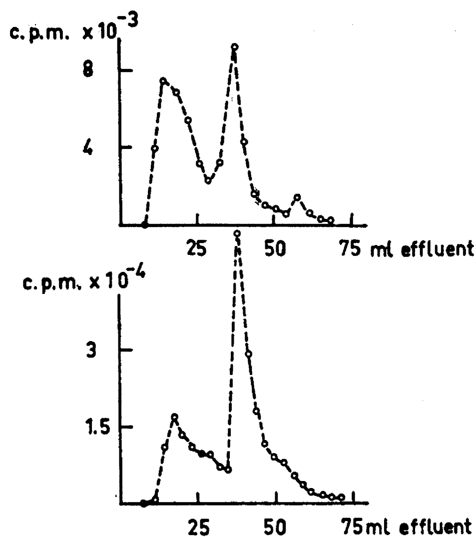

Fig. 4. Rechromatography of "acidic" and "trioxy neutral" fractions from incubation A (upper curve) and C (lower curve). 4.5 g hydrophobic Hyflo Supercel. Phase system III.

In view of the stability of cholesterol towards autoxidation *in vivo*, it appeared possible that the observed occurrence of autoxidation in the mitochondrial system might be due to incomplete penetration of the mitochondrial membrane by the extramitochondrially added cholesterol. As circumstantial evidence for such a concept could be taken the fact that efforts of procuring weighable amounts of the different more polar products by incubating mitochondria from several hundred rat livers did not yield the amounts that could be calculated from the magnitude of conversion of the labeled cholesterol added in these incubations and the amount of endogenous cholesterol present in the mitochondria. Experiments on the distribution of the added cholesterol between the mitochondria and the suspending medium would appear at first sight a more direct approach to this problem. After the termination of a usual 4 h incubation, the mitochondria were isolated again by centrifugation and the amounts of labeled cholesterol and conversion products were assayed both in the mitochondria and in the supernatant fluid. About 75—80 % of the total activity was found associated with the mitochondria. The percentage of more polar products of the total radioactivity was approximately the same in the mitochondria as in the supernatant fluid. Experiments on the rate of association of the added labeled cholesterol with the mitochondria demonstrated that after 15 min incubation 25 %, after 1 h 50 % and, as stated above, after 4 h 75—80 % of the originally added radioactivity was recovered from the mitochondria. That so much of the label was associated with the mitochondria when isolated after a 4 h incubation is by no means any proof, that the label

actually is present inside the mitochondria, as it might just as well be partly or wholly on the surface of the mitochondria. At this time no comparatively simple means of differentiating between these two possibilities could be visualized.

The presence of cupric ions has been shown to be an absolute requirement for the autoxidation of cholesterol in aqueous colloidal solutions [15] and the use of heavy metal complex formers in the incubation mixtures could conceivably reduce the autoxidation occurring in the mitochondrial system. Attempts to achieve a reduction of autoxidation merely by preparing the homogenate and cofactors in redistilled water and carrying out the incubations in flasks carefully rinsed with redistilled water were unsucessful. This is not unexpected since heavy metal ions already occur in the liver. However, in the presence of 10^{-3} M EDTA in the incubation mixture, a considerable reduction of the autoxidation occurred. The conversion to more polar products was in this case 6.4 % compared to 10.8 % in a parallel incubation without EDTA. The distribution of the more polar products between the "acidic", "trioxy neutral" and "dioxy neutral" fractions were in both instances approximately the same, but when the "trioxy neutral" fractions were further analyzed a marked difference was observed. This fraction, as described in detail below, contains 3β, 5α, 6β-trihydroxycholestane, which is the major autoxidation product formed in the present experiments. The content of the cholestanetriol in the "trioxy neutral" fraction from the incubation with EDTA present was only a third (16 %) of that (44 %) found when no EDTA had been added. The used of 10^{-3} M CN^- or 10^{-3} M phenanthroline did not result in the same reduction of autoxidation as the use of EDTA.

