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The Action of Intestinal Microorganisms on Bile Acids

Bile Acids and Steroids 101

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The transformation of cholic-24-^{14}C acid by intestinal microorganisms of the rat in aerobic and anaerobic cultures has been studied. 7-Ketodeoxycholic, deoxycholic, 12-ketolithocholic and 3,12-diketocholanic acids were isolated from the cultures and identified. 7-Ketodeoxycholic acid was the main metabolite formed in the aerobic cultures. The removal of the hydroxyl group at C-7 in cholic acid was mainly effected by anaerobic microorganisms. The ability of different intestinal miroorganisms to transform cholic acid was also tested. Certain strains of *Escherichia coli* and *Clostridium perfringens* oxidize cholic acid to 7-ketodeoxycholic acid.

Numerous bacteria and fungi are known to transform bile acids and related steroids into a variety of metabolites both *in vivo* and *in vitro* (see, *e.g.*, the reviews by Haslewood [1] and Talalay [2]). When labelled bile acids are given to rats, their radioactive metabolites occur as a complicated mixture of unconjugated compounds in the feces [3,4]. The products of purely microbial transformation of cholic acid have been isolated from the feces of rats with biliary fistula following the injection of cholic-24-^{14}C acid into the caeca. The main metabolites have been identified as 3α, 12α-dihydroxy-7-ketocholanic, deoxycholic and 3α-hydroxy-12-ketocholanic acids [5,6]. Small amounts of at least four unidentified labelled products were also isolated from the feces specimens.

In the present investigation we have studied the microbial transformation of cholic acid *in vitro*. The metabolites of the acid formed in cultures of both aerobic and anaerobic intestinal microorganisms were isolated and the major metabolites were identified. Also different bacterial strains isolated from the caecum of the rat were tested for their ability to transform cholic acid.

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EXPERIMENTAL

Bile acids. 24-^{14}C-Labelled cholic, deoxycholic and chenodeoxycholic acids were prepared by the procedure of Bergström, Rottenberg and Voltz [7].

Taurocholic-24-^{14}C and glycocholic-24-^{14}C acids were synthesized as described by Bergström and Norman [24]. The conjugated bile acids were isolated from the products of the reactions by reversed-phase partition chromatography [8].

7-Ketodeoxycholic acid (3α,12α-dihydroxy-7-ketocholanic acid) was obtained by oxidizing cholic acid with N-bromosuccinimide [9] and was crystallized in the form of its methyl ester from ethyl acetate-light petroleum. The melting point of the methyl ester was 153—155°C.

7-Ketolithocholic acid (3α-hydroxy-7-ketocholanic acid) was synthesized as described by Samuelsson [10] and was kindly supplied by him.

12-Ketolithocholic acid (3α-hydroxy-12-ketocholanic acid) was prepared according to Bergström and Haslewood [11]. Its melting point was 160—161°C.

3,12-Diketocholanic acid was synthesized by chromic acid oxidation of deoxycholic acid. It melted at 183—185°C.

Column chromatography. The technique of reversed-phase partition chromatography developed by Bergström, Sjövall and Norman [12-14] was employed using the following phase systems:

Phase system	Moving phase (ml)	Stationary phase (ml)
C 1	methanol-water 150—150	chloroform-*iso*octanol 15—15
C 2	methanol-water 144—156	chloroform-*iso*octanol 15—15
C 4	methanol-water 165—135	chloroform-*iso*octanol 15—15
F 1	methanol-water 165—135	chloroform-heptane 45— 5
F 3	methanol-water 180—120	chloroform-heptane 45— 5.

Four ml of the stationary phase was supported on 4.5 g of hydrophobic Super Cel.

Paper chromatography. The routine method employed to isolate the metabolites of cholic acid-24-^{14}C produced by different microorganisms was the paper chromatographic method developed by Sjövall [15,16]. Specimens of broth culture media, 0.05 ml in volume, were run on strips of Whatmann 3 MM paper 1.5 cm wide. Cholic acid and 7-ketodeoxycholic acid were separated by descending paper chromatography with *iso*propyl ether-heptane (50:50 v/v) as the moving phase. The more hydrophobic metabolites, including deoxycholic acid, were separated from unchanged cholic acid by ascending paper chromatography with 70 % acetic acid as the stationary phase and *iso*propyl ether-heptane (85:15 v/v) as the moving phase.

