Triketocholanoic (Dehydrocholic) Acid

HEPATIC METABOLISM AND EFFECT ON BILE FLOW AND BILIARY LIPID SECRETION IN MAN

ROGER D. SOLOWAY, ALAN F. HOFMANN, PAUL J. THOMAS, LESLIE J. SCHOENFIELD, and PETER D. KLEIN

From the Gastroenterology Unit, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55901 and the Argonne National Laboratories, Argonne, Illinois 60439

ABSTRACT [24-^{14}C]Dehydrocholic acid (triketo-5β-cholanoic acid) was synthesized from [24-^{14}C]cholic acid, mixed with 200 mg of carrier, and administered intravenously to two patients with indwelling T tubes designed to permit bile sampling without interruption of the enterohepatic circulation. More than 80% of infused radioactivity was excreted rapidly in bile as glycine- and taurine-conjugated bile acids. Radioactive products were identified, after deconjugation, as partially or completely reduced derivatives of dehydrocholic acid. By mass spectrometry, as well as chromatography, the major metabolite (about 70%) was a dihydroxy monoketo bile acid (3α,7α-dihydroxy-12-keto-5β-cholanoic acid); a second metabolite (about 20%) was a monohydroxy diketo acid (3α-hydroxy-7,12-diketo-5β-cholanoic acid); and about 10% of radioactivity was present as cholic acid. Reduction appeared to have been sequential (3 position, then 7 position, and then 12 position) and stereospecific (only α epimers were recovered).

Bile flow, expressed as the ratio of bile flow to bile acid excretion, was increased after dehydrocholic acid administration. It was speculated that the hydroxy keto metabolites are hydrocholeretics. The proportion of cholesterol to lecithin and bile acids did not change significantly after dehydrocholic acid administration. In vitro studies showed that the hydroxy keto metabolites dispersed lecithin poorly compared to cholate; however, mixtures of cholate and either metabolite had dispersant properties similar to those of cholate alone, provided the ratio of metabolite to cholate remained below a value characteristic for each metabolite. These experiments disclose a new metabolic pathway in man, provide further insight into the hydrocholeresis induced by keto bile acids, and indicate the striking change in pharmacologic and physical properties caused by replacement of a hydroxyl by a keto substituent in the bile acid molecule.

This work was presented in part at the meeting of the American Gastroenterological Association, Bal Harbour, Fla., 9–15 May 1971 (1).

Received for publication 23 May 1972 and in revised form 13 November 1972.

INTRODUCTION

3,7,12-Triketo-5β-cholanoic acid,[1] a triketo bile acid, is the major reaction product when cholic acid, a trihydroxy bile acid, is oxidized with chromic acid (2). This compound (referred to in this paper as Tri-K-C) does not occur in bile under physiologic conditions but has been used for many years in man to stimulate bile flow (3) and to measure the circulation time (4).

Secretion of phospholipids in bile appears to be induced by bile acid secretion (5–9). Elucidation of the amphipathic properties of bile acids (10, 11) and the structure of the bile acid-lecithin micelle (12) provided a molecular explanation for the phospholipid-bile acid coupling observed during bile secretion and gave rise to the hypothesis, recently discussed in detail (8, 13), that micelle formation was a prerequisite for phospholipid secretion. Since Tri-K-C does not form micelles at physiologic concentrations (14, 15), it was

[1] Bile acids referred to in the text by trivial names or abbreviations have the following systematic names: dehydrocholic acid (Decholin) or triketocholanoic acid (Tri-K-C), 3,7,12-triketo-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; monohydroxy diketo metabolite (OH-di-K-C), 3α-hydroxy-7,12-diketo-5β-cholanoic acid; dihydroxy monoketo metabolite (Di-OH-K-C), 3α,7α-dihydroxy-12-keto-5β-cholanoic acid.

reasoned that experiments defining the effect of Tri-K-C on biliary phospholipid secretion might provide a test of the hypothesis. Indeed, evidence that Tri-K-C was less effective than cholic acid in stimulating phospholipid secretion was reported (13, 16).

Another explanation for these observations was suggested by Hardison (16) who showed that, when Tri-K-C was given intravenously, it was excreted in bile largely as unidentified Tri-K-C metabolites which formed micelles poorly. Thus, both the decreased phospholipid coupling and the well-known hydrocholeretic effect of Tri-K-C (3,17) could be related to properties of the metabolites rather than to those of Tri-K-C. The speculation that Tri-K-C was an osmotic choleretic (18, 19) because it did not form micelles could not be valid if Tri-K-C were not present in bile.

In this paper, we define the hepatic metabolism of Tri-K-C in man and characterize its effect on biliary lipid secretion. In addition, we performed limited studies on the ability of the Tri-K-C metabolites which we identified to disperse lecithin in micellar form in vitro.

METHODS

Radioactive bile acids

[24-^{14}C]Tri-K-C. [24-^{14}C]Cholic acid (New England Nuclear Corp., Boston, Mass.) was methylated with ethereal diazomethane. The labeled methyl cholate and 1 g of carrier methyl cholate were dissolved in 40 ml of acetone and cooled to 10°C. Chromic acid (as Kiliani's reagent) (20), 16 ml, was added slowly; after 15 min, sucrose was added until all the chromic acid had been reduced (solution became deep green). Water, 10 ml, was added and the pH was adjusted to 3–4 with NaOH. The solution was extracted three times with equal volumes of methyl ethyl ketone-ethyl acetate (1:1, vol/vol). The upper phases were pooled, washed with small volumes of water to neutrality, and then evaporated. The residue was dissolved in benzene and chromatographed on a 1.5 by 30 cm column of silicic acid (particle size 0.05-0.20 mm, Brinkmann Instruments, Inc., Westbury, N. Y.). The column had been packed in hexane, and elution was carried out with 1.4 liters of ether-hexane in a 40:60 to 100:0 gradient. Fractions containing pure methyl Tri-K-C by thin-layer chromatography (TLC)[2] (21) were pooled and evaporated, and the residue was saponified in 40 ml of absolute methanol-1 N NaOH (1:1, vol/vol) for 1–3 h at 37°C. The saponification mixture was acidified and extracted three times with equal volumes of ether-methyl ethyl ketone (1:1, vol/vol). The upper phases were pooled, washed with small volumes of water to neutrality, and then evaporated. By TLC (21), zonal scanning (22), and gas-liquid chromatography (GLC) (23), the [^{14}C]Tri-K-C was more than 98% radiopure. The free acid of Tri-K-C was converted to the sodium salt by titration with dilute sodium hydroxide, and the solution (pH 8–9) was diluted with sterile saline and passed through a membrane filter of 0.45 μm pore size (Nalge Co., Rochester, N. Y.) for sterilization.