Isolation and partial identification of the products of oxidation of cholesterol under non-enzymatic and "enzymatic" conditions

Before undertaking the experiments described below, and the analyses of the products formed in these, a number of testruns and testanalyses were performed. The same chromatographic pattern of products was obtained, whether mitochondria isolated from a 10 % or a 33 % homogenate were employed in incubations with 4-^{14}C-cholesterol together with boiled juice prepared from a water extract of whole liver or from the supernatant fluid obtained after removal of mitochondria. As it was found more practicable to use a 33 % homogenate and boiled juice isolated after spinning down the mitochondria, this method was employed in experiments aiming at isolation of products. When this system was tested in a few experiments for $^{14}CO_2$-production from 26-^{14}C-cholesterol, negligible amounts of labeled CO_2 could be recovered. Chromatography of such incubations demonstrated the presence of the same products found in 4-^{14}C-cholesterol incubations. Two incubations, kindly performed by Dr. M. G. Horning, were run with 26-^{14}C-cholesterol with the aim of also analyzing for $^{14}CO_2$ using a 10 % homogenate and boiled juice from a water extract of whole liver. The yield of labeled CO_2 was 2.5 and 0.4 %, respectively. Due to the use of too small an amount of 26-^{14}C-cholesterol in the first experiment, the conversion products could not be isola-

ted. In the second experiment the conversion to polar products was 10 %. Chromatography of this material did not demonstrate any noticeable difference in the pattern of products compared to, *e.g.*, the ones isolated in the experiments described below.

Isolation and partial identification of products formed from 4-^{14}C-cholesterol under non-enzymatic and "enzymatic" conditions were performed on four separate incubations using: A, cofactors only (boiled juice, AMP and DPN); B, mouse liver mitochondria; C, mouse liver mitochondria plus cofactors; D, rat liver mitochondria. Analyses were performed in the same manner for all four incubations unless otherwise stated.

Chromatography of the butanol-extracts on phase system I allowed calculations of the amount of the more polar products and of cholesteryl esters formed, which latter are retained in the stationary phase. The conversions are given at the top of Table 4. The amount of cholesteryl esters in incubation B was 0.2 %, in C 1.6 % and in D 0.7 %. The more polar products were saponified with 10 % methanolic potassium hydroxide for 1 h. This procedure, which facilitates the further isolation of the products, does not appear to change the chromatographic properties of these. After saponification the polar material was chromatographed on phase system III to separate it into the three fractions earlier discussed. Some 20 % of the radioactivity was retained in the stationary phase and was found to consist of cholesterol as shown by rechromatography on phase system I. An explanation for the presence of cholesterol in this eluate could be that a small part of the cholesterol in the original

Table 4. Fractionation of oxidation products of cholesterol formed under non-enzymatic and "enzymatic" conditions. 33 % homogenate. Amounts of mitochondria and cofactors as given in Experimental. 100 μC of 4-^{14}C-cholesterol was added to each incubation.

	A (Boiled juice, AMP, DPN)	B (Mousemitochondria)	C (Mousemitochondria + boiled juice, AMP, DPN)	D (Rat mitochondria)
Conversion to more polar products in % (before saponification)	4.4	8	15.5	5
Conversion to more polar products in % (after saponification)	3.2	6.7	13.1	3.6
% of total more polar products (after saponification) as Acids	22	20	17	21
3β, 5α, 6β-trihydroxycholestane	14	18	24	13
Ia	6 (Ia + Ib)	14	19	12
Ib		3	5	5
7α-Hydroxycholesterol	12	5	5	20 (7α-, 7β-Hydroxycholesterol and unidentified material)
7β-Hydroxycholesterol	26	8	6	
Unidentified material in "dioxy"-fraction (cf. text)	9	7	4	
III	0	18	14	25
7-Ketocholesterol	11	7	6	4

