MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

V. ON A PRINCIPAL INTERMEDIATE IN THE BREAKDOWN OF CHOLIC ACID BY STREPTOMYCES

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A microbiological transformation of cholic acid into a bile acid containing α, β-unsaturated ketone group was reported independently by Hayakawa (1-3) and Halperin et al. (4). Halperin et al. have demonstrated that a degradation product of cholic acid by Nocardia sp. was probably 3-keto-Δ⁴-cholanic acid derivative and this reaction was catalyzed by DPN-linked dehydrogenase. Hayakawa et al. (1-3, 5), on the other hand, have separated a new unsaturated C-22 acid of m.p. 280-282° (decomp.) as an oxidation product of cholic acid by S. gelaticus 1164, and this C-22 acid has been defined to be 7α-hydroxy-3,12-diketo-Δ⁴-bisnorcholenic acid. Though the exact position of one double bond remained to be determined, this observation was the first to demonstrate a β-oxidation of the side chain of cholic acid by microorganisms. In a previous paper (6) of this series, seven species of Streptomyces which utilize cholic acid as the sole source of carbon have been selected and identified. It then became of interest to expand our studies to the metabolism of various bile acids by these species. In the present communication, the following studies were performed:

1) The best condition of the culture media for the formation of the C-22 acid by S. gelaticus 1164; 2) The spectrophotometric analyses of the degradation products of cholic acid by the seven species of Streptomyces; 3) Effect of several bile acids other than cholic acid on the growth of the seven species of Streptomyces.

EXPERIMENTAL AND RESULTS

Effect of Cholate Concentration and Incubation Time on the Formation of the C-22 Acid by S. gelaticus 1164—Into the basal mineral medium ( (NH₄)₂-
SO\textsubscript{4} 2.0 g., K\textsubscript{2}HPO\textsubscript{4} 1.0 g., MgSO\textsubscript{4}-7H\textsubscript{2}O 0.5 g., FeCl\textsubscript{3}-6H\textsubscript{2}O 0.01 g., distilled water 1000 ml.) sodium cholate was added in final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.8 g. per dl., and their pH were adjusted to 7.2. Hundred milliliters, each of these media placed in 500 ml. Sakaguchi's flask was autoclaved at 110\degree for 20 minutes. S. gelaticus 1164 from Czapeck's slant culture (three days' incubation at 27\degree) was inoculated with a platinic loop in these media. All the six flasks were shaken for 20 days at 27\degree on a reciprocal shaker (120 r.p.m., 5 cm. amplitude). After 3, 4, 5, ... and 22 days' incubation, an aliquot of the culture was centrifuged at 3,000 r.p.m. for one minute. The optical density at 246 m\textmu of the supernatant fluid was measured by Beckman spectrophotometer, type DU II. The C-22 acid showed the absorption maximum at 240.3 m\textmu in alcohol and at 246 m\textmu in water within the pH range of 5 to 9, and the optical density of this compound, within the range of concentrations likely to be encountered, obeyed the Beer's law. Effect of the cholate concentration and incubation time on the formation of the C-22 acid is given in Table I. In order to prove the degradation of cholic acid and the production of the C-22 acid, Pettenkofer's test and acid precipitation test of the culture medium were performed in parallel with the measurement of ultraviolet absorption at 246 m\textmu throughout the incubation period. The data are shown in Fig. 1.

As seen in Table I and Fig. 1, 1) a rapid increase in optical density at 246 m\textmu was observed, when the cholate concentration was 0.2 g. per dl. or less, while at 0.3, 0.4 and 0.8 g. per dl. the optical density increased more slowly; 2) the cholate concentration and incubation time had no effect on the absolute amount of the C-22 acid formed, and about 18.8 to 23.9 per cent of the original cholate was converted into the C-22 acid in all the flasks; 3) the C-22 acid formed in these media was gradually degraded, especially at high cholate concentrations; 4) about one half of the amount of the C-22 acid calculated from its molecular extinction coefficient was isolated as crystals. This fact suggests that the ultraviolet absorption maximum at 246 m\textmu is not only due to the C-22 acid but also to some unidentified \(\alpha, \beta\)-unsaturated ketone derivatives formed from cholic acid in the culture medium; 5) Pettenkofer's test and acid precipitation test disappeared in parallel with the formation of the C-22 acid; 6) shaking culture shortened the incubation time for the formation of the C-22 acid in about one half of that of a stationary culture (1-3).

