The *in vitro* Catabolism of Cholesterol: Formation of 3α,7α,12α-Trihydroxycoprostane from Cholesterol in Rat Liver*

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An enzyme system has been found in rat liver which is capable of converting cholesterol to 3α,7α,12α-trihydroxycoprostane. This indicates that 3α,7α,12α-trihydroxycoprostane is one of the intermediates directly on the pathway of catabolism of cholesterol to bile acids. The same enzyme system was also able to degrade cholesterol into a number of products apparently different from 3α,7α,12α-trihydroxycoprostane. It is tentatively suggested that one of these compounds is 7α-hydroxycholesterol, which can be formed enzymatically from cholesterol in rat liver.

The sequence and detailed mechanisms of the reactions in the degradation of cholesterol to bile acids in mammalian liver have not yet been fully elucidated. Two main pathways are possible in the conversion of cholesterol to bile acids. Either hydroxylation and saturation of the steroid nucleus occur before the side-chain is oxidized, or oxidation of the side-chain and removal of the terminal isopropyl group precedes any change in the nucleus. In the former case 3α,7α,12α-trihydroxycoprostane (THC)[1] would be an expected intermediate; in the latter case, 3β-hydroxy-5-cholenic acid would be expected. A number of reports have suggested that THC might be a metabolic intermediate in this series of reactions in rat liver. Bergström *et al.* (1954) and Bergström (1955) noted that, after the administration of THC-4-C^{14} and 3β-hydroxy-5-cholenic acid-24-C^{14} to rats with bile fistulas, only the former compound was rapidly converted to cholic acid isolated from the bile. This finding also indicated that hydroxylation of the nucleus probably preceded oxidation of the side-chain. The observation by Whitehouse *et al.* (1961) that rat liver mitochondria readily oxidized the terminal isopropyl group of the THC side-chain to carbon dioxide lends further support to the idea that THC might be on the pathway of metabolism of cholesterol to form cholic acid.

In this communication evidence will be presented that rat liver possess an enzyme system which is able to metabolize cholesterol to THC.

EXPERIMENTAL PROCEDURE

Materials.—ATP (disodium salt), glutathione, NAD, NADP, NADPH were products of the Sigma Chemical Company. 3α,7α,12α-Trihydroxycoprostane was synthesized according to the procedure of Staple and Whitehouse (1959). Cholesterol-26-C^{14} (specific activity 37 μc/mg) was obtained from the New England Nuclear Corporation. Prior to use the radioactive cholesterol was purified by elution with benzene through a neutral alumina column (Woelm, activity grade III).

Fractionation of Rat Liver Homogenates.—Livers from 10- to 12-week-old male Sprague-Dawley rats were homogenized in cold phosphate buffer (Frantz and Bucher, 1954), *p*H 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 minutes to remove nuclei, cell debris, and unbroken cells, and the supernatant suspension was then centrifuged at 20,000 × *g* for 20 minutes. The supernatant from the latter centrifugation was used as the source of enzyme in the incubations. All manipulations were carried out at 0°.

Incubation with Cholesterol-26-C^{14}.—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 20 (Atlas Powder Company, Wilmington, Del.) and 0.25 mg THC 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, *p*H 7.4, and the mixture was emulsified by shaking. After cooling, ATP (25 mg), NAD (5 mg), trisodium citrate dihydrate (22 mg), glutathione (15 mg), and EDTA (10 mg) were added to each flask followed by 6 ml of enzyme, equivalent to 4 g of rat liver. Flasks prepared in an identical manner, except that they contained enzyme which had been boiled for 10 minutes, acted as controls. Incubations were conducted aerobically for 1 hour at 37° with constant mechanical shaking.

At the conclusion of the incubation period, 40 ml of 95% ethanol was added to each flask. The precipitate was removed by centrifugation and washed once with an equal volume of ethanol, and the combined supernatant solutions were evaporated to dryness *in vacuo* at 37°. After saponification of the residue by refluxing with 50 ml of 5% potassium hydroxide in methanol for 1 hour, the cooled digest was diluted with 60 ml of water and extracted 4 times with 40 ml of petroleum ether (b.p. 40–60°). Less than 1% of the total radioactivity remained in the aqueous methanol layer. This procedure produced a residue that was essentially free of any contaminating pigments. The combined petroleum ether extracts were taken to dryness in a stream of nitrogen, and the residue was subjected to reversed phase partition column chromatography (Danielsson, 1958). 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

Initially, experiments were performed to determine

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[1] The abbreviations used are: THC, 3α,7α,12α-trihydroxycoprostane (5β-cholestane-3α,7α,12α-triol); ATP, adenosine 5′-triphosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); EDTA, ethylenediamine tetraacetic acid.