Radioassays. ^{14}C was determined with a Tracerlab end-window or a gas-flow counter. The radioactivity on the paper chromatograms was measured with an automatic scanning device with a G.M. tube.

Bacteriological procedures. The culture medium employed was a placenta digest broth prepared according to Erlansson [17] to which 10 μg of labelled bile acid had been added per ml. After autoclaving, the broth media were inoculated with different strains of intestinal microorganisms or fecal suspensions and incubated at 37°C under aerobic or anaerobic conditions. The anaerobic cultures were incubated in an anaerobic jar of the Fildes-McIntosh type.

RESULTS

Transformation of cholic acid by intestinal microorganisms in vitro. A loopful of the caecal content from rats was transferred to broth containing cholic-24-^{14}C acid. After either aerobic or anaerobic incubation for seven days, the labelled compounds in the broth were separated by column chromatography.

Fig. 1 shows chromatograms of the metabolites formed in anaerobic cultures of caecal microorganisms. Chromatographic analysis of the ether extract

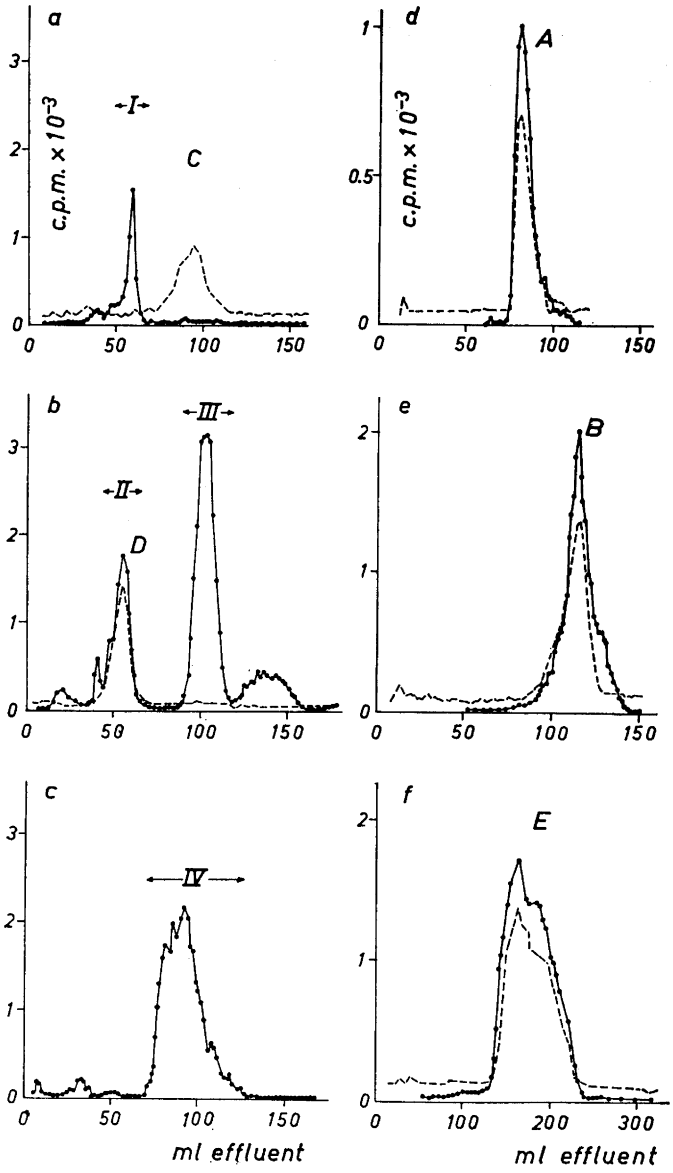

Fig. 1. Separation of metabolites of cholic acid formed in anaerobic cultures of caecal microorganisms. The following fractions were subjected to chromatography. *a*: Ether extract of the broth. Phase system C 1. *b*: Labelled compounds remaining in the stationary phase after the preceding chromatographic elution. Phase system F 1. *c*: Labelled compounds remaining in the stationary phase after the preceding chromatographic elution. Phase system F 3. *d*: Radioactive band I. Phase system C 2. *e*: Radioactive band III. Phase system F 1 *f*: Radioactive band IV. Phase system C 4. Reference substances: cholic (C), deoxycholic (D), 7-ketodeoxycholic (A), 12-ketolithocholic (B), 3, 13-diketocholanic (E) acids. ●—●—●, radioactivity; ————, titration.