[2] *Additional abbreviations used in this paper:* GLC, gas-liquid chromatography; PEG, polyethylene glycol; TLC, thin-layer chromatography.

Di-OH-K-C and OH-di-K-C. Methyl-^{3}H esters of these compounds were prepared by using ^{3}H-labeled methanol (New England Nuclear Corp.) saturated with boron trifluoride.

Reference compounds

Di-OH-K-C was prepared from cholic acid as described previously (24), using procedures described by Fieser and Rajagopalan (25) and Sato and Ikekawa (20). The product was recrystallized from ethyl acetate by the addition of ligroine (petroleum ether).

OH-di-K-C was synthesized by chromic acid oxidation of methyl 3α-carbethoxy-7α,12α-dihydroxy-5β-cholanoate (25) followed by saponification, methylation, and chromatography on silica gel with a gradient of acetone-benzene used for elution. Pure fractions were pooled, and the compound was recrystallized from 95% ethanol by the addition of water. Both compounds were pure by TLC and GLC.

Chromatography

TLC of whole bile was performed on 35 by 20-cm silica gel H plates with a solvent system of isoamyl acetate-propionic acid-*n*-propanol-water (40:30:20:10, vol/vol per vol per vol) (21). Bands were identified by relating their mobility to that of standards (two specimens of the same bile were chromatographed simultaneously, one on either side of the standards). The region of one specimen and the standards were sprayed with 2,4-dinitrophenylhydrazine (0.3% in sulfuric acid-ethanol) for sample detection. The corresponding bands with the mobility of free bile acids or glycine- and taurine-conjugated bile acids were scraped from the unsprayed portion of the plate into counting vials; 3 ml of absolute ethanol was added to elute the compound, a toluene-base scintillation cocktail was then added, and radioactivity was determined by liquid scintillation spectroscopy. Conjugated bile acids were saponified with 1 N NaOH in a nickel-lined bomb (Parr Instrument Co., Moline, Ill.) or with a crude preparation of cholyl amidase (26) extracted from *Clostridium perfringens* (generously provided by our colleagues in the Department of Microbiology). The free bile acids were extracted into ether and methylated with ethereal diazomethane.

Methyl esters of free bile acids were chromatographed on 5 by 20-cm plates of silicic acid developed with a solvent system of chloroform-acetone-methanol (70:25:5, vol/vol per vol) (21). The location of radioactivity was determined by using a zonal scanning device (22), which transfers 4-mm bands of the adsorbent to counting vials; ethanol and a toluene-based scintillation cocktail were added and radioactivity was determined by liquid scintillation spectroscopy.

GLC of the methyl esters of the free bile acids was carried out at 250°C on 4-ft columns packed with 3% QF-1 (methyl fluoroalkyl silicone) on Gas-Chrom S; helium was the carrier gas and a hydrogen flame detector was used. Bile acids were also esterified with methanol and acetylated (27). Reference compounds were esterified with [^{3}H]-methanol by using boron trifluoride catalysis and acetylated. GLC was carried out on columns packed with 2% AN-600 (Analabs Inc., North Haven, Conn.) on Gas-Chrom S (23).

Radio-GLC was accomplished by replacing the flame ionization detector with a nondestructive argon detector and trapping the compounds in a toluene-based scintillation cocktail which was collected directly into counting vials (28). Combined GLC/mass spectrometry was carried out on the methyl ester acetates (previously isolated by column chromatography as described above for the specific refer-

ence compounds) at the Argonne National Laboratory. Samples were injected into a GLC system coupled to a Perkin-Elmer DF/MS-270 mass spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) at 70 eV with an accelerating voltage of 2,000 V and a scan time of 10–30 s/decade (29).

Solubility studies

A series of mixtures of the sodium salts of cholic acid, OH-di-K-C, and Di-OH-K-C with highly purified egg lecithin (Pierce Chemical Co., Rockford, Ill.) was prepared. Bile acid concentrations were 0–30 mM; lecithin concentrations were varied reciprocally so that the total concentration of bile acids and lecithin was 30 mM. In the triangular phase diagram of the ternary system of bile acid-lecithin-water, the series of mixtures falls on a line parallel to the base opposite the water corner; this experimental design has been described elsewhere in more detail (30). To each vial 1 ml of sterile N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (A grade, Calbiochem, Los Angeles, Calif.) was added and the volume was adjusted to 5 ml with sterile isotonic saline. After equilibration with nitrogen, each vial was flame sealed. Vials were shaken vigorously for at least 24 h on a Dubnoff metabolic shaker (Precision Scientific Co., Chicago, Ill.). Turbidity was estimated visually. The vials were then opened and the contents were centrifuged at 2,000 rpm for 30 min. The supernate, which was free from large particles of lecithin, was then centrifuged in a type 50 or 50Ti rotor in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 100,000 *g* (50,000 rpm) for 2 h at 25°C. The clear supernate, containing the micellar phase, was then removed from the cellulose nitrate tubes by puncturing the side of the tube with a syringe and needle. The concentration of phospholipids in the micellar phase was measured by the Fiske and Subbarow method (31).

Physiologic studies

Metabolism after intravenous administration. After their informed consent was obtained, two women were studied 2 wk after cholecystectomy and common duct exploration for cholesterol gallstones. A balloon-occludable reinfusion T tube (32) was inserted. The enterohepatic circulation was restored for at least 48 h before the study by clamping the drainage limb of the T tube. After the balloon was inflated to achieve occlusion, a complete collection of hepatic bile was obtained. The enterohepatic circulation was maintained by reinfusion of bile-salt-enriched bile (previously obtained from other bile fistula patients) through another lumen of the T tube, which bypassed the balloon and emptied into the distal common bile duct.

