METABOLISM OF EPICHOLESTEROL-4-C$^{14}$ IN THE RAT*

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(Received for publication, November 12, 1954)

Recent studies on the catabolism of cholesterol-4-C$^{14}$ in the rat have shown that the isotopic carbon is eliminated from the body almost entirely with the feces and that up to 90 per cent of the fecal C$^{14}$ enters the intestine via the bile. Not more than 10 per cent of the C$^{14}$ is excreted directly through the intestinal wall. Approximately 90 per cent of the C$^{14}$ recovered in bile is in the form of bile acids, whereas the remainder consists of non-saponifiable steroids. Three of the bile acids have now been identified as taurocholic, taurochenodeoxycholic, and (tauro?) lithocholic acids (1-5).

The present report deals with the fate of C$^{14}$-labeled epicholesterol in the rat. This compound is of interest because it differs from cholesterol only in the steric configuration of the OH group on carbon 3. In cholesterol this group has a β configuration, but in epicholesterol it is in the α position, which is also characteristic of most of the naturally occurring bile acids. The possibility was, therefore, considered that epicholesterol is an intermediate in the conversion of cholesterol to bile acids.

EXPERIMENTAL

Epicholesterol-4-C$^{14}$—The preparation of this labeled steroid has been described (6). Since radioactive steroids deteriorate on standing (7), the epicholesterol-4-C$^{14}$ was purified immediately before use. β-Steroids were removed by precipitation with digitonin, and the supernatant solution was evaporated. The residue was dissolved in pyridine, and the free digitonin was precipitated with ethyl ether and removed by centrifugation. The supernatant solution was evaporated, and the epicholesterol residue was purified by chromatographing it twice on an alumina column (Merck). The epicholesterol was applied to the column in hexane and eluted with 15 per cent ethyl ether in hexane. The melting point of the eluted material was 140°.

Unlabeled epicholesterol was synthesized (8), and its purity was established by melting point and infra-red spectroscopy.

* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.
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Taurochenodeoxycholic acid was isolated from goose bile (9). After several reprecipitations from a mixture of methanol and ethyl ether, the acid was chromatographically pure and was identical with synthetic taurochenodeoxycholic acid. Hydrolysis with alkali gave chenodeoxycholic acid, m.p. 137-139°C, chromatographically identical with authentic chenodeoxycholic acid.

Treatment of Animals—Male rats of the Long-Evans strain, weighing 200 to 300 gm., were lightly anesthetized with ether. Cannulas were inserted into the common bile ducts (1). The rats received by injection into the tail vein 1 mg. of epicholesterol (specific activity, $3.4 \times 10^6$ c.p.m. per mg.) which was prepared for injection as follows: It was dissolved in ethyl ether, and to this solution were added 1 drop of Tween 20 and 0.5 ml. of 0.9 per cent NaCl solution. The ether was removed by evaporation on a steam bath; a clear emulsion remained. The animals were placed in restraining cages and allowed free access to food and water. Bile was collected continuously for 48 hours, the collection intervals ranging from 2 to 10 hours. Feces excreted during the entire period were combined for analysis.

Fractionation Procedures—Aliquots of bile (generally 50 µl.) were mounted directly on aluminum disks, and $^{14}C$ was determined in a gas flow counter to ±5 per cent. Mass correction was negligible.

The fractionation of the bile is outlined in Diagram 1. 1 ml. was added to 3 ml. of 4 N NaOH, and the mixture was autoclaved for 4 hours at 120°C. After cooling, the mixture was acidified with H$_2$SO$_4$ and extracted with several portions of petroleum ether. Since radioactivity in the fatty acids has previously been shown to be negligible (2), the radioactivity found in this petroleum ether extract was attributed to neutral steroids. Further fractionation into α- and β-steroids was accomplished by precipitation with digitonin.

The aqueous phase (referred to as PE-insoluble in Diagram 1) was extracted three times with ethyl ether to remove bile acids. Aliquots of the ether extract were mounted, and their $^{14}C$ content was determined.

Feces were extracted with ethyl alcohol until all radioactivity was removed. Suitable aliquots were mounted, and the $^{14}C$ content was determined. The alcohol extract was saponified and extracted as described above. The neutral steroids were examined in the manner described for bile.

Liver was hydrolyzed with dilute sodium hydroxide, and the neutral steroids were isolated by extraction with petroleum ether. The petroleum ether extract was washed several times with aqueous ethanol and evaporated to dryness. The subsequent treatment of the residue is described below.
Filter Paper Chromatography of Bile—Whole bile samples were chromatographed on Whatman No. 1 filter paper strips as described in detail elsewhere (3). The solvents used were (a) collidine-water, (b) butanol saturated with water in an ammonia atmosphere, and (c) n-hexanol-butylamine-water, 100:45:16. The compounds on the paper were located both by spraying with antimony trichloride and by preparing radioautographs from the chromatograms.

