hyaluronic acid did not occur until the 2nd and 3rd hour following intravenous injection. Only traces of intravenously injected hyaluronic acid appeared in the synovial space, skin or vitreous humor.

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In vitro Formation of Deoxycholic and Lithocholic Acid by Human Intestinal Microorganisms.*† (27577)

ARNE NORMAN AND MARY S. SHORB‡
Department of Clinical Chemistry, Stockholms Läns Centrallasarett, Danderyd, Sweden

Elimination of the 7-hydroxysubstituent of the bile acids by intestinal microorganisms is a common reaction occurring in many species (1) and has been reproduced *in vitro* by a mixture of anaerobic intestinal microorganisms from the rat(2). Samuelsson has demonstrated that the reaction mechanism is a dehydration to a Δ^6-derivative, followed by reduction(3).

Human bile contains 3 main bile acids, cholic, chenodeoxycholic and deoxycholic acid, with a mean ratio of 1.1:1.0:0.6(4). Cholic and chenodeoxycholic acids are formed directly from cholesterol in the liver (1). Due to the action of microbial enzymes the 7-hydroxyl group of cholic acid is removed and the "secondary" bile acid deoxycholic acid is formed(5). Contrary to the rapid transformation of cholic acid, chenodeoxycholic acid is transformed slowly during the enterohepatic circulation. Three days after administration of chenodeoxycholic acid-24-C^{14} only 3% of the isotope in the human bile was recovered as lithocholic acid(6). Although definitive proof is lacking, investigation so far indicates that deoxycholic and lithocholic acids are not metabolized by the human liver to a significant extent(6,7). The different concentration of deoxycholic and lithocholic acids in human bile, therefore, might be explained by a difference in ability of the intestinal flora to remove the 7-hydroxyl group from cholic and chenodeoxycholic acid or by a difference in accessibility for absorption of the "secondary" bile acids.

The aim of the present investigation was to study the formation of deoxycholic and lithocholic acid in subcultures of mixed human intestinal microorganisms.

Experimental. The 24-C^{14}-labeled cholic, deoxycholic and chenodeoxycholic acids were prepared according to Bergström *et al.*(8).

Labeled bile acids as sodium salts dissolved in 80% ethanol were added to thick walled centrifuge tubes (inside diameter 13 mm, height 100 mm). After evaporation, the

* Bile acids and steroids 122.

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‡ On sabbatical leave from Poultry Husbandry Dept., Univ. of Maryland.

residue was taken up in 4 ml of culture medium. The bile acid concentration was always 0.2 mM/l broth. The tubes were plugged with cotton and autoclaved for 20 minutes. The first subculture was made by inoculating one loop of human feces into each experimental tube and into a tube without bile acid. Subsequent serial stock subcultures of mixed microorganisms were made in medium free of bile acids. One drop from a serial stock subculture was inoculated into the respective set of experimental tubes containing the bile acids. For aerobic incubation the tubes were cotton-stoppered. For anaerobic incubation the excess cotton of the plug was burned off, the plugs carefully pushed one-quarter inch into the tubes, 4 drops of 2% NaOH and 6 drops of 40% pyrogallic acid were placed on the cotton and the tubes immediately sealed with rubber stoppers. All subcultures were incubated at 37°C for the time indicated in each experiment, then centrifuged for one hour at 25,000 $\times$ *g*, and the supernatant decanted. The sediment was washed with 1/15 M phosphate buffer (pH 7.4) and centrifuged. The washed sediment was extracted by refluxing in acetone. The isotope content of the supernatant, phosphate washing and acetone extract was determined.

The bile acids were separated with reversed phase column chromatography(9,10) using the following solvent systems:

Phase system Moving phase (ml) Stationary phase (ml)

C 1 Methanol-water 150:150 Chloroform-isooctanol 15:15

F 1 Methanol-water 165:135 Chloroform-heptane 45:5

F 2 Methanol-water 180:120 Chloroform-heptane 45:5

Four ml of stationary phase were supported on 4.5 g of hydrophobic Supercel. The fractions collected from the columns were titrated and the isotope content determined on aliquots after plating.

Results. Removal of the 7-hydroxyl group of bile acids by subcultures of mixed human intestinal microorganisms. The ability of the fecal microorganisms responsible for elimination of the 7-hydroxyl group to survive in subculture was tested with different media. The best medium was Trypticase soy broth (Baltimore Biological Laboratories) with anaerobic incubation. The time-curve for formation of lithocholic acid from chenodeoxycholic acid in this medium by mixed fecal microorganisms (anaerobic subculture 5) is shown in Fig. 1. This experiment as well as a similar one with cholic acid showed that elimination of the 7-hydroxyl group occurred mainly after 24-48 hours incubation. In subsequent experiments subcultures were incubated for 3 days.