On the day of the study, a solution containing 200 mg and 20 μCi of the sodium salt was infused intravenously for 15 min. (This is equivalent to the amount of Tri-K-C used in the test for circulation time; however, the rate of delivery was much slower in this study.) Bile flow was measured in graduated cylinders to the nearest 0.1 ml in 30-min collection intervals for 10 h. Lipids in bile were measured as previously described (33). A 24 h collection of urine was obtained from patient 2.

Metabolism after duodenal administration. A cholecystectomized woman was intubated with a double-lumen polyvinyl tube. The perfusion lumen was positioned in the second portion of the duodenum and the collection lumen was located 40 cm distally. Saline containing polyethylene glycol (PEG, mol wt 4000), 5 g/liter, was infused for 100 min to establish a base line. Then, the same solution containing ^{14}C-labeled Tri-K-C (220 mg; 6 μCi) was infused for 10 min. After this, perfusion was continued for 4 h with the PEG saline solution, but an essential amino acid mixture, known to evoke cholecystokinin-pancreozymin release, was added to simulate the postprandial condition and ensure a good bile flow (34). Absorption of Tri-K-C in the 40 cm test segment and biliary secretion of Tri-K-C metabolites were calculated from the concentration changes relative to the PEG.

RESULTS

Metabolism of Tri-K-C after intravenous administration

Time-course and route of excretion. 82 and 86% of the administered dose was recovered in bile during the 10 h after administration in the two patients; 91 and 96% of the total biliary radioactivity recovered was secreted within 2 h after the dose (Fig. 1). 10% of the administered dose was present in a 24 h collection of urine in the one patient (patient 2) examined.

Chemical form of radioactivity in whole bile. All radioactivity present in bile during peak excretion had the TLC mobility of conjugated bile acids, 79 and 89% as glycine-conjugated and 21 and 11% as taurine-conjugated bile acids, respectively, in the two patients (Table I). After incubation of whole bile with cholyl amidase (bile acid deconjugase) activity (26), the

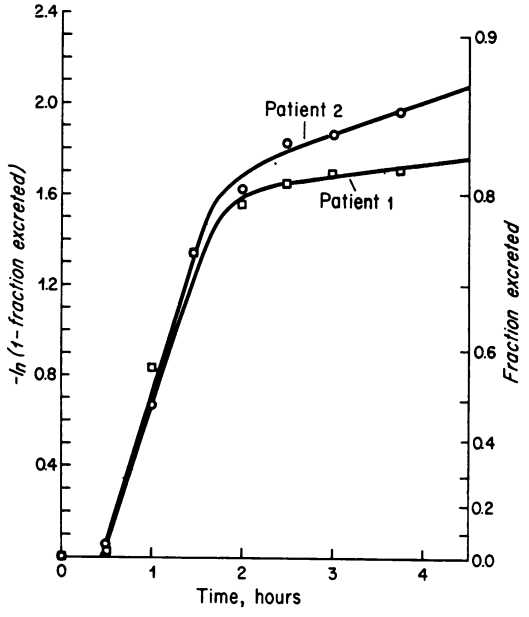

FIGURE 1 Cumulative excretion of ^{14}C radioactivity into bile after intravenous administration of ^{14}C-labeled Tri-K-C. 82 and 86% of the administered dose was recovered in bile during the 10 h after administration. The half-time for the period of rapid excretion is about ½ h in each patient; for the period of slow excretion, it is 11.5 h in patient 1 and 5 h in patient 2.

TABLE I

Radioactivity in Bile or Intestinal Content after Administration of [24-^{14}C]Triketocholanoic Acid

	Mode of administration*		
	Intravenous		
	Exp. 1	Exp. 2	Intraduodenal
Recovery of dose, %	82	86	96‡
Distribution by TLC, %§			
Glycine conjugates	79	89	69
Taurine conjugates	21	11	31
Free	0	0	0‖
Distribution by TLC after deconjugation, %§			
OH-di-K-C	26	20	29
Di-OH-K-C	68	73	61
Cholic	6	7	11

* Administered dose, 200 mg.

‡ Based on assumption that recycling did not occur during the collection period.

§ During period of highest radioactivity in bile.

‖ Free bile acid was exclusively Tri-K-C which was assumed to be unabsorbed material.

mobility of the radioactivity changed to that of unconjugated bile acids.

Unconjugated bile acids. After deconjugation by alkaline or enzymatic hydrolysis, the methyl esters of the liberated free bile acids were examined by zonal scanning. Compounds with the mobilities of cholic, chenodeoxycholic, and deoxycholic acids, the predominant bile acids of human bile, were present. Tri-K-C could not be detected. However, two additional substances were present, one with an R_f slightly less than that of chenodeoxycholic acid and one with an R_f greater than that of deoxycholic acid. The slower migrating spot had the mobility of a dihydroxy monoketo bile acid, and the faster that of a monohydroxy diketo acid. By zonal scanning, the majority of radioactivity was present in these two spots, although radioactivity having the mobility of cholic acid also was present (Fig. 2).

Radio-GLC. Two peaks were clearly separated from the usual biliary bile acids and had retention times corresponding to synthetic standards (as acetates) of 3α-hydroxy-7,12-diketo-5β-cholanoic acid (the monohydroxy diketo metabolite, OH-di-K-C) and 3α,7α-dihydroxy-12-keto-5β-cholanoic acid (the dihydroxy monoketo metabolite, Di-OH-K-C) (Fig. 3). The majority of the radioactivity was present in the first two metabolites, with a small amount in cholic acid. No radioactivity was present in fractions with retention times corresponding to those of Tri-K-C and chenodeoxycholic and deoxycholic acids.

Co-chromatography. Synthetic Di-OH-K-C and OH-di-K-C were esterified with [^{3}H]methanol and mixed with the biliary bile acid methyl esters (which contained ^{14}C in the carboxyl group). Chromatographic mobilities of the two isotopes were identical on adsorption (TLC) and partition (GLC) chromatography, providing further evidence for the chemical identity of the metabolites.

