The *in Vitro* Catabolism of Cholesterol. Formation of Cholic Acid from Cholesterol in Rat Liver*

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ABSTRACT: The interrelationship that exists between cholesterol and cholic acid has been investigated in rat liver. An enzyme preparation, which is capable of converting cholesterol to cholic acid *in vitro*, is described in this paper. It is suggested that enzymes from both the mitochondria and the microsomes are required for the complete metabolic degradation of cholesterol to bile acids in rat liver. An *in vitro* system capable of transforming cholesterol to cholic acid should considerably aid investigations concerned with the sequence and chemical mechanisms of bile acid formation in mammalian liver.

The complex series of reactions which occur when cholesterol is degraded to bile acids has not yet been fully described. Both *in vivo* and *in vitro* techniques have been used in an attempt to elucidate this problem. While the former approach has yielded important information regarding this metabolic pathway (see review by Bergström *et al.*, 1960), it is apparent that a more comprehensive understanding of the chemical and metabolic reactions involved will be obtained from *in vitro* studies. However, results employing *in vitro* methods have on the whole proved rather disappointing. Recently, we have described a preparation from rat liver which is able to metabolize cholesterol to a number of degradation products including cholest-5-ene-3β,7α-diol (Mendelsohn *et al.*, 1965a), 5β-cholestane-3α,7α-diol (Mendelsohn *et al.*, 1965b), and 5β-cholestane-3α,7α,12α-triol (Mendelsohn and Staple, 1963). All three compounds are known to be converted to cholic acid in the bile fistula rat (Bergström *et al.*, 1960), thus implicating them as intermediates in the sequence cholesterol to cholic acid. Since an *in vitro* system capable of transforming cholesterol to cholic acid would offer an excellent opportunity for the study of this reaction sequence, and since our *in vitro* approach appeared to be a useful one in this respect, the authors attempted to investigate the problem using our previously described procedure.

The present communication describes the enzymatic conversion of cholesterol to cholic acid by a rat liver preparation *in vitro*. A preliminary report of this work has already appeared (Mendelsohn and Mendelsohn, 1965).

Experimental Section

Materials. ATP[1] (disodium salt), glutathione, NAD, and coenzyme A were obtained from the Sigma Chemical Co. Cholic acid (Hopkin and Williams, Ltd., England) was recrystallized four times from ethyl acetate. After the fourth recrystallization the cholic acid melted at 198.5–199° (lit. (Shoppee, 1964) mp 196–198°). [4-^{14}C]Cholesterol (sp act. 54.5 μc/mg) was obtained from the Radiochemical Center, Amersham, England. Immediately prior to use the radioactive cholesterol was purified by elution with benzene through a neutral alumina column (activity grade III).

Fractionation of Rat Liver Homogenates. Livers from 10- to 12-week-old male Wistar rats were homogenized in 3 volumes of cold phosphate buffer (Frantz and Bucher, 1954), pH 7.4, containing 3.6 mg/ml of nicotinamide with a loose-fitting glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 500*g* for 10 min to remove nuclei, cell debris, and unbroken cells, and the supernatant suspension was then centrifuged at 20,000*g* for 20 min. The supernatant layer from the latter centrifugation was used as the source of enzymes in the incubations. All manipulations were carried out at 0°.

Incubation with [4-^{14}C]Cholesterol. The substrate, cholesterol, dissolved in benzene, was added in the amounts specified into individual 50-ml erlenmeyer flasks. After addition of a solution of 4 mg of Tween 80 (L. Light and Co., Ltd., England) and 0.25 mg of cholic acid in methanol as trapping agent, the solvents were evaporated in a stream of nitrogen at 40–50°. To the warm residue was added 5 ml of phosphate buffer, pH 7.4, and the mixture was well emulsified by shaking. After cooling, ATP (25 mg), NAD (5 mg), coenzyme A (1 mg), trisodium citrate dihydrate (22 mg), glutathione (15 mg), and EDTA (10 mg)

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[1] Abbreviations used: ATP, adenosine triphosphate; NAD, nicotinamide–adenine dinucleotide; tlc, thin layer chromatography.

were added to each flask followed by 6 ml of enzyme, equivalent to 2 g of rat liver. Flasks prepared in an identical manner, except that they contained enzyme which had been boiled for 10 min, acted as controls. The incubations were conducted aerobically for 90 min at 37° with constant mechanical shaking.