Results

Recovery of C\textsuperscript{14} in Liver, Bile, and Feces

By the end of 48 hours, approximately 50 per cent of the injected C\textsuperscript{14} was recovered in the bile (Fig. 1). In the same period no more than 0.6 per cent was recovered in the feces. Most of the remaining C\textsuperscript{14} was found in the liver.

Liver—The neutral steroids of the liver were isolated as described above.
About 90 per cent of the C\textsuperscript{14} in this fraction is precipitable with digitonin. Since cholesterol is the major neutral steroid in liver, it was of interest to determine whether C\textsuperscript{14} had been incorporated into cholesterol. The digitonides were therefore dissolved in pyridine, the digitonin was removed by precipitation with ethyl ether, and the supernatant solution was evaporated. The cholesterol was precipitated as the dibromide, as described by Schwenk \textit{et al.} (10). Several recrystallizations of the dibromide (from ethanol, methanol, and aqueous acetone) yielded a product virtually free of C\textsuperscript{14}, thereby indicating that epicholesterol is not converted to cholesterol.

C\textsuperscript{14}-Epicholesterol is, however, converted to a neutral sterol that is precipitable with digitonin and that does not form an insoluble dibromide. This observation suggested that the sterol might be dihydrocholesterol. The following experiments demonstrate that epicholesterol is, indeed, converted to dihydrocholesterol in the liver. The supernatant solution from the bromination was diluted with ethyl ether and washed thoroughly, first with a dilute solution of sodium bisulfite, to remove excess bromine.

![Cumulative excretion of C\textsuperscript{14} in bile as a percentage of the administered Epicholesterol-4-C\textsuperscript{14}](http://www.jbc.org/)

\textbf{Fig. 1}
and then with water. The ether phase was evaporated, excess carrier dihydrocholesterol was added to the residue, and the mixture was recrystallized successively from ethanol, aqueous acetone, and methanol. No difference in the specific activities of the initial and final products was observed. Two derivatives were then prepared. Part of the dihydrocholesterol was oxidized to cholestanone, m.p. 131° (11), and the remainder was acetylated with pyridine-acetic anhydride (m.p. of acetate, 110°). The corrected specific activities of the two derivatives were identical with those of the initial product. It would therefore appear that dihydrocholesterol is the major neutral sterol formed from epicholesterol in the liver and accounts for at least 90 per cent of the digitonin-precipitable C\(^14\).

**Bile and Feces**—The distribution of C\(^14\) among the various fractions prepared from bile and feces is shown in Diagram 1. For convenience of presentation, the injected dose has been assigned an arbitrary value of 10\(^6\) c.p.m. Not more than 10 per cent of the total C\(^14\) found in saponified bile was recovered in the petroleum ether-soluble fraction (referred to as the neutral steroid fraction). The C\(^14\) in this fraction was about equally distributed between digitonin-precipitable and non-precipitable steroids. Carrier cholesterol was added to the digitonin-precipitable fraction, and the \(\beta\)-sterols were analyzed as described above for liver. Again, no C\(^14\) was found in cholesterol, and the major C\(^14\)-\(\beta\)-sterol was identified as dihydrocholesterol.

The remaining 90 per cent of the C\(^14\) present in the hydrolyzed bile was extractable with ether, and practically all of the ether-extractable material was precipitated with FeCl\(_3\) by the method of Doubilet (12). Thus it would appear that bile acids constitute the major excretion product of epicholesterol.

The C\(^14\) found in saponified extract of feces was completely extracted with petroleum ether. About two-thirds of the C\(^14\) was precipitated with digitonin, but again no C\(^14\) was found in cholesterol.

**Bile Acid End-Products of Epicholesterol Metabolism**

To identify the acidic biliary products of epicholesterol metabolism the bile was chromatographed as described above. The positions of the color bands brought out by antimony trichloride were compared with those of the radioactive bands as revealed by radioautography.

**Color Bands**—Fig. 2, A is a diagram of a typical chromatogram of rat bile developed in the collidine-water system. The identification of some of the various color bands was dealt with in a previous communication (3). In descending order, the bands are cholesterol and other neutral steroids \((R_P 0.98\) to 1\)), taurochenodeoxycholic acid together with small amounts of (tauro?) lithocholic acid \((R_P 0.82\)\), and taurocholic acid \((R_P 0.73\)\).
Radioactive Bands—Radioautographs of bile samples obtained at different times after the injection of epicholesterol-4-C\textsuperscript{14} are presented in Fig. 2, B to D. In the bile sample obtained during the first 2 hours, four C\textsuperscript{14} bands are visible (Fig. 2, B). To identify these bands the following experiments were carried out.