Mass spectrometry. Mass spectra of the methyl ester acetates (Fig. 4) of the two metabolites isolated from the biliary bile acids were identical to those of synthetic OH-di-K-C and Di-OH-K-C, establishing their

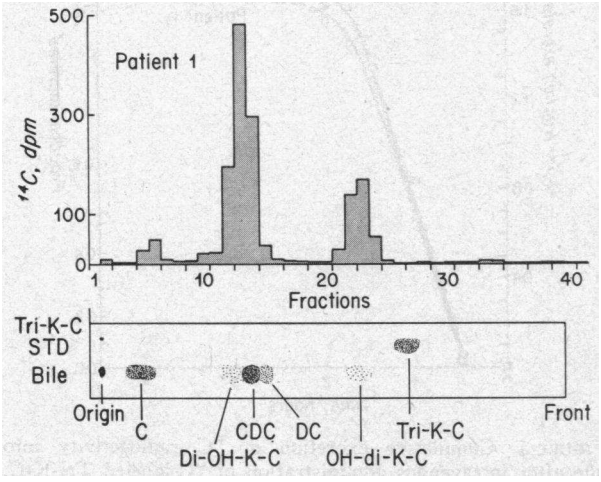

FIGURE 2 Thin-layer chromatography (below) and distribution of radioactivity by zonal scanning (above) of the methyl esters of biliary bile acids, showing the presence of three Tri-K-C metabolites and the absence of unaltered Tri-K-C. The origin is at the left.

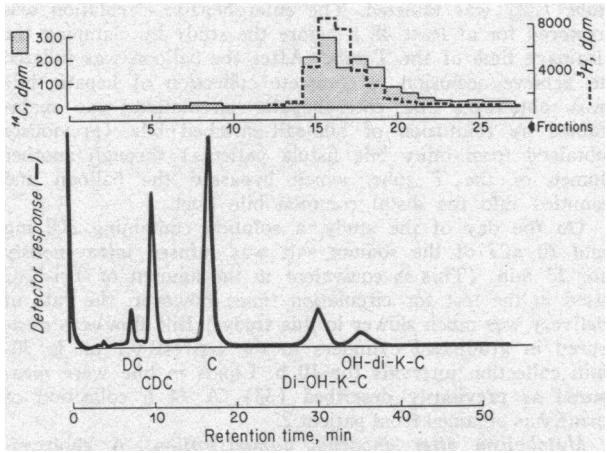

FIGURE 3 Radio-GLC of the acetates of the methyl esters of the ^{14}C-labeled biliary bile acids with the diacetate of the [^{3}H]methyl ester of synthetic Di-OH-K-C. The distribution of radioactivity shows that the [^{14}C]di-OH-K-C metabolite is identical to the ^{3}H-labeled synthetic standard.

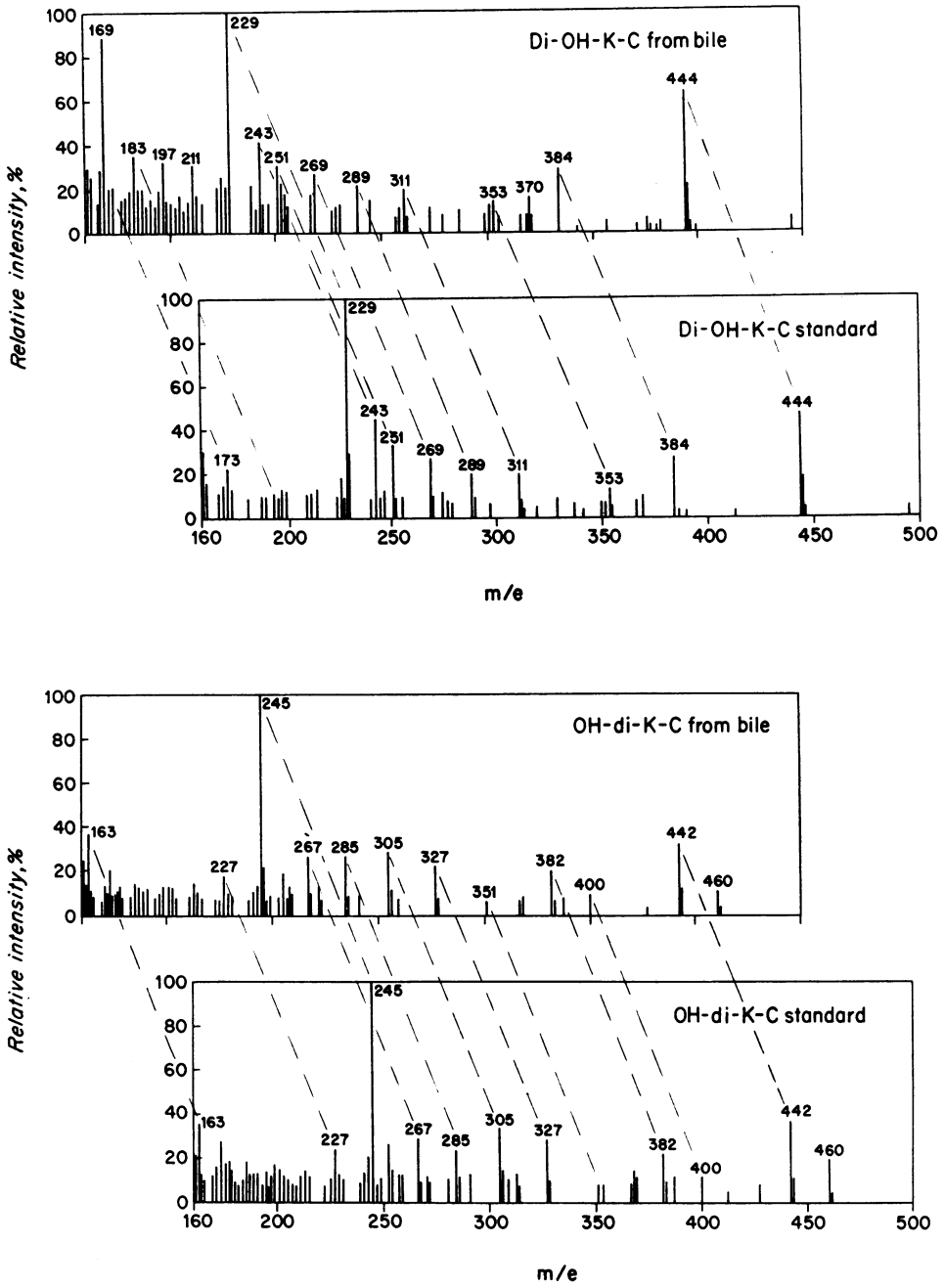

FIGURE 4 Mass spectra of acetates of the methyl esters of the free bile acids from chromatographic fractions representing the two major biliary metabolites (above) compared with mass spectra of synthetic standards (below).

chemical identity. The mass spectra agreed with those previously reported by Egger (35).