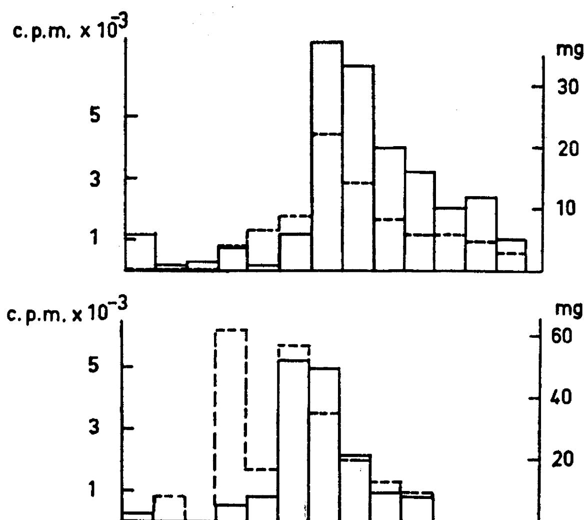

Fig. 5. Chromatography of acetylated "trioxy neutral" fractions of A and C together with unlabeled 3*β*, 5*α*, 6*β*-trihydroxycholestane. *Cf.* Fig. 4. 100 ml fractions. Each bar represents one fraction. 20 g Woelm I. Broken line: radioactivity. Solid line: weight.

butanol-extract was in the form of a complex with lipid material of such a polarity, that it is eluted early with phase system I. After saponification this complex would dissociate and the free cholesterol formed is retained in the stationary phase of the chromatogram with phase system III.

The "acidic" and the "trioxy neutral" fractions were combined and rerun to obtain a more distinct separation of these fractions. This chromatogram is shown in Fig. 4. The upper curve is the one obtained from incubation A (cofactors only) and the lower from incubation C (full system). All subsequent figures will also each show chromatograms from incubation A (upper curves) and C (lower curves).

"Acidic" fraction. The acidic material was chromatographed together with unlabeled cholic acid with phase system C1. In all cases there was a front peak. In material from incubations B, C and D there was also obtained a peak appearing shortly after cholic acid. The eluates of these columns containing any dihydroxy- and monohydroxy acids were chromatographed together with unlabeled chenodeoxycholic acid on phase system F1. In all four chromatograms two radioactive bands appeared after the titration curve of the added carrier.

"Trioxy neutral" fraction. The "trioxy neutral" fractions were acetylated together with 200 mg 3*β*, 5*α*, 6*β*-cholestanetriol each and chromatographed on aluminium oxide (Woelm, Eschwege, W-Germany, grade 1) using increasing concentrations of ethyl acetate (from 0 to 5 %) in benzene as eluant. In the chromatograms of this fraction from incubation C, as shown in Fig. 5, a large radioactive peak, designated in Table 4 as Ia, appears before the 3,6-diacetate

of the carrier is eluted. Comparatively small amounts of activity is eluted at this place in the chromatogram of the same fraction from incubation A, *cf.* Fig. 4. The same early radioactive peak, although smaller, was observed in chromatograms of B and D. Radioactivity was in all cases eluted together with the carrier. At the end of the peak of the carrier the specific activity per mg carrier rose, and to find out if this represented another radioactive compound than the cholestanetriol, the carrier peaks were divided into two fractions, that were crystallized separately to constant specific activity (four crystallizations from methanol/water, acetone/water, hexane and methanol/water). It was found that the late increase in specific activity of the carrier observed in the chromatogram represented radioactive material, designated in Table 4 as Ib, not identical with the 3,6-diacetate of the cholestanetriol. As this occurred also in A, it was thought that this material might be the 3-acetate of 3β-, 5α-dihydroxy-6-ketocholestane, but cocrystallization of the radioactive material with this compound failed to establish any such identity. As Ib represented only a small amount of the total "trioxy neutral" fraction any further characterization was not pursued.

Some information on the structure of compound Ia was obtained by its metabolism in the bile-fistula rat. When injected intraperitoneally it was rapidly excreted, 60—70 % the first 24 h, and had been transformed into chenodeoxycholic acid, the identity of which was established by cocrystallization with unlabeled chenodeoxycholic acid, and into two other acids with chromatographic properties identical to those of the 6β-hydroxylated acids formed from chenodeoxycholic acid.

