Stero-Bile Acids and Bile Sterols

LIV.* Studies on the Bile of Carp

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In a previous paper (XLVI of this series), the isolation of cyprinol and other three new bile sterols from the alkaline hydrolyzate of carp bile was reported. Also it was suggested that the chemical constitution of one of these new bile sterols would correspond to 3α,7α, 12α-trihydroxy-26,27-epoxycholestane and the natural form of this new bile sterol might be 3α,7α,12α,26,27-pentahydroxycholestanate sulfate (1).

The present paper is a report of our study of bile salts isolated from the acidic hydrolyzate of bile with an aim to clarify the natural form of bile sterols of carp.

**MATERIALS AND METHODS**

Carp bile (300 ml. from 500 carp) was extracted with 95% ethanol and the ethanol extract was evaporated to dryness under a reduced pressure. For the preliminary examination of bile salts, thin layer chromatography was performed. Glass plate (20×5 cm.) was coated with silica gel containing 5% calcium sulfate in a layer of 0.25 mm. thick. The solvent system was n-butanol: acetic acid: water (85:10:5 v/v). The spots were detected by spraying concentrated sulfuric acid followed by heating. Thin layer chromatogram of the extract of carp bile gave a faint spot which moved at the same rate as tauro-cholate and a faster-running strong spot due to cyprinol sulfate as shown in Fig. 1.

The bile salt obtained from carp bile was partially acetylated with acetic acid and acetic anhydride, then hydrolyzed with trichloroacetic acid in dioxane solution according to the method of Bridgwater et al. (2). The hydrolyzate was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was washed with water, 2% sodium carbonate solution, water and evaporated, leaving a brown gum, containing partially acetylated bile acids or conjugated bile acid mixture.

The acetylated bile sterol mixture was hydrolyzed with N sodium hydroxide solution. The hydrolyzate was poured into a large amount of water to separated bile sterol mixture and then extracted with n-butanol: ethyl acetate (1:1). Evaporation of the extract left crude bile sterol mixture as a gelatinous material (5.2 g.).

The bile acid mixture was hydrolyzed with 2.5 N sodium hydroxide by heating in a sealed metal container for 8 hours at 160°C. The hydrolyzate was diluted with water and acidified with dilute hydrochloric acid to give precipitate. The precipitate was digested with ethyl acetate and the extract was washed

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* LIII, Yashima, H., J. Biochem., 54, 47 (1963)
with water, dried with sodium sulfite. Evaporation of the extract left crude bile acid mixture as a brown gum (150 mg.).

The bile acid and sterol mixtures were subjected to reversed phase partition chromatography according to the same method as previously described (1).

RESULTS

I. Bile Acids of Carp Bile—One hundred and fifty milligrams of the bile acid mixture was chromatographed with solvent system C, and a single band appeared at the position of cholic acids as shown in Fig. 2.

![Fig. 2. Chromatography of bile acid mixture. Column, 13.5 g. of Hostalen; phase system C.](image)

The effluents from 90 ml. to 180 ml. were combined and concentrated by evaporation on a water bath. The remaining aqueous solution was acidified with dilute hydrochloric acid and extracted with ethyl acetate:ether (1:1). The extract was made free from hydrochloric acid by repeated washing with water and then evaporated to dryness. The residue (20 mg.) was recrystallized from ethyl acetate and then from methanol to give crystals melting at 196°C. The melting point of this crystals was not depressed when mixed with authentic cholic acid. Its infrared spectrum and the behaviour on paper chromatogram completely coincided with those of cholic acid.

The material retained in the stationary phase of the column was rechromatographed using a suitable solvent system for the separation of more hydrophobic substances and by this procedure a band was obtained whose position corresponded to that of chenodeoxycholic acid. The effluents of this band were combined and then the solvent was evaporated to dryness. Although the residue was not crystallized from any solvent so far employed, it showed only one spot at the same position of chenodeoxycholic acid when subjected to paper chromatography.

II. Bile Sterols of Carp Bile—Five grams of the bile sterol mixture was chromatographed by use of solvent system C. As shown in Fig. 3, a main peak appeared at 4000 ml.

![Fig. 3. Chromatography of bile sterol mixture. Column, 450 g. of Hostalen; phase system C.](image)

The effluents from 3000 ml. to 5000 ml. were combined and evaporated to dryness. The residue (3.6 g.) was crystallized from acetone, then from methanol-ethyl acetate and...
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finally from methanol to give rods melting at 242°C. This substance was identical with cyprinol previously isolated by Haselowood (3). Paper chromatogram of this sterol showed the same Rf value with that of 3α, 7α, 12α, 26, 27-pentahydroxycholestanone (4). The infrared and nuclear magnetic resonance spectra of cyprinol are shown in Fig. 4 and 5C.

![Infrared spectrum of cyprinol, Nujol.](image)

Cyprinol gave a positive Hammarsten test and did not reacted with lead tetraacetate in benzene.

Analysis Calcd. for C_{27}H_{48}O_{5}  C 71.64 H 10.69
Found  C 71.77 H 10.61

The material that stayed in the stationary phase of the column chromatography was eluted with ethanol and evaporation of the ethanol eluate left a small amount of yellow gum (200 mg.). Thin layer chromatogram with solvent system of ethyl acetate:acetone (70:30) of this material showed several spots at the position of tetrahydroxy derivatives as shown in Fig. 6. Rf value of none of these spots agreed with those of the sterols previously isolated by the alkaline treatment from carp bile.