Comment. All chromatographic methods used demonstrated that, after Tri-K-C administration, the metabolites were conjugated with glycine and taurine and consisted of a dihydroxy monoketo acid, a monohydroxy diketo acid, and cholic acid (Table I; Fig. 5). Only 3 of 27 possible hydroxy keto metabolites were produced in detectable amounts.

Metabolism of Tri-K-C after intraduodenal instillation

Of the 6.03 μCi infused, 0.54 μCi was withdrawn for analysis, leaving 5.49 μCi available (91%) for intes-

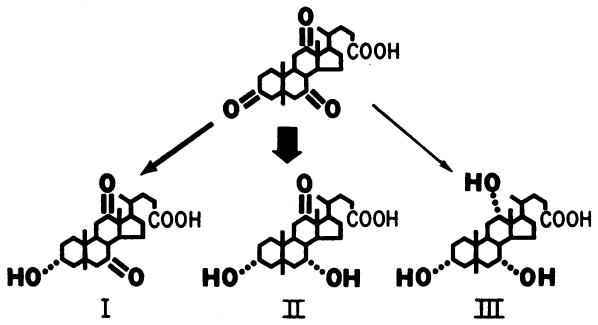

FIGURE 5 Metabolism of Tri-K-C in man (200 mg given intravenously). Tri-K-C is metabolized by the liver to OH-di-K-C (I), Di-OH-K-C (II), and cholic acid (III).

tinal absorption (maximal absorption). Since Tri-K-C, if absorbed, would be metabolized and conjugated by the liver and since Tri-K-C was present in jejunal samples predominantly in unconjugated form, all Tri-K-C present in jejunal samples was considered to represent unabsorbed Tri-K-C. From PEG recovery and TLC separation of Tri-K-C from conjugated metabolites, it could be calculated that about 2.45 μCi (41%) of infused Tri-K-C was absorbed in the 40 cm test segment, but additional absorption probably occurred distally.

Chemical form of radioactivity. Radioactivity present as conjugated bile acids appeared promptly in aspirates (within 10 min). The rate of secretion of radioactivity was highest during the 1st h, but secretion was maintained throughout the entire 3 h study. Conjugated bile acid radioactivity had the mobility of glycine conjugates or taurine conjugates and, after hydrolysis, was present predominantly as cholic acid and hydroxy keto bile acids identical to those previously identified in bile after intravenous administration of Tri-K-C (Table I). The ratio of hydroxy keto metabolites to cholic acid was similar to that observed in bile after intravenous administration of Tri-K-C. Since the total radioactivity recovered in conjugated bile acids was about 96% of that in the Tri-K-C available for absorption (infused minus aspirated), we have no proof that Tri-K-C metabolites circulated enterohepatically during the 3 h of our experiment. Only 2% of infused radioactivity was excreted in urine during the 24 h after infusion.

Hydrocholeretic effect of Tri-K-C

Bile flow correlated positively with bile acid output ($r = 0.56$, patient 1; $r = 0.71$, patient 2). However, bile flow was strikingly greater ($P < 0.01$) for samples containing Tri-K-C metabolites during periods when total bile acid concentrations were similar (Table II).

Failure of Tri-K-C to decrease biliary lipid secretion

The ratio of the bile acids plus phospholipids to cholesterol (lipid ratio) for each 30 min collection period was measured before, during, and after intravenous Tri-K-C infusion. In patient 1, the lipid ratio increased even when Tri-K-C metabolites represented 21% of biliary bile acids. In patient 2, the lipid ratio remained unchanged despite Tri-K-C metabolites increasing to 32% of the biliary bile acids (Fig. 6). Outputs of phospholipids and cholesterol were maintained or increased during these periods.

In vitro solubilization of lecithin by Tri-K-C metabolites

The monohydroxy diketo metabolite, OH-di-K-C, possessed little ability to solubilize lecithin. Although the dihydroxy monoketo metabolite, Di-OH-K-C, had somewhat greater solubilizing properties, it was strikingly inferior to cholate. With Tri-K-C metabolites, turbidity, indicating that lecithin was present in excess of its micellar solubility, was present at a lecithin-bile acid

TABLE II
*Presence of Tri-K-C Metabolites in Bile and Bile Flow**

	Patient 1		P for	Patient 2		P for
	A	B	A vs. B	A	B	A vs. B
Tri-K-C metabolites, range, %	0.9–21	0		1.5–32	0	
Number of collections	6	19		5	16	
Bile acid concentration, *mM*	45.0±3.4	44.0±1.5		26.6±2.2	30.3±0.9	
Bile acid output, *μmol/min per kg*	0.723±0.098	0.336±0.044	<0.01	0.218±0.026	0.197±0.021	<0.05
Bile flow, *μl/min per kg*	15.7±1.2	7.3±0.9	<0.01	8.1±0.7	7.1±0.3	<0.02
Bile flow/bile acid concentration, *μl/μmol*	0.363±0.023	0.165±0.019	<0.01	0.312±0.030	0.234±0.009	<0.01

* Bile flow in relation to bile acid output (last row) is compared for periods with similar bile acid concentrations (third row) when bile contained (column A) or did not contain (column B) Tri-K-C metabolites. Data are shown as mean ±SE except where indicated otherwise.