"Dioxy neutral" fraction. The "dioxy neutral" fraction was subjected to additional chromatography on Hyflo Supercel columns using phase system III. By this method of chromatography this fraction could be separated into two distinct peaks in the case of B, C and D, while A yielded only one main peak, *cf.* Fig. 6. The material in the first peak, present in A, B, C and D, had the same elution volume as 7α- and 7β-hydroxycholesterol, which are difficult to separate with this phase system. The method of choice for achieving a separation of these epimers appears to be to form the dibenzoates, which are reported to be separable by chromatography on aluminium oxide [21] and which in addition are easier to crystallize than the free sterols, anyway in the case of the β-epimer. Accordingly the radioactive material was diluted with 100 mg of unlabeled 7β-hydroxycholesterol and benzoylated in the usual manner. The ether extract of the benzoylation mixture was distributed between hexane and 70 % aqueous methanol, a procedure that was found to remove byproducts, which seriously interfered with the chromatography. The hexane-phase containing the hydroxycholesterol dibenzoates was evaporated to dryness and the residue chromatographed on aluminium oxide. In spite of considerable efforts to separate the epimers by careful chromatography on both Woelm, grade I, and Merck aluminium oxide, no such separation could be obtained. However, it was found that in B and C, but not in A (D was lost in an earlier chromatogram), a radioactive peak appeared in the chromatogram just before the elution of the hydroxycholesterol dibenzoates, but whether this early peak represented some unknown compound or just by chance a partial separation of the α-epimer from the β-epimer cannot be stated at present. All material

eluted in respective columns was combined and crystallized to constant specific activity (from methanol once and acetone/water twice) and the amount of 7β-hydroxycholesterol could thus be calculated. The first mother liquor containing the α-epimer, was then diluted with 80 mg unlabeled 7α-hydroxycholesterol dibenzoate and crystallized to constant specific activity (from methanol once and acetone/water twice) giving the amount of this epimer in the original mixture. Isotope dilution as analysis for the two hydroxycholesterol dibenzoates in a mixture of both has been used earlier by Dauben and Payot [25]. In the case of A there remained 20 %, in B 36 % and in C 30 %, of the original radioactivity not accounted for as the 7-hydroxycholesterol dibenzoates. During the course of the attempts to separate the epimeric dibenzoates, it was observed that limited hydrolysis of the dibenzoates occurred, especially when the chromatograms had to be run over several days. Whether the radioactivity not identified as 7α- and 7β-hydroxycholesterol dibenzoate could be accounted for as partly or wholly saponified dibenzoate or represented a new compound(s) was not further investigated with this material.

In the chromatograms of the "dioxy neutral" fraction (*cf.* Fig. 6), as mentioned earlier, another compound, designated as III in Table 4, was eluted after the 7α- and 7β-hydroxycholesterol in B, C and D. The formation of this unknown metabolite has been observed only in "enzymatic" incubations and its nature and further metabolism is presently being studied.

In all four chromatograms of the "dioxy neutral" fraction on Hyflo Supercel columns (Fig. 6) part of the radioactivity was retained in the stationary