of the broth medium with phase system C 1 yielded the chromatogram shown in Fig. 1 a. A separation of 3,12-dihydroxy-7-ketocholanic acid (in effluent fraction 60—70 ml), 3-hydroxy-7,12-diketocholanic acid (80—110 ml) and 3,7,12-triketocholanic acid (110—150 ml) is effected by this phase system. The only labelled compound (I) was found in the effluent fraction where 7-ketodeoxycholic acid is eluted. Most of the radioactive compounds were retained in the stationary phase. When the chromatographic analysis of these metabolites was continued with phase system F 1 (Fig. 1 b) three radioactive bands were eluted; the first two emerged in the effluent fractions where a dihydroxycholanic acid (II) and a monoketomonohydroxycholanic acid (III) were expected. The remaining labelled material in the stationary phase was eluted at 70—100 ml (IV) when phase system F 3 was employed (Fig. 1 c). Lithocholic and 3,12-diketocholanic acids are eluted at about the same rate (70—100 ml) by phase system F 3. To separate these we employ *iso*octanol-chloroform instead of heptane-chloroform as the stationary phase because *iso*octanol retains hydroxy derivatives of cholanic acid more effectively than it retains the keto derivatives of the acid. When phase system C 4 is employed, 3,12-diketocholanic acid is eluted in the effluent fraction 100—210 ml, whereas lithocholic acid stays in the stationary phase. Rechromatography of compound IV with phase system C 4 showed that it behave like 3,12-diketocholanic acid (Fig. 1 f).

The analyses thus showed that no unchanged cholic acid was left in the anaerobic cultures of caecal microorganisms, but instead five metabolites had been formed. Radioactive bands appeared in the effluent fractions where 7-ketodeoxycholic acid (I), 3,12-diketocholanic acid (IV), a dihydroxycholanic acid (II) and a monoketomonohydroxycholanic acid (III) appear. These radioactive metabolites were rechromatographed together with appropriate inactive carriers: fraction I with 7-ketodeoxycholic acid, fraction II with deoxycholic acid, fraction III with 12-ketolithocholic acid, and fraction IV with 3,12-diketocholanic acid. As seen from Fig. 1, the titration peaks coincided with the radioactive peaks. The identities of the metabolites were also confirmed by determining the specific activities after they had been recrystallized together with the inactive reference compounds. The identity of the fifth metabolite (effluent fraction 120—160 ml, Fig. 1 b) was not established.

Chromatographic separations of metabolites formed in aerobic cultures of caecal microorganisms showed that a complete transformation of cholic acid had occurred also in these cultures. The separated metabolites appeared in the same places in the chromatograms as the metabolites produced in the anaerobic cultures. The quantitative distribution, however, was different. More than 80 % of the cholic acid was transformed into deoxycholic, 12-ketolithocholic and 3,12-diketocholanic acids in the anaerobic cultures, whereas only 10 % was transformed into these metabolites in aerobic cultures of the same inocula. It is thus obvious that anaerobic microorganisms are responsible for the removal of the 7α-hydroxyl group. The main metabolite formed in the aerobic cultures was 7-ketodeoxycholic acid. Oxidation of cholic acid to 3-hydroxy-7,12-diketocholanic or 3,7,12-trihydroxycholanic acid was not found to occur.