Formation of lithocholic acid in anaerobic and aerobic subcultures in Trypticase soy broth was compared. Seven serial anaerobic subcultures had about the same activity as the first one. In contrast, this ability was lost in the second aerobic subculture.

So far the microorganism(s) responsible for elimination of the 7-hydroxyl group has not been obtained in pure culture.

Nature of labeled bile acids in supernatant and sediment after centrifugation of the subcultures. The distribution of labeled metabolites of chenodeoxycholic acid between supernatant and sediment was determined in 7 serial anaerobic subcultures. As seen in Table I, 47-63% of the isotope was recovered in the sediments after acetone extraction. In contrast, subcultures originally containing cholic or deoxycholic acid-24-C^{14} had labeled metabolites only in the supernatant.

The labeled metabolites of chenodeoxycholic acid in the supernatants and sediments of the 7 subcultures from the above experiments were chromatographically analyzed for labeled lithocholic acid. Results are summarized in Table I. Fig. 2 gives the chromatograms of the 7th anaerobic subculture. The labeled bile acids in the sediment consist mainly of lithocholic acid, whereas the supernatant contains only a small amount of lithocholic acid. The labeled products in the supernatant were chromatographically separated with phase system F 1. All chenodeoxycholic acid has been transformed (Fig. 3, right part). The supernatant contains several metabolites which also are formed in the 7th aerobic subculture (Fig. 3, left part).

Anaerobic subcultures also give a complete

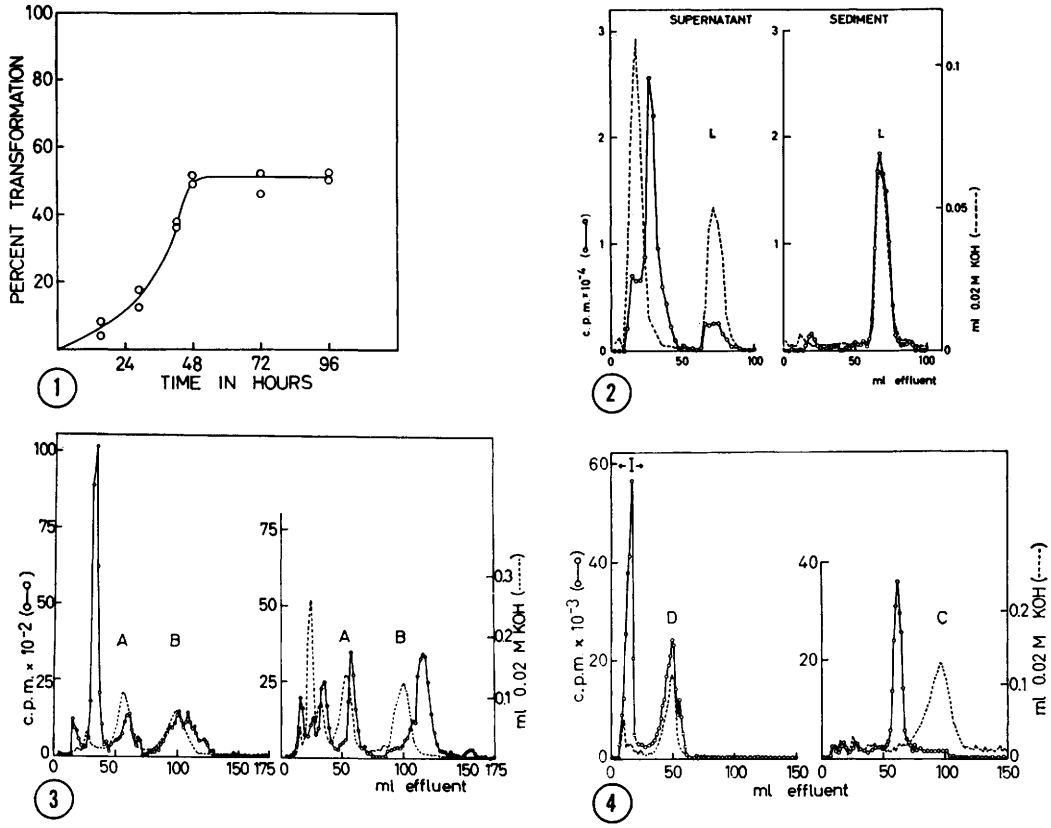

FIG. 1. Removal of 7-hydroxyl group from chenodeoxycholic acid in 5th subculture of human fecal microorganisms incubated anaerobically.