Extraction Procedure. At the conclusion of the incubation period, 40 ml of 95% ethanol was added to each of ten flasks. The precipitate was removed by centrifugation and washed once with an equal volume of warm ethanol, and the combined supernatant solutions were evaporated to dryness *in vacuo* at 37°. Cholic acid (10 mg) was added to the residue before saponification with 50 ml of 7 N aqueous sodium hydroxide for 1 hr. The cooled digest was diluted with 50 ml of water and extracted four times with 40 ml of petroleum ether (bp 30–60°) to remove nonsaponifiable material. The aqueous phase was acidified to pH 1 with concentrated hydrochloric acid and extracted four times with 40 ml of petroleum ether to remove fatty acids. Finally, the acidified digest was extracted six times with 40 ml of diethyl ether. After drying the combined ether extracts over anhydrous sodium sulfate the solvent was removed *in vacuo*. The residue was dissolved in methanol and further purified using the technique of preparative thin layer chromatography (1.0-mm thick silica gel; Kieselgel-DS-5 Camag, Switzerland, which had previously been purified by soxhlet extraction with redistilled methanol for 2 days and dried overnight at 110° before use). After developing the plates three times in the same solvent (benzene–isopropyl alcohol–acetic acid, 30:10:1) a small edge of each plate was sprayed lightly with anisaldehyde reagent (Neher and Wettstein, 1951) in order to locate the position of the cholic acid band. During the latter operation care was taken to protect the rest of the plate from the anisaldehyde spray. An area of silica gel corresponding to the position of the cholic acid was scraped off each plate into a funnel containing three thicknesses of Whatman No. 42 filter paper. The silica gel was washed with boiling methanol (usually 100 ml/plate), and the clear filtrate was collected and evaporated to dryness *in vacuo*. After resubjecting the residue to tlc using solvents benzene–ethyl acetate (2:1) followed by benzene–isopropyl alcohol–acetic acid (30:10:1), the cholic acid was eluted from the silica gel and recrystallized from ethyl acetate and from ethyl acetate–petroleum ether (bp 80–100°) to constant specific activity (see Table I). In all, 25 incubations were performed. At the end of this time the combined silica gels and filter papers were subjected to further soxhlet extraction with redistilled methanol for 2 days and the methanol extract was treated as described above. A total of 110 mg of pure cholic acid was isolated from the incubations.

In order to provide further evidence for the identity of the recrystallized material, its behavior on tlc in three different chromatographic systems, benzene–isopropyl alcohol–acetic acid (30:10:1); benzene–ethyl acetate (2:1); isooctane–isopropyl ether–acetic acid (50:25:25), was compared with that of pure cholic acid. After identification of the spots by exposing them to iodine vapor for a few minutes, they were eluted from the silica gel with methanol, plated, and dried, and their radioactivity was assayed. In all instances the material behaved exactly like pure cholic acid and, furthermore, radioactivity was detected only in those areas occupied by the material and nowhere else on the chromatogram. The substance was further characterized by its melting point, mixture melting point behavior with pure cholic acid, and comparison of its infrared spectrum with that of authentic cholic acid.

TABLE I: Melting Points and Specific Activities of Cholic Acid Isolated from the Incubation Mixtures.[a]

	Recrystn	Wt (mg)	Mp (°C)	Sp Act. (cpm/mmole × 10^4)
1.	Ethyl acetate–petroleum ether	130	196–197	12.24
2.	Ethyl acetate	110	197–198	12.48
3.	Ethyl acetate[b]	250	198–199	4.40
4.	Ethyl acetate–petroleum ether	234	198–199	4.33

[a] See text for details. [b] The 110 mg of cholic acid from system 2 was diluted with 200 mg of nonradioactive cholic acid to give a total weight of 310 mg which was then recrystallized from ethyl acetate. Conditions of incubation as described in the text. [4-^{14}C]Cholesterol, 250,000 cpm/flask. Ten flasks were used for each incubation.