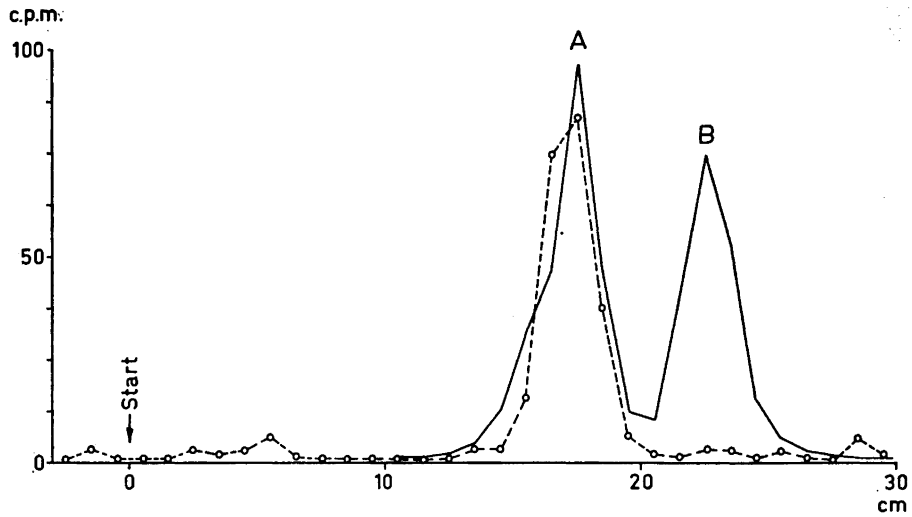

Fig. 2. Descending paper chromatography with *iso*propyl ether-heptane (50—50) as moving phase. ————, 24-^{14}C-labelled 7-ketodeoxycholic (A) and cholic (B) acids. O—O—O, 0.05 ml of a broth originally containing cholic acid-24-^{14}C incubated seven days after inoculation with *E. coli*.

Transformation of cholic acid by microorganisms isolated from the rat caecum. A number of aerobic and anaerobic intestinal microorganisms were isolated from the rat caecum. A loopful or organisms from a blood agar plate was inoculated into the broth and incubated for seven days. The ability of the organisms to transform cholic acid was determined by paper-chromatographic analysis. Of the isolated strains the following were found to be inactive: various strains of aerobic and anaerobic streptococci, *B. proteus*, *Pseudomonas pyocyanea*, *Aerobacter aerogenes*, *Staphylococcus aureus* and *albus*, *B. subtilis*, *diphtheroids* and some *candida* and *streptomyces* strains.

Different strains of *E. coli* transformed cholic acid completely into a labelled compound that appeared in the chromatogram in the same place as 7-ketodeoxycholic acid (Fig. 2). The same transformation was effected by strains of *Clostridium perfringens*. No intestinal microorganisms have yet been isolated that are capable of removing the 7α-hydroxyl group from cholic acid.

Oxidations of bile acids by E. coli and Clostridia. Fig. 3 shows chromatograms of broth specimens containing cholic acid that had been incubated 24 and 72 h after inoculation with *E. coli*. After three days the cholic acid had been completely transformed into a compound with the same chromatographic behaviour as 7-ketodeoxycholic acid (Fig. 4 a). An aliquot of the labelled metabolite was treated with chromic acid under conditions known to oxidize 7-ketodeoxycholic acid to dehydrocholic acid. After rechromatography the radioactivity then moved with dehydrocholic acid added as carrier. After its reduction with sodium borohydride the labelled compound exhibited the same

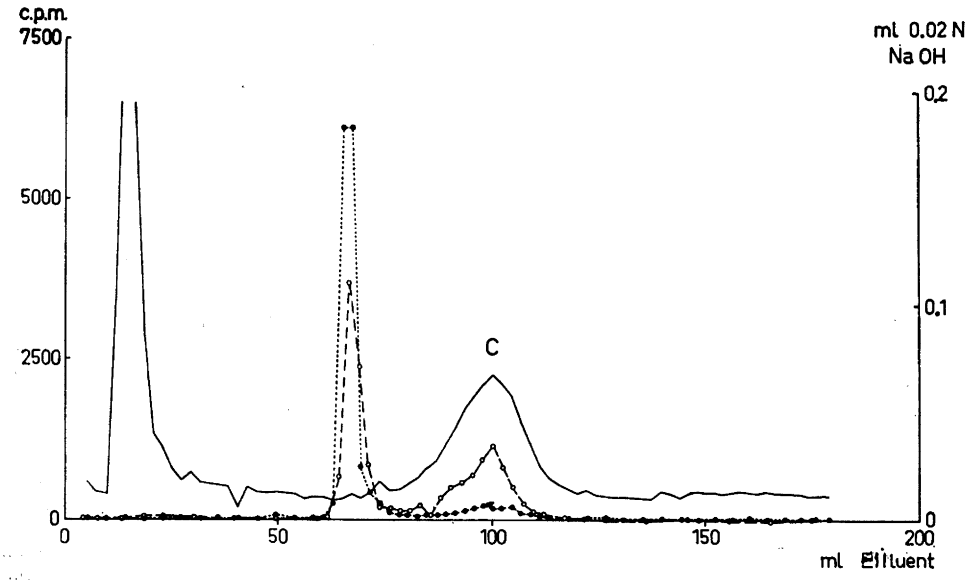

Fig. 3. Separation of labelled products formed in a broth originally containing cholic acid-24-^{14}C and inoculated with a strain of *E. coli* after 24 (O—O—O) and 72 (●—●—●), hours incubation. Phase system C 1. Reference substance: cholic acid (C). ———— titration.