FIG. 2. Separation of metabolites of chenodeoxycholic acid-24-C^{14} present in supernatant (left curve) and sediment (right curve) after centrifugation of 7th anaerobic subculture of human fecal microorganisms. Phase system F 2. Reference substance: lithocholic acid (L).

FIG. 3. Separation of metabolites of chenodeoxycholic acid-24-C^{14} present in supernatant after centrifugation of 7th subculture of human fecal microorganisms. Left curve: aerobic incubation. Right curve: anaerobic incubation. Phase system F 1. Reference substances: chenodeoxycholic (A) and 7-ketolithocholic (B) acids.

FIG. 4. Left curve: Separation of metabolites of cholic acid-24-C^{14} formed in 7th anaerobic subculture of human fecal microorganisms. Phase system F 1. Right curve: Rechromatography of radioactive band I, left curve. Phase system C 1. Reference substances: deoxycholic (D) and cholic (C) acid.

transformation of cholic acid. The metabolites, which are all present in the supernatant, appear on the chromatograms at the places of deoxycholic acid (Fig. 4, left part) and 7-keto-3, 12-dihydroxycholanic acid (Fig. 4, right part).

The main transformation of cholic and chenodeoxycholic acids by subcultures of human fecal microorganisms is the removal of the 7-hydroxyl group. Lithocholic acid is the only metabolite present in the sediment after centrifugation of the cultures. The sodium salt of this acid is sparingly soluble in water (11) and its solubility in Trypticase soy broth is below 0.02 mM/l. Different methods for extraction of the isotope from the sediment have been tested. The results show that it is not solely a precipitation of lithocholic acid from the broth, since extraction of the sediment suspended in phosphate buffer, pH 6.0, with ethyl ether gives an incomplete recovery of the isotope. Therefore an intracellular localization of lithocholic acid is possible.

Conclusions. The human intestinal microorganism responsible for removal of the 7-hydroxyl group from the bile acids can be subcultured anaerobically in Trypticase soy broth. The intestinal microorganisms have a

TABLE I. Transformation of 24-C^{14}-Labeled Cholic, Deoxycholic and Chenodeoxycholic Acids in Anaerobic Subcultures of Mixed Human Fecal Microorganisms.

Serial subculture from feces	Bile acid .2 mM/l Trypticase soy broth	pH of broth after 3 days incubation	% distribution of isotope in Supernatant broth	Phosphate buffer washing	Acetone extract of sediment	Labeled metabolities without hydroxyl or keto group at C-7, as % of total amt of labeled material present in: Supernatant	Sediment
1	Chenode-oxycholate	7.2	35	2	63	0	98
2	"	6.9	48	2	50	4	96
3	"		49	4	47	8	98
4	"		44	2	54	8	97
5	"	6.8	48	2	50	9	97
6	"	6.8	46	2	52	9	97
7	"	7.2	46	2	52	10	95
7	Cholate	7.2	97	1	2	70	—
7	Deoxycholate	7.2	96	2	2	—	—

high ability for removal of the 7-hydroxyl group from cholic as well as from chenodeoxycholic acid. Centrifugation of the subcultures at 25,000 × *g* for 60 minutes reveals that the 2 reaction products appear in a different physical state. Deoxycholic acid appears in the supernatant whereas lithocholic acid is recovered mainly in an acetone extract of the sediment. These results indicate that lithocholic acid formed *in vivo* in the human intestine probably is less accessible for absorption than deoxycholic acid and therefore is prevented from entering the enterohepatic circulation. This might also explain why lithocholic acid is found only in trace amounts in the human bile.

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Cell Division and Phagocytic Activity in Liver Reticulo-Endothelial Cells. (27578)

LOLA S. KELLY, BARBARA A. BROWN AND ERNEST L. DOBSON
Donner Laboratory, University of California, Berkeley

Evidence has been presented that stimulation to hyperactivity of the reticulo-endothelial (R. E.) system by estradiol or zymosan and recovery of the R. E. system from "blockade" by colloids are both accompanied by liver littoral* cell proliferation(1). A

* The term littoral cell rather than reticulo-endothelial cell has been used here after Abercrombie and Harkness(2), because some uncertainty has existed as to whether or not all the sinusoidal lining cells are capable of phagocytosis and thus whether or not they belong to the reticulo-endothelial system.