Spectrophotometric Analyses of the Degradation Products of Cholic Acid
### Table I

**Effect of Cholate Concentration and Incubation Time on the Formation of the C-22 Acid by S. gelaticus 1164**

<table>
<thead>
<tr>
<th>Cholate concn. (g./dl.)</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>0.05</td>
<td>7</td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>0.2</td>
<td>22</td>
</tr>
<tr>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>0.4</td>
<td>33</td>
</tr>
<tr>
<td>0.8</td>
<td>2</td>
</tr>
</tbody>
</table>

The amount of the C-22 acid formed is given γ/ml. and per cent of the C-22 acid formed to the original cholic acid added is given in parentheses.

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**Fig. 1.** Degradation of cholic acid and production of the C-22 acid by *S. gelaticus* 1164.

(Sodium cholate: 0.3 g./dl.).
by the Seven Species of Streptomyces—The degradation products of cholic acid by the seven *Streptomyces* spp., which were confirmed to utilize cholic acid as the sole source of carbon in the previous paper (6) were investigated by the following methods: Dulaney's mineral solution (6) containing 0.1 g. of sodium cholate per dl. was distributed in small test tubes, each receiving 2 ml., and autoclaved at 110° for 20 minutes. Each of the tubes was inoculated with one of the seven *Streptomyces* spp. After stationary incubation for 5 days at 27° in darkness, the culture fluid was centrifuged at 3,000 r.p.m. for one minute, and the resulting supernatant was divided into two parts after 40-fold dilution. The ultraviolet absorption spectra of the one part were measured. All of the seven *Streptomyces* tested gave an absorption peak at 246 mμ as shown in Fig. 2. To the aliquots of the other part was added separately a tenth volume of aqueous N NaOH or concentrated hydrochloric acid and the mixture was heated in a boiling water bath for ten minutes. After chilling, the ultraviolet spectra of each culture were measured. All of the seven *Streptomyces* tested gave an absorption peak at 290 mμ as shown in Figs. 3 and 4.

As controls, these *Streptomyces* spp. were cultured in a medium containing glucose (1.0 g. per dl.) in place of sodium cholate for ten days at 27°. The supernatants obtained according to the procedure described above showed no absorption peak at 246 mμ, but showed a peak at 270 mμ in the cases of *S. gelaticus* 1164, *S. flavogriseus*, *S. californicus* and *S. rubescens*. An absorption peak at 240 mμ was observed only for *S. halstedii*. Such evidences suggest that an absorption peak at 246 mμ is due to the degradation products of cholic acid in the culture medium. Also the shift of λ<sub>max</sub>*water* by alkali or acid from 246 mμ to 290 mμ suggests that all of the seven *Streptomyces* may degrade cholic acid through an intermediate having the same chromophore.

*Utilization of Various Kinds of Bile Acids by these Seven Cholic Acid Utilizers*—Autoclaved Dulaney's mineral solution (6) was used as a basal medium and to the solution were added sodium salts of eleven kinds of bile acid. The concentration of these bile salts was made up to 0.1 per cent and the resulting culture media were sterilized fractionally at 100°. These media were tubed in 1 ml. quantity in small test tubes and the seven cholic acid utilizers were inoculated from Czapeck's agar (7 days' incubation at 27°). The growth of *Streptomyces* spp. was examined for ten days. The results are shown in Table II.

The position and the number of hydroxyl group and the side chain...
length of the various kinds of bile acids played an important role for the utilization of bile acid by Streptomyces spp. tested. Chenodesoxycholate and desoxycholate were considered to be toxic against all of the seven Streptomyces tested because they showed no growth even when cultivated in the medium containing glucose (1.0 g. per dl.), and homocholate and α-hyodesoxycholate against some of the species, but norcholate had no toxicity for all of the seven species.

**DISCUSSION**

Various microbiological transformations of steroids were reviewed by Peterson (7), but he did not describe in his review the conversion of bile acid by microbes. However, it has been demonstrated that S.
gelaticus 1164 is capable of converting cholic acid into a C-22 acid, namely, 7α-hydroxy-3,12-diketo-Δ4-bisnorcholenic acid (1–3,5). The C-22 acid shows an ultraviolet absorption maximum at 246 mμ in water and is transformed by treating with caustic alkali or sulfuric acid into 3,12-diketo-Δ4,6-bisnorcholadienic acid (5) which has an absorption maximum at 290 mμ in water.