The total amount of radioactive cholic acid isolated from the incubations was diluted with 200 mg of nonradioactive cholic acid. After recrystallization to constant specific activity (Table I) 234 mg of the diluted radioactive cholic acid was obtained. The sample was then utilized to form a number of derivatives of this bile acid.

Methyl 3α,7α,12α-Trihydroxy-5β-cholanoate (I). Cholic acid (234 mg) was methylated in 2 ml of a freshly prepared 1% solution of hydrochloric acid in methanol according to Fieser and Rajagopalan (1950). After standing at 4° for 3 hr the product was collected and washed with ice-cold methanol. The solid, 172 mg (mp 156–157°, lit. (Fieser and Rajagopalan, 1950) mp 156–157°), was recrystallized from boiling methanol to constant specific activity (Table II).

Methyl 3α-Carbethoxy-7α,12α-dihydroxy-5β-cholanoate (II). Compound I (30 mg) was dissolved in 0.4 ml of dry pyridine and 0.05 ml of ethyl chloroformate was added gradually. After 12 hr at room temperature the solution was diluted with ten times the volume of water and allowed to stand 30 min. The precipitate was then collected, washed well with water, and recrystal-

TABLE II: Melting Points and Specific Activities of Derivatives Obtained from Radioactive Cholic Acid.[a]

Derivative	Recrystzn	Wt (mg)	Mp (°C)	Sp Act. (cpm/mmole × 10^4)
I	1. Methanol	172	156–157	4.47
	2. Methanol	154.6	156–157	4.30
II	1. Acetone–water	20.3	176–177	4.15
	2. Ethyl acetate	14	177–178	4.44
III	1. Acetone–water	6.3	156.5–158	4.38
	2. Methanol–water	4.1	157.5–158.5	4.58
IV	1. Methanol–water	44.1	185–186	4.25
	2. Acetone–water	36.7	186–187	4.35
V	1. Methanol–water	20.6	176–179	4.19
	2. Acetone–water	11.1	177–179	4.29

[a] See text for details.

lized from ethyl acetate and acetone–water. It exhibited a slight shrinking at 148° and melted at 177–178°. (The same characteristics were reported by Fieser and Rajagopalan, 1949.) See Table II.

Methyl 3α-Carbethoxy-7-oxo-12α-hydroxy-5β-cholanoate (III). Compound II (14 mg) was dissolved in 0.2 ml of a solution containing 133.6 mg of sodium acetate trihydrate in 0.65 ml of acetic acid and was treated gradually with 0.03 mg of a solution containing 95.7 mg of potassium chromate in 0.25 ml of water. After standing overnight at room temperature ten times the volume of water was added, and the precipitate was collected and washed with water until colorless. The solid (mp 157–158°, lit. (Fieser and Rajagopalan, 1950) mp 157–158°) was recrystallized from methanol–water and acetone–water (Table II).

Methyl 3α,7α-Diacetoxy-12α-hydroxy-5β-cholanoate (IV). Compound I (124 mg) was dissolved with warming in 2 ml of benzene in a 50-ml glass tube fitted with a ground-glass stopper. After the addition of 0.075 ml of dry pyridine and 1.0 ml of acetic anhydride the solution was allowed to stand overnight at room temperature. A further 2 ml of benzene was added before diluting the solution with about 35 ml of water. The mixture was shaken well and then centrifuged for a few minutes to produce a clear separation between the benzene and aqueous layers. After removal of the benzene layer, the aqueous solution was again extracted with 2 ml of benzene; the combined benzene extracts were first washed three times with water and then evaporated to dryness. The oily residue was washed once with 4 ml of petroleum ether (bp 30–60°) and then recrystallized from methanol–water and acetone–water. The solid melted at 185–186° (lit. (Fieser and Rajagopalan, 1950) mp 187–188°).