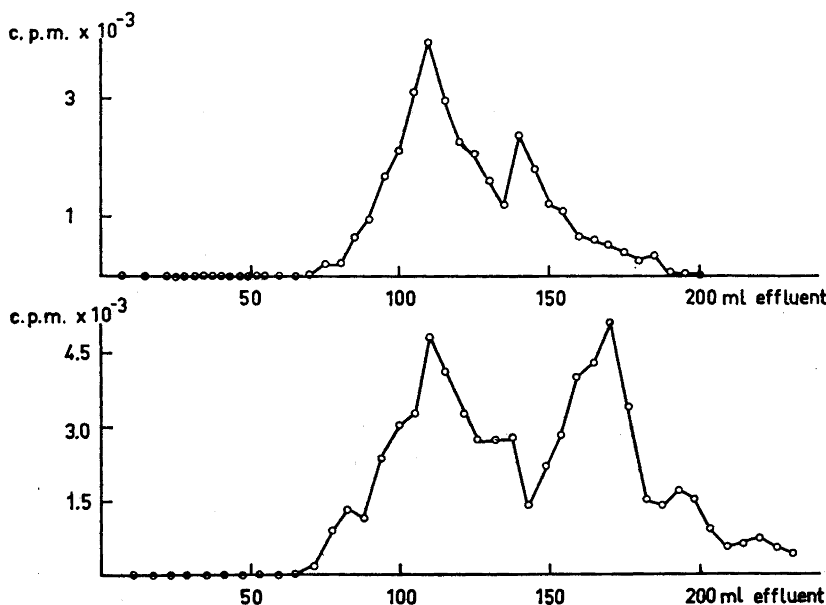

Fig. 6. Chromatograms of "dioxy neutral" fractions of A and C. *Cf.* Fig. 4. 4.5 g hydrophobic Hyflo Supercel. Phase system III.

phase. This material was rechromatographed on phase system I and found to have the same elution volume as 7-ketocholesterol. The identity of the labeled material with 7-ketocholesterol was established by isotope dilution.

DISCUSSION

Prior to the present work only one investigation, that by Fredrickson [10], on the oxidation of 4-^{14}C-cholesterol in a fortified mouse liver mitochondrial system has been published. The results obtained in the two studies are entirely at variance with each other and no explanation can be offered. While Fredrickson found products that appeared to have arisen by enzymatic oxidation only, the results now presented, corroborated by further analyses of the experiments reported by Danielsson and Horning [12], emphasize first of all that a considerable degree of autoxidation of the added 4-^{14}C-cholesterol indubitably occurs. Thus, there appears to be no reason to invoke enzymatic action as an explanation for the formation of such compounds as 3β, 5α, 6β-cholestanetriol, 7β-hydroxycholesterol and 7-ketocholesterol, which were formed in all incubations examined. These compounds are all typical autoxidation products and would be illogical and improbable intermediates in the catabolism of cholesterol to bile acids, which latter have normally a 7α-hydroxyl-group and invariably lack a hydroxyl at position 5. The possibility that 7α-hydroxycholesterol in part was formed enzymatically should not be excluded expecially as the evidence so far available makes it a highly probable early intermediate in the formation of bile acids from cholesterol [3]. On the other hand, it must also have been formed by autoxidation, which yields both epimers. Any possibility of establishing if 7α-hydroxycholesterol was formed partly by enzymatic action on cholesterol in the present experiments appears remote.

Based on the nature of the polar products and the respective amounts of these the extent of autoxidation in the fortified mouse liver mitochondrial system amounted to approximately 50 % of the total amount of labeled cholesterol oxidized.

Although the occurrence of autoxidation in the mitochondrial system cannot be regarded as unexpected in view of the well-documented instability of cholesterol in polar solvents upon exposure to oxygen *, it would seem possible that the mode of solubilizing the cholesterol could be of importance. Attempts to reduce autoxidation by emulsifying with Tween instead of serum albumin were not successful. Recently Avigan [29] published a method for preparing cholesterol solutions with the aid of serum lipoproteins and this method merits to be tested as a possible means of reducing autoxidation in the mitochondrial system. Another possibility could be to run short time incubations. The shape of the time curve was such (Fig. 3) that it had some resemblance to that of an enzymatic process in mitochondria or microsomes during the first 2 h of incuba-

* The radiation of ^{14}C in ^{14}C-labeled cholesterol stored in the dry state in presence of air for long periods of time, 1 month—2 years, has been shown [25] to induce autoxidation proceeding in a manner identical with that of unlabeled cholesterol in colloidal suspensions. The contribution, if any, of the radiation of ^{14}C in the labeled cholesterol to the magnitude of autoxidation in the present experiments was not investigated.

tion. However, some preliminary analyses of polar products formed during this early phase of the incubation did not reveal any striking differences.