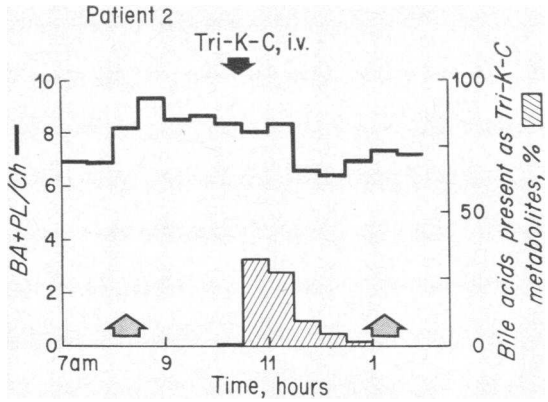

FIGURE 6 Ratio of bile acids (BA) plus phospholipids (PL) to cholesterol (Ch) before, during, and after intravenous Tri-K-C administration (30-min periods). The arrows pointing upward indicate the ingestion of breakfast and lunch.

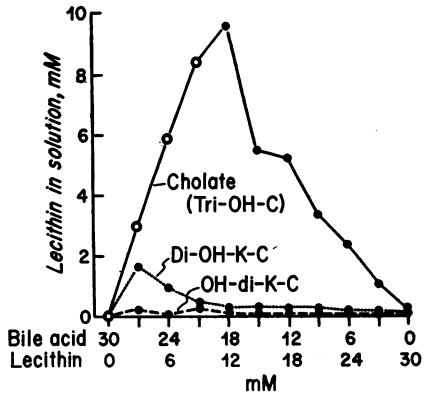

FIGURE 7 Solubility of lecithin in mixtures containing various molar ratios of bile acids and lecithin; sum of bile acids and lecithin was 30 mM in all instances. Open circles indicate visually clear (micellar) solutions. At low lecithin-bile acid ratios, lecithin was completely solubilized. At high lecithin-bile acid ratios, excess lecithin is present in liquid crystalline aggregates (11) containing an unknown amount of bile acid.

ratio of 1:9 whereas with cholate, turbidity did not occur until a ratio of 1:1.5 (Fig. 7).

In a second group of experiments, cholate was replaced stoichiometrically and stepwise by OH-di-K-C or Di-OH-K-C, and the ability of the resultant mixtures to disperse 6 mM lecithin was assessed (Fig. 8). Mixtures of Di-OH-K-C and cholate were as effective as cholate alone until more than 60% of the cholate had been replaced by the dihydroxy monoketo derivative; after that, the solubilizing properties of the bile acid mixtures decreased markedly. Mixtures with OH-di-K-C were equivalent to cholate until more than 40% of the cholate had been replaced by the Tri-K-C metabolite. Replacement ability was inversely proportional to the number of keto groups in the Tri-K-C metabolite.

DISCUSSION

Biotransformations. The nearly complete recovery of radioactivity in only three metabolites, all possessing one or more α-hydroxy constituents, suggests that Tri-K-C was sequentially and stereospecifically reduced. It seems likely, based on animal models (16), that the liver was the site of the observed biotransformations. It is possible that the pattern of metabolites observed in our studies is dose-related, and our findings are not necessarily in conflict with those of Ellin, Mendeloff, and Turner (36) who detected Tri-K-C in bile, apparently in unconjugated form, after 1 g of Tri-K-C was given intravenously. Since the intestinal absorption and hepatic metabolism of the two metabolites has not been characterized and since no information is available on bacterial metabolism of the metabolites or on the intestinal absorption and hepatic metabolism of the products resulting from bacterial biotransformations, our data cannot be used to predict the effect of continuous ingestion of Tri-K-C on biliary bile acid composition.

Our data are in general agreement with qualitative observations on the hepatic metabolism of Tri-K-C in rats (16), rabbits (37, 38), guinea pigs (39), and dogs (40). Bile acids possessing a keto group at the 3 position have been shown to be reduced by rat liver in vitro and in vivo (41–45). There is limited evidence suggesting that the 7-keto group of bile acids is reduced during hepatic passage (46), and recently evidence for hepatic reduction of the 12-keto group of Di-OH-K-C has been reported (47).

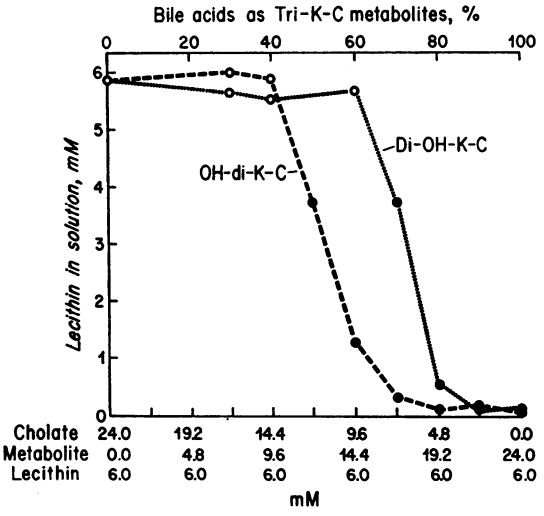

FIGURE 8 Solubilization of lecithin (6 mM total) by bile acid mixture of various compositions. Cholate was replaced stoichiometrically by either of the two major Tri-K-C metabolites. Open circles indicate visually clear (micellar) solutions.

The enzyme system mediating a reduction of the 3-keto group of bile acids in man has not been characterized, although information on a steroid 3-hydroxy dehydrogenase present in rat liver has been published (45); the enzyme described in these studies may or may not be the same enzyme involved in reduction of the 3-keto group of bile acids. To our knowledge, there has been no attempt to characterize the enzyme which reduces the 7-keto group, but a 12-keto reduction has been shown to be present in rat liver microsomes (47). In our experiments, reduction appeared to be sequential. Reduction at the 3 position occurred first and the rate was significantly greater than at the 7 position where it occurred second and in turn was greater than at the 12 position (ratio of rates at 3, 7, and 12 positions = 17: 10:1); no quantitative interpretation of these data is possible.

The duodenal infusion experiment indicates that Tri-K-C is absorbed from the proximal small intestine, and that small intestinal absorption of Tri-K-C, before hepatic passage, does not substantially alter the pattern of metabolites appearing in bile. The most reasonable explanation of this finding is that Tri-K-C was not reduced during passage through the mucosal cell, although intestinal reduction similar to that occurring in the liver cannot be excluded.