Methyl 3α,7α-Diacetoxy-12-oxo-5β-cholanoate (V). A solution of 36 mg of IV in 0.8 ml of acetic acid was treated gradually with 35.8 mg of potassium chromate in 0.6 ml of water and allowed to stand overnight at room temperature. After dilution with water to distinct turbidity, the ketone was collected and washed well with water until colorless. The solid (mp 177–179°, lit. (Fieser and Rajagopalan, 1950) mp 177–179°) was recrystallized from methanol–water and acetone–water (Table II).

The behavior of each of the derivatives of cholic acid on tlc in four different solvent systems (the three already mentioned together with 1-propanol–ammonia–water (90:5:5) for I) was compared with that of the authentic compounds. In all instances the substances behaved like the reference compounds and radioactivity was observed only in the area occupied by the material and nowhere else on the chromatogram. This was assessed by extracting the silica gel from the various areas on the plate with boiling methanol, plating, and counting.

Radioactivity of all samples was assayed at "infinite thinness" in a windowless gas-flow counter. Counting was continued until the error was less than ±3%.

Results

Purity of the Cholic Acid. Commercial samples of cholic acid contained a number of impurities when they were chromatographed in various solvent systems on tlc. After four crystallizations from ethyl acetate the melting point of the commercial cholic acid used in this study was raised from 193 to 198.5–199°. The purity of the cholic acid was further assessed by running this compound in three different solvent systems (see Experimental Section) using the tlc technique. Relatively large amounts of material (0.5–5 mg) were chromatographed in order to be able to detect small quantities of impurities. In each instance a single band was observed after spraying the developed plate with anisaldehyde reagent.

Validity of the Extraction Procedure. The validity of the extraction procedure outlined above for cholic

TABLE III: Distribution of Radioactivity during the Various Stages of the Extraction Procedure.[a]

Total number of counts added to incubation medium	2.5×10^6
Number of counts recovered after extracting the alkaline digest with petroleum ether	1.9×10^6
Number of counts recovered after extracting the acidified digest with petroleum ether	5.6×10^5
Number of counts recovered after extracting the acidified digest with diethyl ether	3.5×10^4
Total	2.495×10^6

[a] See text for details. These figures were obtained from a typical incubation in which 2.5×10^6 cpm was distributed equally among ten flasks.

acid was satisfactory. In three different experiments the average recovery of 20 mg of cholic acid was better than 90%. Since a relatively large number of counts (2.5×10^6) in the form of the substrate [4-^{14}C]cholesterol was employed in each incubation, the distribution of the radioactivity during each stage of the extraction procedure at the conclusion of the incubation was observed in order to determine the efficacy of the method in removing the bulk of the unreacted labeled cholesterol. It can be seen from Table III that less than 2% of the total counts in the incubation medium were recovered in the combined ether extracts which contained the cholic acid. When an aliquot of the ether extract was chromatographed using tlc virtually all the radioactivity on the plate could be accounted for in compounds which were more polar than cholesterol.

Table I shows the melting points and specific activity data of cholic acid isolated from the incubations. The percentage conversion of cholesterol to cholic acid under the incubation conditions described above varied from 0.5 to 0.75.

In order to reduce the possibility of a contaminant being responsible for the radioactivity in the cholic acid isolated from the incubations, a number of derivatives of the latter was prepared as described above. Table II demonstrates the melting point behavior and specific activities of these substances, in which it can be seen that no loss of radioactivity occurred after the formation of each derivative.