In the present study six species of Streptomyces were studied in this respect along with S. gelaticus 1164. Cultures of all of the seven cholic acid utilizers showed an absorption maximum at 246 mμ (Fig. 2) and their culture supernatants which were heated with caustic alkali or hydrochloric acid indicated an absorption maximum at 290 mμ (Figs. 3 and 4). These findings, therefore, indicate that the six cholic acid

![Graph showing ultraviolet absorption spectra](image-url)
utilizers tested are able to degrade cholic acid to an unidentified derivative containing an \( \alpha, \beta \)-unsaturated ketone grouping which is probably 7\( \alpha \)-hydroxy-3,12-diketo-\( \Delta^4 \)-bisnorcholenic acid, and seems to afford a substance containing \( \Delta^{4,6} \)-3-ketone grouping by treating with alkali or acid.

Gallagher et al. (8) showed that the oxidation of cholic acid by chromic acid proceeds most easily at C7, and least at C3. This order has been suggested previously by the work of Kaziro et al. (9) and Hoehn et al. (10). Haslewood (11, 12) likewise confirmed this finding. On the other hand, Schmidt et al. (13) stated that cholic acid added to cultures of a strain of Alcaligenes faecalis or E. coli isolated from human faeces, was converted into different ketocholanic acids among which they isolated 3,7,12-triketocholanic (dehydrocholic) acid, and further they (14, 15) demonstrated that 3\( \alpha \),12\( \alpha \)-dihydroxy-7-ketocholanic acid and 3\( \alpha \)-hydroxy-7,12-diketocholanic acid were two of the intermediate derivatives formed during bacterial oxidation of...
cholic acid to dehydrocholic acid. Recently, Hayakawa et al. (16) found that $3\alpha,12\alpha$-dihydroxy-7-ketocholestanic acid was formed when cholic acid was incubated with a culture of *E. coli*. These findings are of interest, for they show that microbiological oxidation follows the same path as chemical oxidation and affords the $3\alpha,12\alpha$-dihydroxy-7-keto acid, the $3\alpha$-hydroxy-7,12-diketo acid, and finally dehydrocholic acid, namely, the order of oxidation of the hydroxyl groups in cholic acid is $C_7 > C_{12} > C_3$.

The formation of $7\alpha$-hydroxy-3,12-diketo-$A^4$-bisnorcholestanic acid from cholic acid by *S. gelaticus* 1164 and the data as seen in Figs. 2, 3 and 4 may suggest two possible pathways in the microbial oxidation of

### Table II

**Utilization of Various Kinds of Bile Acids by Seven Cholic Acid Utilizers**

<table>
<thead>
<tr>
<th>Bile acids (sodium salt)</th>
<th>Cholic acid utilizers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Position of OH groups</td>
</tr>
<tr>
<td>Tauro-cholate</td>
<td>3,7,12</td>
</tr>
<tr>
<td>Glyco-cholate</td>
<td>3,7,12</td>
</tr>
<tr>
<td>Lithocholate</td>
<td>3</td>
</tr>
<tr>
<td>$\alpha$-Hydoxycholate</td>
<td>3,6</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>3,7</td>
</tr>
<tr>
<td>Desoxycholate</td>
<td>3,12</td>
</tr>
<tr>
<td>Cholate</td>
<td>3,7,12</td>
</tr>
<tr>
<td>Dehydrocholate</td>
<td>-</td>
</tr>
<tr>
<td>Bisnorcholate</td>
<td>3,7,12</td>
</tr>
<tr>
<td>Norcholate</td>
<td>3,7,12</td>
</tr>
<tr>
<td>Homocholate</td>
<td>3,7,12</td>
</tr>
</tbody>
</table>

Figures refer to period necessary for positive growth (in days) and minus signs mean non-growth.
cholic acid.