Tri-K-C conjugated with glycine is not metabolized during passage through the rabbit liver (48). Thus, in this species, conjugation appears to render the keto groups of certain bile acids inaccessible to hepatic reductases.

The biologic significance of reduction of keto groups on bile acids is unclear. In man, keto bile acids have been identified in stool (49) but are rare in bile (50, 51). Although it has been assumed that absorption of keto bile acids from the colon is negligible, perhaps the magnitude of such absorption may be greater than previously suspected. Parenthetically, it should be noted that 7-keto acids compose a significant fraction of bile in a number of mammals (52).

Tri-K-C choleresis. Our work confirms a large body of experimental evidence in animals and limited evidence in man that Tri-K-C or its metabolites increase bile flow more than the usual biliary bile acids—that is, cause a hydrocholeresis. This hydrocholeretic effect cannot be explained by osmosis, even if the metabolites were not participating in micelle formation, because the increased flow occurred at a time when the Tri-K-C metabolites accounted for a small proportion of the bile salts in bile. Forker (53) showed that Tri-K-C infusion increased biliary clearance of mannitol and erythritol in the guinea pig but that secretin did not; his data suggest that Tri-K-C increases canalicular bile production. By definition, the hydrocholeresis induced by the Tri-K-C metabolites represents the "bile salt dependent fraction" of bile flow but it seems more reasonable to assign the increment in water flow to a "secretory" fraction as distinguished from an "osmotic" fraction.

Bargeton, Salesse, Barber, and Delavierre (54) made a systematic comparison of the choleretic potency of a number of tri-substituted hydroxy keto bile acids given intravenously to anesthetized rabbits. Their results are difficult to interpret since hepatic metabolism was not defined, but they did show that Di-OH-K-C had a choleretic potency identical to that of Tri-K-C, and that OH-di-K-C was twice as potent. These experiments indicate that hydrocholeresis can be induced by metabolites and that the presence of an intact Tri-K-C molecule is not essential.

Our experiments shed no light on the mechanism of hydrocholeresis induced by Tri-K-C. Recent work has shown that dihydroxy bile acids, but not trihydroxy bile acids, induce water secretion by the human colon (55) and jejunum (56), and the action of Tri-K-C metabolites on bile flow could be related to this poorly understood secretory property of bile acids. The dihydroxy bile acids activate the Na^+-activated ATPase of intestinal mucosa, but OH-di-K-C is the most potent activator of this brush border enzyme observed to date (57). If OH-di-K-C is shown to have a potent secretory effect in a variety of tissues, detailed studies of the pharmacology of this compound would appear indicated.

Tri-K-C excretion and bile acid-phospholipid coupling. The seeming discrepancy between our results, which indicate no change in bile salt-phospholipid coupling after Tri-K-C administration, and those of Hardison (16), Wheeler and King (8), and Young and Hanson (48), which indicate that Tri-K-C infusions decrease phospholipid and cholesterol output, was clarified by the in vitro solubility studies which showed that the ratio of Tri-K-C metabolites to cholate determines the degree of lecithin solubilization. In those studies, Tri-K-C metabolites composed the majority of bile acids in bile, precluding efficient micellar solubilization of lecithin. In our study, the enterohepatic circulation was intact, with the result that the amount of Tri-K-C infused was small relative to that of circulating bile acids. Lipid output was not depressed because the metabolites acted cooperatively with endogenous di- and trihydroxy bile acids to solubilize lecithin.

Structure-function relationships. These studies point out the striking effects caused by alteration in the ring substituents of bile acids and complement previous studies indicating the importance of the steric configuration of the A/B ring juncture (58–60). The special properties of Tri-K-C and its metabolites appear to be caused by the presence of a keto group rather than the absence of a hydroxyl group.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Mrs. Judith Ann Duenes and Miss Patricia Szezepanik, as well as that of Miss Janet A. Carter and Mrs. Linda Moskalik, and aid in patient studies from Richard Tucker and Miss Lee E. Fast, R. N.

Investigation at Mayo Clinic was supported in part by Research Grant AM-6908, Training Grant T1-AM-5259, and Clinical Research Center Grant RR 585 from the National Institutes of Health, U. S. Public Health Service; a grant-in-aid from the Mead Johnson Laboratories; and work at Argonne National Laboratories was supported by the U. S. Atomic Energy Commission.