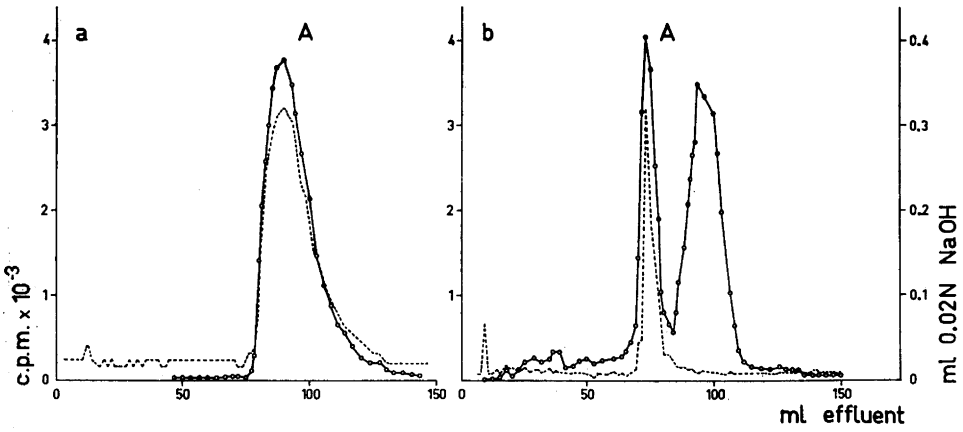

Fig. 4. a: Rechromatography with phase system C 2 of labelled material appearing before cholic acid in the chromatogram shown in Fig. 3.
Fig. 4. b: Chromagraphy of metabolites of cholic acid-24-^{14}C formed by *Clostridium perfringens* type E during seven days' incubation. Phase system C 1. Reference substance: 7-ketodeoxycholic acid (A). ————, radioactivity; — — — — —, titration.

Table 1. Transformation of cholic acid into 3,12-dihydroxy-7-ketocholanic acid by different strains of *E.coli* isolated from human feces. 5 ml of inoculated placenta broth containing 0.1 mg of cholic acid-24-^{14}C were cultured one and seven days. The quantitative estimates were based on paper-chromatographic analyses.

All the *E.coli* strains produced acid from arabinose, glucose, maltose and mannitol (also gas) and also produced indole.

No. of strain	Per cent of cholic acid transformed after 1 day	7 days	Biochemical reactions Sucrose	Dulcitol	Sorbitol	Salicin
I	61	100	−	−	+	−
II	71	100	−	−	+	−
III	79	100	−	−	+	−
V	62	100	−	−	+	−
VI	81	100	−	−	+	−
VII	75	100	−	−	+	−
VIII	79	100	−	−	+	−
IX	71	100	−	−	+	−
X	67	100	−	+	+	−
XI	74	100	+	−	+	+
XII	0	0	−	−	−	−
XIII	69	100	−	−	+	+
XIV	60	100	−	+	+	−
XV	68	100	−	+	+	−
XVI	88	100	−	−	+	−
uninoculated control	0	0				

chromatographic behaviour as cholic acid. The identity of the metabolite was confirmed further by recrystallizing it together with unlabelled 7-ketodeoxycholic acid. The specific activity remained constant through five recrystallizations.

E. coli strains are capable of oxidizing only the hydroxyl group at C-7 as no oxidation to 3-hydroxy-7,12-diketocholanic or 3,7,12-trihydroxycholanic acid was established. Chenodeoxycholic acid was transformed in cultures of *E. coli* into a compound that behaved like 12-ketolithocholic acid (Fig. 5 a) in chromatography and which was further identified by cocrystallization with unlabelled 12-ketolithocholic acid. No oxidation of deoxycholic acid occurred (Fig. 5 b) in similar tests. Experiments with 24-^{14}C-labelled glycocholic and taurocholic acids have shown that these conjugates undergo a similar oxidation by *E. coli*. No splitting of peptide bonds was observed.

Sixteen strains of *E. coli* isolated from human feces were tested for their ability to oxidize cholic acid to 7-ketodeoxycholic acid (Table 1). All the strains except one effected a rapid and complete oxidation of the cholic acid.