1) Cholic acid $\rightarrow 3\alpha,12\alpha$-Dihydroxy-7-ketocholestanic acid $\rightarrow 3\alpha$-Hydroxy-7,12-diketocholestanic acid $\rightarrow 3,7,12$-Triketoocholestanic (Dehydrocholic) acid $\rightarrow$

2) Cholic acid $\rightarrow 3\alpha,7\alpha$-Dihydroxy-12-ketocholestanic acid (or $7\alpha$-12\alpha$-Dihydroxy-3-ketocholestanic acid) $\rightarrow 7\alpha$-Hydroxy-3,12-diketocholestanic acid $\rightarrow (?) \ldots \rightarrow 7\alpha$-Hydroxy-3,12-diketo-\(\Delta^4\)-bisnorcholestanic acid $\rightarrow$

The first pathway is the above-described Alcaligenes faecalis or E. coli type and is the same as observed in the chemical oxidation by chromic acid. The second pathway is postulated for our Streptomyces type but the chemical oxidation of this type is not yet known. However, it is well known that in Oppenauer oxidation of bile acid the first step of the oxidation of the secondary hydroxyl groups in cholic acid (17) or desoxycholic acid (18, 19) is not at C\(_7\) or C\(_{12}\) but at C\(_3\), and also that the oxidation product of methyl cholate by potassium permanganate is not methyl $3\alpha$-hydroxy-7,12-diketocholestanate but methyl $12\alpha$-hydroxy-3,7-diketocholestanate (20). Such evidences suggest the presence of the second oxidative pathway of cholic acid by microorganisms.

In our experiments, the (axial) $7\alpha$-hydroxyl group in cholic acid is not attacked even after the $\beta$-oxidation of a side chain of it. This finding indicates that the first oxidation product of cholic acid by S. gelaticus 1164 may be either $3\alpha,7\alpha$-dihydroxy-12-ketocholestanic acid or $7\alpha,12\alpha$-dihydroxy-3-ketocholestanic acid. Also it is known that the $3\alpha$-hydroxyl group in cholic acid is an equatorial bond and the $12\alpha$-hydroxyl group is an axial bond. Furthermore, Magasanik et al. (21) has discovered a most interesting correlation between the conformations of cyclitols and their liability to oxidation by Acetobacter suboxidans and stated that only axial hydroxyl groups were oxidized. On the basis of these evidences, it is tentatively suggested that the first step of the oxidation of cholic acid in the second pathway is $3\alpha,7\alpha$-dihydroxy-12-ketocholestanic acid. While, as well as Oppenauer oxidation (17–19) and permanganate oxidation (20) it may be suggested that $7\alpha$, $12\alpha$-dihydroxy-3-ketocholestanic acid is the first mirobial oxidation product from cholic acid in the second pathway. In both cases, the second oxidation product is probably $7\alpha$-hydroxy-3,12-diketocholestanic acid and its $7\alpha$-hydroxyl group seems to be intact until $\beta$-oxidation of the side chain of this acid occurs.

Therefore, such observations suggest that an intermediate containing $7\alpha$-hydroxyl group or $7\alpha$-hydroxy-3-keto-\(\Delta^4\)-ene grouping plays
an important role for the degradation of cholic acid by our seven Streptomyces spp. In order to confirm this pathway by Streptomyces, the isolation of the intermediate derivatives formed during the oxidation of cholic acid to $7\alpha$-hydroxy-3,12-diketo-$\Delta^4$-bisnorcholestenic acid is under way. The expected intermediates, namely, $7\alpha,12\alpha$-dihydroxy-3-ketocholestanic acid, $3\alpha,7\alpha$-dihydroxy-12-ketocholestanic acid, $7\alpha$-hydroxy-3,12-diketocholestanic acid and some unidentified substances have been isolated from the culture filtrate obtained after incubation of cholic acid with S. gelaticus 1164. These data will be reported in elsewhere (22).

SUMMARY

1. The culture conditions for the formation of a new unsaturated C-22 acid (presumably $7\alpha$-hydroxy-3,12-diketo-$\Delta^4$-bisnorcholestenic acid) from cholic acid by S. gelaticus 1164 were investigated by shake bottle culture.

2. All of the seven Streptomyces spp. which were identified in the previous paper (6) degraded cholic acid through an intermediate containing $7\alpha$-hydroxy-3-keto-$\Delta^4$-ene grouping. This fact was confirmed through the spectrophotometric analyses of the cultures.

3. Ability of the above seven Streptomyces spp. to utilize the various kinds of bile acids was investigated and it was found that the bile acids containing three functional groups at C$_3$, C$_7$ and C$_{12}$ were utilized except norcholic and homocholic acids. This fact suggests that the utilization of bile acids by Streptomyces is dependent on both the nucleus constitution and the length of side chains of bile acid.

4. An oxidative pathway of cholic acid by Streptomyces was discussed.

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