A more direct approach to the problem of reducing autoxidation seems to be to incubate in the presence of heavy metal complex formers, as the autoxidation of cholesterol in aqueous colloidal solutions has been shown to require the presence of cupric ions [15]. The use of 10^{-3} M EDTA in the incubation mixture resulted in a considerable but not total reduction of the autoxidation. Further work is needed in order to be able to closer evaluate the possibilities in this method.

The emphasis should, however, not be put only on the occurrence of autoxidation of the added labeled cholesterol in the mitochondrial preparations. Approximately half of the products isolated must have been formed by the action of enzymes. As basis for this conclusion is taken that products formed only in the presence of mitochondria are enzymatic products. The two main enzymatically formed products were Ia, which is identical with the compound earlier reported on by Danielsson and Horning [12], and III (*cf.* Table 4). As earlier stated both compound Ia and III must have a C_{27}-side-chain, as they were formed also from 26-^{14}C-cholesterol, but the detailed structures of these have not been established. Ia had chromatographic properties similar to those of a trihydroxylated neutral C_{27}-sterol both on reversed phase and as acetylated on aluminium oxide columns. It forms probably a triacetate. as it had the same mobility on aluminium oxide as the triacetate of 3α, 7α, 27-trihydroxycoprostane. The manner it was metabolized in the bile-fistula rat, yielding chenodeoxycholic but not cholic acid, indicated that the hydroxyls could be in positions 3, 7 and 27, as 3α, 7α-dihydroxycoprostane is metabolized to both cholic and chenodeoxycholic acid, while 3α, 7α-dihydroxycoprostanic acid gives chenodeoxycholic but not cholic acid [30], *i.e.* the presence of oxygen functions in the side-chain of a steroid with a 3,7-hydroxylated nucleus makes a 12-hydroxylation in the rat impossible.

Compound III showed chromatographic properties similar to those of a saturated dihydroxy neutral C_{27}-sterol or of an unsaturated hydroxyketone with considerably greater polarity than 7-ketocholesterol. Work on the structure and further metabolism of this compound is in progress and will be the subject of a future communication.

Among the acidic products at least one acid, the one which was eluted shortly after cholic acid with phase system C1, was an enzymatic product, but no further characterization of this acid has been carried out. The presence of labeled acidic compounds in the non-enzymatic incubations was unexpected, as such products have not been demonstrated earlier in autoxidized cholesterol. Further work on the nature of these must be done before their chromatographic counterparts in the "enzymatic" incubations can be studied.

In discussing the labeled enzymatic products formed from labeled cholesterol in the mitochondrial preparations, it must be stressed that there is no evidence, either for or against, that these compounds are formed *directly* from cholesterol, as they just as well could be enzymatically transformed autoxidation products, in the case of Ia and III most probably from 7α-hydroxycholesterol and in the case of the acidic compound(s) from any of the autoxidation products.

Whether or not any $^{14}CO_2$ was formed from 26-^{14}C-cholesterol, the same general pattern of polar products was obtained. The results presented therefore have the same bearing on incubations performed with 26-^{12}C-cholesterol as with 4-^{14}C-cholesterol. There can be no doubt that the formation of $^{14}CO_2$ from 26-^{14}C-cholesterol is an enzymatic process but the nature of the substrate in this oxidation is in more doubt, anyway as judged from the results of this investigation. Using only $^{14}CO_2$-formation from 26-^{14}C-cholesterol as an assay it appears that results of studies of the *quantitative* aspects on the conversion of cholesterol to bile acids should be interpreted with caution, especially in incubations of long duration.

Acknowledgments. The skillful technical assistance of Miss Birgitta Holmberg and Miss Electra Pascalides is gratefully acknowledged.

This work is part of investigations supported by *Statens Medicinska Forskningsråd, Knut och Alice Wallenbergs Stiftelse, Karolinska Institutets Reservationsanslag* and *The National Institutes of Health,* Bethesda, Md, USA (H 2842).

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Received December 17, 1959.