In a previous study [18] it was found that different strains of *Clostridium perfringens* quickly hydrolyze conjugated bile acids and transform a minor part of cholic acid into a more polar compound. Fig. 4 b shows a chromatogram of a broth culture of *Clostridium perfringens* type E originally containing cholic-24-^{14}C acid. The metabolite formed was eluted at the same rate as 7-ketodeoxycholic acid. It was identified as 7-ketodeoxycholic acid in the manner described above.

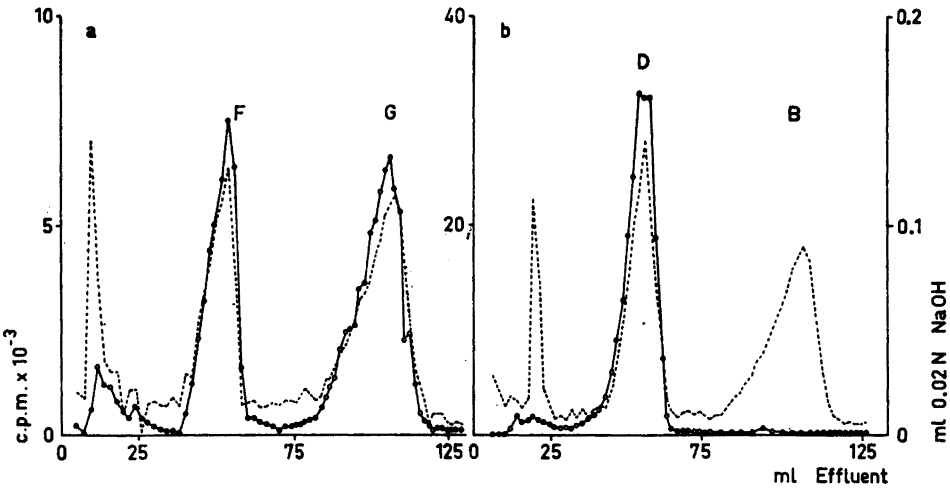

Fig. 5. Chromatographic analyses of three-day-old broth cultures of *E. coli*. *a*: broth originally contained chenodeoxycholic acid 24-^{14}C. *b*: broth originally contained deoxycholic acid-24-^{14}C. Phase system F 1. Reference substances: deoxycholic (D), chenodeoxycholic (F), 12-ketolithocholic (B) and 7-ketolithocholic (G) acids. ————, radioactivity; — — — — —, titration.

DISCUSSION

A transformation of cholic acid similar to that taking place *in vivo* is effected by anaerobic caecal microorganisms *in vitro*. The 7*α*-hydroxyl group is removed for the most part. Whereas the greater part of deoxycholic acid is excreted unchanged by the rat, the acid is for the most part oxidized by caecal microorganisms in vitro to 12-keto-3-hydroxycholanic and 3,12-diketocholanic acids. However, the caecal microorganisms more quickly remove the 7*α*-hydroxyl group than oxidize the hydroxyl groups at C-3 and C-12.

Schmidt and Hughes [19,20] demonstrated that a rapid and extensive decomposition of cholic acid occurs in the caecum of the guinea pig. They assumed this to be mainly due to an oxidative breakdown of the acid by strains of *Alcaligenes faecalis via* 7-keto-3,12-dihydroxycholanic and 7,12-diketo-3-hydroxycholanic acids to 3,7,12-triketocholanic acid. We have found that a rapid oxidation of cholic acid to 7-ketodeoxycholic acid occurs in aerobic cultures of microorganisms isolated from the rat caecum where the removal of the 7-*α*-hydroxyl group takes place only in minor degree. No further oxidation of the hydroxyl groups at C-3 and C-12 in cholic acid has been demonstrated. Neither 7,12-diketo-3-hydroxycholanic nor 3,7,12-triketocholanic acids has been isolated from the rat feces.

Turfitt [21] and Hayakawa *et al.*[22] have reported that the bile acid side chain is degraded by *Proactionomyces* and *Streptomyces gelatinus* 1164, respectively. Our experiments with 24-^{14}C-labelled cholic acid did not provide any evidence showing that a degradation of the bile acid side chain occurs in cultures of

microorganisms isolated from the rat caecum. Several investigators [22,23] have shown that cholic acid is oxidized to derivates of 3-keto-Δ^4-cholanic acid by different microorganisms. The formation of such compounds by intestinal microorganisms of the rat seems to be of minor degree.

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