<table>
<thead>
<tr>
<th>COMPOUND ON FILTER PAPER CHROMATOGRAM</th>
<th>Typical Rat Bile Chromatogram Sprayed with SbCl\textsubscript{3}</th>
<th>Radioautographs of Rat Bile Samples Obtained After Injection of Epicholesterol-4-C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, etc.</td>
<td>Front</td>
<td>0-2 2-6 23-48</td>
</tr>
<tr>
<td>Taurochenodesoxycholic Acid</td>
<td>0.82</td>
<td>--- --- ---</td>
</tr>
<tr>
<td>Taurocholic Acid</td>
<td>0.73</td>
<td>--- --- ---</td>
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<tr>
<td>Compound y\textsuperscript{†}</td>
<td>0.59</td>
<td>--- --- ---</td>
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<tr>
<td></td>
<td>0.39</td>
<td>--- --- ---</td>
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<tr>
<td>Origin</td>
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Fig. 2. Chromatogram and radioautographs of rat bile after injection of epicholesterol-4-C\textsuperscript{14}. (*), as pointed out in the text, the position of this compound overlaps, but does not coincide with, that of taurocholic acid. For purposes of presentation this displacement has been exaggerated. (†), Compound Y was observed in our earlier experiments with cholesterol-4-C\textsuperscript{14} (3). A similarly placed band has been observed in the present experiments with epicholesterol-4-C\textsuperscript{14}, but it should not be inferred that these two bands are necessarily identical.

First C\textsuperscript{14} Band—The band at the front corresponds to the position of neutral steroids, such as cholesterol and dihydrocholesterol.

Second C\textsuperscript{14} Band ($R_f$ 0.82)—Superimposition of the films upon the filter paper chromatograms indicated, for all three solvents used, that this C\textsuperscript{14} band corresponded exactly to the color band of taurochenodeoxycholic acid. This suggested that taurochenodeoxycholic acid is a product of epicholesterol metabolism, a suggestion which proved incorrect on further study. A sample of bile collected during the first 12 hours after injection of the epicholesterol was hydrolyzed with 2 N NaOH and acidified, and the
bile acids were fractionated by the method of Bergström and Sjövall (13). Two large radioactive peaks were obtained, the first corresponding to the band at \( R_F 0.72 \) and the second to the band at \( R_F 0.82 \). Excess chenodeoxycholic acid was added to a sample of the second peak, and the mixture was then recrystallized six times from the following solvents: (1) ethyl acetate and petroleum ether, (2) chloroform and petroleum ether, and (3) benzene and methanol. The specific activity of the product obtained after six recrystallizations did not differ from that of the starting material. The final product was then acetylated with pyridine-acetic anhydride on a steam bath for 3 hours, and the acetate was recrystallized from the following solvents: aqueous ethanol, aqueous methanol, and aqueous acetone. After six recrystallizations, the product was virtually free of radioactivity. It must therefore be concluded that the band at \( R_F 0.82 \) does not represent chenodeoxycholic acid. By a similar procedure, it was established that the \( ^{14}C \) compound under consideration is not deoxycholic acid. The chromatographic behavior of this substance suggests, however, that it is the taurine conjugate of an unidentified bile acid possessing two hydroxyl groups (3).

Third \( ^{14}C \) Band (\( R_F 0.72 \))—Superimposition of the films upon the filter paper chromatograms revealed that the \( ^{14}C \) band overlapped the color band, but they did not coincide. Indeed, good separation of the two bands was achieved by using the butanol-water and hexanol-butylamine systems. Further evidence that the \( ^{14}C \) substance at \( R_F 0.72 \) is not identical with taurocholic acid was obtained by crystallization experiments. A sample of bile collected during the first 12 hours after injection of \( ^{14}C \)-epicholesterol was hydrolyzed, and the free bile acids were fractionated by column chromatography as described in the preceding section. A sample of the first peak eluted from the column by the technique of Bergström and Sjövall was mixed with carrier cholic acid, and the mixture was recrystallized from ethyl acetate. Several recrystallizations sufficed to remove all but a small fraction of the initial radioactivity. Thus, while some of the radioactivity in this band may be due to taurocholic acid, the major \( ^{14}C \) component is neither taurocholic nor any other conjugate of cholic acid. The position of this band on the paper suggests that the compound responsible is the taurine conjugate of a compound very similar to cholic acid, perhaps an isomer (3).