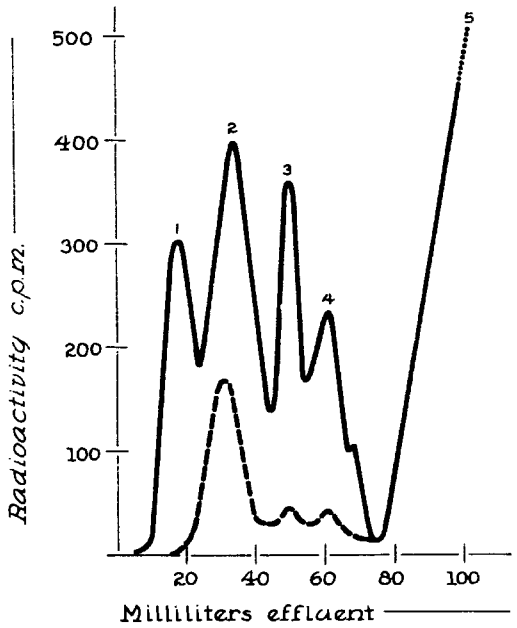

FIG. 1.—Reversed phase column partition chromatography of extract from incubation of cholesterol-26-C^{14} with rat liver enzyme system. Moving phase: 55% (v/v) aqueous isopropanol; stationary phase: 20% (v/v) chloroform in heptane; 4 ml stationary phase supported on 4.5 g hydrophobic supercel. Peak 5 corresponds to unreacted cholesterol-26-C^{14}. ——, enzyme; ---, control.

whether THC might be an intermediate in the breakdown of cholesterol to bile acids by rat liver *in vitro*. Figure 1 summarizes the results obtained in five different experiments. In all cases studied, four radioactive peaks were eluted prior to the large cholesterol peak. Cholesterol, 50,000 cpm, was added to each flask. The average of total counts recovered from peaks 1 to 4 was 4420 (8.8%) and 1220 (2.4%) for the enzyme and control flasks, respectively. Duplicate determinations indicated an average error of ±3.5% in the counts recovered under each peak. The elution volumes (Danielsson, 1958) of the four peaks suggested that they might contain the following compounds: peak 1, THC; peak 2, 7α-hydroxycholesterol; peak 3, 3α,7α-dihydroxycoprostane; and peak 4, 3α,12α-dihydroxycoprostane. Since it has been well documented (Wintersteiner and Bergström, 1941; Danielsson, 1960) that cholesterol readily undergoes nonenzymatic autoxidation to 7α-hydroxycholesterol, a further indication that peak 2 might contain this compound is evidenced by the fact that it was only in this position that significant radioactivity was observed in the control. Furthermore, the difference in radioactivity under peak 2 between test and control indicates that, whatever the exact nature of this compound, it was produced enzymatically from cholesterol. Studies are at present in progress to elucidate this problem and also to determine the nature of the other radioactive components. The isolation of labeled THC is described below.

Coenzyme Requirements.—Table I indicates the relative stimulating effect of various coenzymes upon the incorporation of cholesterol-26-C^{14} into its breakdown products. Whereas a slight enhancement of activity was observed in the presence of NADP or NADPH, respectively, NAD alone had the greatest stimulatory effect. The addition of NADP or NADPH together with NAD caused an inhibition of enzyme activity when compared with NAD alone.

Buffer and Hydrogen Ion Concentration Requirements.—Increasing the *p*H of the buffer from 7.4 to 8.4 markedly inhibited the enzyme system. The use of either Krebs bicarbonate buffer or Tris buffer (both at *p*H 7.4) also led to a significant decrease in the incorporation of labeled cholesterol into its degradation products (Table II).

TABLE I

EFFECT OF VARIOUS COENZYMES ON INCORPORATION OF CHOLESTEROL-26-C^{14} INTO DEGRADATION PRODUCTS

Conditions of incubation as described in the text. NAD added only as indicated in the Table. Cholesterol-26-C^{14}, 50,000 cpm per flask.

Coenzyme	Concentration (μmoles per flask)	Increased Incorporation of Labeled Cholesterol into Degradation Products (%)
NADP	7.0	9
NAD	7.0	16.5
NAD + NADP	3.5, 3.5	12.2
NAD + NADPH	3.5, 3.5	12.9
NADPH	6.5	8.4

TABLE II

EFFECT OF BUFFER, *p*H, AND EDTA ON INCORPORATION OF CHOLESTEROL-26-C^{14} INTO DEGRADATION PRODUCTS

Conditions of incubation as described in the text. Cholesterol-26-C^{14}, 50,000 cpm per flask. Mean values are given.

No. of Expts.	Buffer	*p*H	EDTA	Incorporation of Cholesterol into Degradation Products[a]
5	Phosphate	7.4	+	4420
4	Phosphate	7.4	−	3172
2	Bicarbonate	7.4	+	2903
2	Tris	7.4	+	2714
2	Tris	8.3	+	2087
2	Phosphate	8.3	+	2842

[a] Counts recovered under peaks 1–4; Fig. 1.