**DISCUSSION**

Infrared spectrum of cyprinol showed the peaks at 885, 953, 1005, 1031 and 1075 cm⁻¹ which appear to be quite characteristic to allocholic acid nucleus. The existence of allocholane nucleus in cyprinol molecule was showed by the early observation that chromic anhydride oxidation of cyprinol afforded dehydroallocholic acid (5). Although the structure of the terminal part of the side chain has not yet been disclosed, the chemical structure (I) or (II) as shown in Table I has been proposed for cyprinol on the basis of elementary analysis and on the fact that this sterol remained unchanged after lead tetraacetate oxidation.

As shown in the present paper, the hydrolysis of carp bile in dioxan-trichloroacetic acid solution gave cyprinol as a chief component but did not yield the other three bile steroids which could be isolated from the alkaline hydrolysate of the same bile (7). It was already reported that the natural scymnol sulfate is converted to anhydroscymnol on alkaline hydrolysis which possesses four-membered oxide ring in the molecule (6). From these findings, it is highly probable that the new bile sterol previously characterized as 3α, 7α, 12α-trihydroxy-26, 27-epoxycholestanone is "anhydrocyprinol" an artifact of alkaline treatment of the natural cyprinol sulfate. Thus, cyprinol may be shown to be the structure (I). Also this assumption is strongly supported by the following nuclear magnetic resonance spectroscopy*. The nuclear magnetic resonance spectra of tetrahydroxycholestanone and deoxycymnol (Fig. 5A and B) show several
FIG. 5. Nuclear magnetic resonance spectra of tetrahydroxycholane (A), deoxyscymnol (B) and cyprinol (C).

peaks attributable to methyl groups in high-field part. The signals of angular methyl groups (C18 and C19) appear as the intense peak at 9.20Å and 9.02Å respectively. The doublet at 8.80Å in the spectrum of tetrahydroxycholane (Fig. 5A) may be due to C21 methyl group. On the other hand, the doublet peaks at 8.79Å in Fig. 5B come from the almost equally shielded two similar methyl groups.

* All the spectra were taken with a Varian A-60 spectrometer on about 10% solution in pyridine containing about 1% tetramethylsilane as an internal reference. Chemical shifts are expressed in τ-units, and their accuracy limits are about ±0.02.

FIG. 6. Thin layer chromatogram of bile sterols obtained from carp bile.

I: Bile sterols isolated from the alkaline hydrolyzate.
   A: cyprinol. B: the sterol with m.p. 227°C.
   C: anhydrocyprinol. D: the sterol with m.p. 241-2°C.

II: More hydrophobic bile sterol mixture obtained from the acidic hydrolyzate.

(C21 and C26) on deoxyscymnol side chain. The spectrum of cyprinol (Fig. 5C) shows a doublet-like signal 8.80Å due to the methyl group in the side chain in addition to the singlets at 9.20Å and 9.10Å assigned to angular methyl groups (C18 and C19). Approximate area of this doublet corresponds to one methyl group. Also it is to be noted that the area under signals observed in the region around 5.6Å-6.1Å in these spectra are due to the number of protons on hydroxyl-bearing carbon atoms. The relative intensities of the signals in this region of tetrahydroxycholane correspond to five protons and those of deoxyscymnol correspond to six protons. In the spectrum of cyprinol, the number of protons in this region was seven. Therefore, it is apparent that the both hydroxyl groups in the side chain should be primary.
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TABLE I

<table>
<thead>
<tr>
<th>Number of methyl groups in the side chain</th>
<th>Number of protons on hydroxyl-bearing carbon atoms</th>
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<tbody>
<tr>
<td><img src="image1" alt="Diagram of I" /> Cyprinol</td>
<td>1 (C&lt;sub&gt;21&lt;/sub&gt;)</td>
</tr>
<tr>
<td><img src="image2" alt="Diagram of II" /> Tetrahydroxycholone</td>
<td>2 (C&lt;sub&gt;21&lt;/sub&gt; and C&lt;sub&gt;25&lt;/sub&gt;)</td>
</tr>
<tr>
<td><img src="image3" alt="Diagram of IV" /> Deoxysecynol</td>
<td>2 (C&lt;sub&gt;21&lt;/sub&gt; and C&lt;sub&gt;26&lt;/sub&gt;)</td>
</tr>
</tbody>
</table>

The interpretation of the nuclear magnetic resonance spectrum of cyprinol was completely consistent with structure (I), 3α,7α,12α,26,27-pentahydroxycholestane. The same conclusion on the chemical constitution of cyprinol has been obtained by Haslewood*.

Further study of carp bile sterols by means of thin layer chromatography has demonstrated the presence in the bile of several other bile sterols as minor components. They are now under further investigation.

In 1928, Hatakeyama and Okumura reported the isolation of cholic acid from carp bile (7). The presence of this acid is confirmed by the present work. From thin layer chromatographic data, cholic acid may be conjugated with taurine in the natural bile. It is very interesting that "modern" bile acid (cholic acid) is isolated from carp bile, the chief constitution of which is, of course, "most primitive" bile sterol (cyprinol).

SUMMARY

1. Bile salts of carp, Cyprinus carpio, when hydrolyzed with trichloroacetic acid in dioxane solution yielded cyprinol as a chief component, which is believed to be 3α,7α,12α,26,27-pentahydroxycholestane on the basis of the nuclear magnetic resonance spectroscopic data and on the fact that 3α,7α,12α-trihydroxy-26,27-epoxycholestane was derived from cyprinol sulfate.

2. The presence of cholic acid in carp bile was confirmed.

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