Discussion

The existence of a direct metabolic relationship between cholesterol and bile acids was conclusively shown in *in vivo* studies on the dog by Bloch *et al.* (1943). However, previous attempts to demonstrate the *in vitro* conversion of cholesterol to cholic acid in mammalian liver have so far been unsuccessful. Frederickson (1956), employing washed mouse liver mitochondria together with a heat-stable soluble fraction of whole liver homogenate, observed that cholesterol was transformed into at least four distinct acidic steroids. Two of these acids had similar properties to but were not identical with cholic or chenodeoxycholic acid, the primary end products of cholesterol degradation in the rat. The formation of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by rat liver mitochondria fortified with soluble cofactor was described by Briggs *et al.* (1961). Suld *et al.* (1962) also used a mitochondrial system derived from rat liver to study the stages in the conversion of 5β-cholestane-3α,7α,12α-triol to cholic acid. Thus it would appear that in order to metabolize cholesterol to bile acids mitochondrial enzymes are necessary for at least the latter portion of this metabolic pathway.

In a series of earlier reports (Mendelsohn and Staple, 1963; Mendelsohn *et al.*, 1965a,b) the authors have described the *in vitro* conversion of cholesterol to a number of compounds (cholest-5-ene-3α-7α-diol, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3α,7α,12α-triol). The latter are probable intermediates in the cholesterol to bile acids sequence since all three compounds have been shown to give rise to cholic acid in the bile fistula rat (Bergström *et al.*, 1960). In each instance the authors employed essentially the microsomal plus supernatant fraction from rat liver as a source of enzymes. While no attempt has been made here to characterize histologically the enzyme system used in the present study, it is indeed possible that during its preparation in the phosphate buffer the mitochondria were damaged sufficiently to allow "solubilization" of certain enzymes necessary for some of the stages in the transformation of cholesterol to cholic acid. Thus, the authors would have been working with a preparation which contained enzymes from both the mitochondria and the microsomes. The omission of microsomal enzymes from his washed mitochondrial system could have accounted for the failure of Fredrickson (1956) to observe any formation of cholic acid from cholesterol. Further work is in progress to clarify these points.

The elucidation of the sequence and detailed mechanisms in the formation of bile acids from cholesterol has been considerably hampered because of the lack of an *in vitro* system suitable for studying this metabolic pathway. The findings, reported here, of an *in vitro* system capable of converting cholesterol to cholic acid should greatly facilitate investigations into the

chemical reactions involved in bile acid formation in mammalian liver.

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The Biologic Activity of Insulin A and B Chains as Determined by the Rat Diaphragm and Epididymal Fat Pad*

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ABSTRACT: This study demonstrates that both purified insulin A and B chains inhibit the uptake of glucose by the rat hemidiaphragm, but neither chain prevents the expected stimulation of glucose uptake in the presence of unmodified insulin. A chain but not B chain is capable of stimulating the conversion of labeled glucose to carbon dioxide in the rat epididymal fat pad, and this simulates the action of the intact insulin molecule. This effect of A chain on adipose tissue is abolished by the addition of insulin antiserum. The integrity of the A and B chains was established by the demonstration of significant insulinlike activity in both muscle and adipose tissue when the chains were recombined and by the neutralization of this activity by insulin antiserum.

These results suggest that insulin A chain alone is capable of producing an insulinlike action upon rat adipose tissue and that, in the absence of insulin, both A and B chains are capable of inhibiting the uptake of glucose in muscle, but this action does not appear to be dependent upon the actual antagonism of unreduced insulin.

The insulin molecule is composed of two polypeptide units, A chain having 21 amino acids with glycine at its NH_2 terminus, and B chain having 31 amino acids with a phenylalanine residue at its NH_2 terminus. These two chains are linked together by two disulfide bonds, and it had been generally accepted that when these disulfide bonds are broken hormone activity is abolished (du Vigneaud *et al.*, 1931). Dixon and Wardlaw (1960) have reported that separated A or B chains did not stimulate glycogen formation in the mouse diaphragm, nor were they active in the mouse convulsion test. Recently, however, conflicting reports have suggested that synthetic A chain produces slight insulinlike activity upon rat diaphragm muscle (Volfin *et al.*, 1964) and that B chain is capable of stimulating adipose tissue (Langdon, 1960). In addition, it has been suggested that B chain, when in combination with serum albumin, behaves as an insulin antagonist and is capable of inhibiting glucose uptake in muscle but not in adipose tissue (Ensinck and Vallance-Owen, 1963).

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