The addition of EDTA to the system was found to be necessary for optimum enzyme activity (Table II).

Isolation of Labeled THC.—At the conclusion of the incubation period the contents of 10–12 flasks were combined. Then 25 mg of carrier THC was added, followed by 4 volumes of 95% ethanol. The contents were prepared for column chromatography as described above. Initially the labeled compounds obtained after incubation were separated from unreacted cholesterol with phase system I (Danielsson, 1958) (55% [v/v] aqueous ispropanol, moving phase; 20% [v/v] chloroform in heptane, stationary phase; 4 ml stationary phase supported on 4.5 g hydrophobic supercel). The labeled compounds were then rechromatographed with phase system III (50% [v/v] aqueous isopropanol, moving phase; 20% [v/v] chloroform in heptane, stationary phase; 4 ml stationary phase supported on 4.5 g hydrophobic supercel) in order to obtain a more efficient separation of THC from the other radioactive compounds. The effluent fractions, which were thought to correspond to those containing THC (by reference to elution volumes previously determined with authentic THC under the same chromatographic conditions), were combined and evaporated to dryness under nitrogen. The material so obtained was recrystallized three times from aqueous methanol to constant specific activity. The results of a typical experiment are presented in Table III. The isolated material melted at 183–185°, and no depression of the melting

TABLE III
MELTING POINTS AND SPECIFIC ACTIVITIES OF THC ISOLATED FROM THE INCUBATION MIXTURES
(See text for details)

Re-crystal-lization	Weight (mg)	Melting Point	Specific Activity (cpm/mg)
1	15.6	181–184°	60
2	10.7	183–185°	68
3	7.4	183–185°	60

point was observed when this material was mixed with an authentic sample of THC.

In order to provide further evidence for the identity of the material isolated from the column, its behavior in three different chromatographic systems (Sjövall, 1952; Neher and Wettstein, 1956; Kritchevsky *et al.*, in preparation) was compared with that of authentic THC. After identification of the spots by exposing them to iodine vapor for a few minutes, they were eluted from the papers (or the silicic acid when thin-layer chromatography was employed) with chloroform, plated, and dried, and their radioactivity was assayed. In all cases the material behaved exactly like pure THC and, furthermore, radioactivity was detected only in those areas occupied by the material and nowhere else on the chromatogram.

DISCUSSION

Previous investigations by Bergström *et al.* (1954) and Bergström (1955), using *in vivo* techniques, and the *in vitro* studies of Whitehouse *et al.* (1961) suggested that THC might be a metabolic degradation product of cholesterol in mammalian liver. The findings of the present study have established that an enzyme system is present in rat liver which is capable of converting cholesterol to THC. This fact lends considerable support to the idea that THC is probably a normal and obligatory intermediate in the conversion of cholesterol to cholic acid. The exact nature of the metabolic transformations from cholesterol to THC is at present not known. That hydroxylation of the cholesterol nucleus precedes the inversion of the 3β-hydroxyl group and/or the saturation of the Δ^5-double bond is indicated from the work of Bergström (1955) and Harold *et al.* (1955, 1957). It is of interest that the enzyme system reported here was capable of degrading cholesterol into a number of products apparently different from THC. The elution volumes of these metabolites were such as to indicate the following compounds: 7α-hydroxycholesterol, 3α,7α-dihydroxycoprostane, and 3α,12α-dihydroxycoprostane. It seems possible, therefore, that cholesterol is initially hydroxylated in the 7α position. This is followed by inversion of the hydroxyl group at C_3 and saturation of the nucleus to give 3α,7α-dihydroxycoprostane, which is then hydroxylated at C_{12} to form THC. However, since Danielsson (1962) has presented evidence that 12α hydroxylation of cholesterol might be an early step in the formation of cholic acid in the rat, the occurrence of this reaction prior to the introduction of the 7α-hydroxyl group has also to be considered. The mechanism of the inversion of the 3β-hydroxyl group and saturation of the Δ^5-double bond remains to be determined.

A further point arising out of these studies is the fact that the enzyme system seemed to require only NAD for optimal activity. Both NADP and NADPH had very little effect in increasing the incorporation of labeled cholesterol into its degradation metabolites, and in fact when these coenzymes were separately combined with NAD a slight inhibition of enzyme activity was observed. It has been reported that NADPH is required for the enzymic introduction of hydroxyl groups into steroids by tissue homogenates, mitochondria, and microsomes (Grant, 1955; Gloor, 1954). Since the reactions from cholesterol to THC must involve several hydroxylations of the steroid nucleus it is not immediately apparent why NADP has no stimulatory effect on the system reported here. The possibility does exist that pyridine nucleotide transhydrogenases are active in the crude enzyme system employed in this study.

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