Fourth \( ^{14}C \) Band—The \( ^{14}C \) band at \( R_F 0.59 \) has not been identified. It should be recalled that the chromatography of bile from rats injected with cholesterol-4-\( ^{14}C \) (3) also revealed a \( ^{14}C \) band in this position (Compound Y), but we have not been able to decide whether the radioactive compound at \( R_F 0.59 \) is the same in both cases.

As the excretory process is followed for longer periods, radioactivity fades from the band at \( R_F 0.82 \), while the \( ^{14}C \) band at \( R_F 0.72 \) becomes more
prominent (Fig. 2, C, 2 to 6 hours). Eventually this band becomes the predominant radioactive excretion product of epicholesterol-4-C\textsuperscript{14}, accompanied by a faint band at $R_F$ 0.82 (Fig. 2, D, 23 to 48 hours).

**DISCUSSION**

It is shown here that the metabolic fate of epicholesterol resembles that of cholesterol in that the major biliary excretion products are bile acids. A study of the radioactive bile acids found in bile after the intravenous injection of cholesterol-4-C\textsuperscript{14} and epicholesterol-4-C\textsuperscript{14} reveals, however, that the biliary end-products of these two isomeric steroids are not identical. Thus, in the case of the former, taurochenodeoxycholic acid is the major C\textsuperscript{14} bile acid excreted in bile during the early intervals and taurocholic, the major acid during the later intervals. In the case of epicholesterol, two unidentified bile acids are encountered, possessing $R_F$ values close to those of taurochenodeoxycholic acid ($R_F$ 0.82) and taurocholic acid ($R_F$ 0.73), respectively, but not identical with them. These unidentified bile acids appear together during the early intervals, whereas in the later intervals the unidentified acid at $R_F$ 0.72 becomes the major biliary product. On the basis of their chromatographic behavior, it is suggested that these unidentified acids are conjugated with taurine and possess structures resembling those of taurochenodeoxycholic and taurocholic acids, respectively.

The metabolism of cholesterol and epicholesterol also differs in other respects. Thus, it has been observed that the C\textsuperscript{14} of intracardially injected epicholesterol-4-C\textsuperscript{14} is excreted in bile about twice as rapidly as is the C\textsuperscript{14} of cholesterol-4-C\textsuperscript{14}. This difference was not observed after intravenous injection and is not readily explained at present. It may also be recalled that the rate of absorption of epicholesterol in the rat is much lower than that of cholesterol (14).

The structural relations of cholesterol, epicholesterol, and the bile acids suggested the possibility that epicholesterol might be an intermediate in the degradation of cholesterol to cholic acid. The observation that epicholesterol is not converted to either taurocholic or taurochenodeoxycholic acid renders this unlikely. The fact that similar compounds are formed, however, suggests a high degree of stereochemical specificity in the metabolic degradation of steroids.

Under our experimental conditions, epicholesterol was not converted to cholesterol by the rat. Instead, dihydrocholesterol appears to be the major neutral sterol recovered from cholesterol both in liver and in bile. In view of the well established conversion of $\Delta^4$-cholestenone to dihydrocholesterol (15, 16), it is conceivable that $\Delta^4$-cholestenone is an intermediate in the conversion of epicholesterol to dihydrocholesterol.
We are indebted to Dr. W. G. Dauben and Dr. J. F. Eastham for the preparation of epicholesterol-4-C¹⁴, to Dr. S. Abraham for the preparation of unlabeled epicholesterol, and to Dr. M. D. Siperstein and Mr. H. H. Hernandez for many helpful discussions.

SUMMARY

1. The elimination of the C¹⁴ of intravenously injected epicholesterol-4-C¹⁴ from the bodies of rats with cannulated bile ducts has been studied. In the course of 48 hours, approximately 50 per cent of the injected C¹⁴ was excreted via the bile. In that period, only 0.6 per cent was excreted via the feces. 90 per cent of the biliary C¹⁴ was in the form of bile acids and the rest in non-saponifiable steroids.

2. The C¹⁴ bile acids formed resembled taurochenodeoxycholic acid and taurocholic acid, but were not identical with them. It is concluded that epicholesterol is not an intermediate in the conversion of cholesterol to bile acids.

3. Dihydrocholesterol has been shown to be a product of epicholesterol metabolism in the rat.

4. The conversion of epicholesterol to cholesterol could not be demonstrated.

BIBLIOGRAPHY

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