REFERENCES

1. Soloway, R. D., A. F. Hofmann, P. J. Thomas, L. J. Schoenfield, and P. D. Klein. 1971. Dehydrocholic acid: hepatic metabolism and effect on biliary secretion in man. *Gastroenterology.* **60**: 754. (Abstr.)
2. Hammarsten, O. 1881. Ueber Dehydrocholalsäure, ein neues Oxydationsprodukt der Cholalsäure: Vorläufige Mittheilung. *Ber. Deut. Chem. Ges.* **14**: 71.
3. Neubauer, E. 1924. Über die cholagoge Wirkung der Dehydrocholsäure beim Menschen. *Klin. Wochenschr.* **3**: 883.
4. Winternitz, M., J. Deutsch, and Z. Brüll. 1931. Eine klinisch brauchbare Bestimmungsmethode der Blutumlaufszeit mittels Decholininjektion: Kurze Mitteilung. *Med. Klin.* **27**: 986.
5. Nilsson, S., and T. Scherstén. 1969. Importance of bile acids for phospholipid secretion into human hepatic bile. *Gastroenterology.* **57**: 525.
6. Swell, L., C. C. Bell, Jr., and C. Entenman. 1968. Bile acids and lipid metabolism. III. Influence of bile acids on phospholipids in liver and bile of the isolated perfused dog liver. *Biochim. Biophys. Acta.* **164**: 278.
7. Dowling, R. H., E. Mack, and D. M. Small. 1970. Effects of controlled interruption of the enterohepatic circulation of bile salts by biliary diversion and by ileal resection of bile salt secretion, synthesis, and pool size in the rhesus monkey. *J. Clin. Invest.* **49**: 232.
8. Wheeler, H. O., and K. K. King. 1972. Biliary excretion of lecithin and cholesterol in the dog. *J. Clin. Invest.* **51**:1337.
9. Entenman, C., R. J. Holloway, M. L. Albright, and G. F. Leong. 1968. Bile acids and lipid metabolism. I. Stimulation of bile lipid excretion by various bile acids. *Proc. Soc. Exp. Biol. Med.* **127**: 1003.
10. Hofmann, A. F., and D. M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. *Annu. Rev. Med.* **18**: 333.
11. Small, D. M. 1971. The physical chemistry of cholanic acids. *In* The Bile Acids: Chemistry, Physiology, and Metabolism. Vol. 1: Chemistry. P. P. Nair and D. Kritchevsky, editors. Plenum Publishing Corporation, New York. 249.
12. Small, D. M., S. A. Penkett, and D. Chapman. 1969. Studies on simple and mixed bile salt micelles by nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta.* **176**: 178.
13. Hardison, W. G. M., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Am. J. Physiol.* **222**: 61.
14. Ekwall, P. 1951. Association colloids: surface and colloid chemical studies. *Sven. Kem Tidskr.* **63**: 277.
15. Fontell, K. 1971. Micellar behaviour in solutions of bile-acid salts. I. Vapor pressure of the aqueous solutions and the osmotic activity of the bile-acid salts. *Kolloid-Z. Z. Polym.* **244**: 246.
16. Hardison, W. G. 1971. Metabolism of sodium dehydrocholate by the rat liver: its effect on micelle formation in bile. *J. Lab. Clin. Med.* **77**: 811.
17. Grodins, F. S., A. L. Berman, and A. C. Ivy. 1941. Observations on the toxicities and choleretic activities of certain bile salts *J. Lab. Clin. Med.* **27**: 181.
18. Wheeler, H. O. 1968. Water and electrolytes in bile. *Handb. Physiol.* **5**: 2409.
19. Hofmann, A. F. 1965. Clinical implications of physicochemical studies on bile salts. *Gastroenterology.* **48**: 484.
20. Sato, Y., and N. Ikekawa. 1959. Preparation of chenodeoxycholic acid. *J. Org. Chem.* **24**: 1367
21. Hofmann, A. F. 1964. Thin-layer chromatography of bile acids and their derivatives. *In* New Biochemical Separations. A. T. James and L. J. Morris, editors. Van Nostrand Reinhold Company, New York. 261.
22. Snyder, F. 1965. Zonal scanning of thin-layer chromatograms. *Adv. Tracer Methodol.* **2**: 107.
23. Hofmann, A. F., and J. R. Poley. 1972. Role of bile acid malabsorption in pathogenesis of diarrhea and steatorrhea in patients with ileal resection. I. Response to cholestyramine or replacement of dietary long chain triglyceride by medium chain triglyceride. *Gastroenterology.* **62**: 918.
24. Hofmann, A. F. 1963. The preparation of chenodeoxycholic acid and its glycine and taurine conjugates. *Acta Chem. Scand.* **17**: 173.
25. Fieser, L. F., and S. Rajagopalan. 1950. Oxidation of steroids. III. Selective oxidations and acylations in the bile acid series. *J. Am. Chem. Soc.* **72**: 5530.
26. Nair, P. P. 1969. Enzymatic cleavage of bile acid conjugates. *In* Bile Salt Metabolism. L. Schiff, J. B. Carey, Jr., and J. Dietschy, editors. Charles C Thomas, Publisher, Springfield, Ill. 172.
27. Roovers, J., E. Evrard, and H. Vanderhaeghe. 1968. An improved method for measuring human blood bile acids. *Clin. Chim. Acta.* **19**: 449.
28. Thomas, P. J., and H. J. Dutton. 1969. Improved resolution in high-sensitivity dual-label gas radiochromatography. *Anal. Chem.* **41**: 657.
29. Klein, P. D., J. R. Haumann, and W. J. Eisler. 1972. Gas chromatograph-mass spectrometer-accelerating voltage alternator system for the measurement of stable isotope ratios in organic molecules. *Anal. Chem.* **44**: 490.
30. Hofmann, A. F. 1968. Molecular association in fat digestion: interaction in bulk of monoolein, oleic acid, and sodium oleate with dilute, micellar bile salt solutions. *Adv. Chem. Ser.* **84**: 53.
31. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375.
32. Soloway, R. D., H. C. Carlson, and L J. Schoenfield. 1972. A balloon-occludable T-tube for cholangiography and quantitative collection and reinfusion of bile in man. *J. Lab. Clin. Med.* **79**: 500.
33. Thistle, J. L., and L. J. Schoenfield. 1971. Induced alterations in composition of bile of persons having cholelithiasis. *Gastroenterology.* **61**: 488.
34. Go, V. L. W., A. F. Hofmann, and W. H. J. Summerskill. 1970. Pancreozymin bioassay in man based on pancreatic enzyme secretion: potency of specific amino acids and other digestive products. *J. Clin. Invest.* **49**: 1558.

35. Egger, H. 1968. Zur Massenspektrometrie von Gallensäurederivaten. *Montsh. Chem.* **99**: 1163.
36. Ellin, R. I., A. I. Mendeloff, and D. A. Turner. 1962. Quantitative determination of 3,7,12-triketocholanic acid in biological fluids by gas-liquid chromatography. *Anal. Biochem.* **4**: 198.
37. Ogura, M., T. Wakutani, and K. Yamasaki. 1960. The fate of ^{14}C-dehydrocholate administered to the bile-fistula rabbits. *Yonago Acta Med.* **4**: 179.
38. Erlinger, S., M. Dumont, P. Berthelot, and D. Dhumeaux. 1971. Comparison of the choleretic effects of dehydrocholate and glycodeoxycholate in the rabbit. *Digestion.* **4**: 144. (Abstr.)
39. Ogura, M., and K. Yamasaki. 1961. The fate of C^{14}-labeled dehydrocholic acid in guinea pig. *J. Biochem. (Tokyo).* **49**: 649.
40. Desjeux, J.-F., S. Erlinger, and M. Dumont. 1971. Metabolism and choleretic effect of dehydrocholate in the dog. *Gut.* **12**: 757. (Abstr.)
41. Kallner, A. 1967. On the reduction of 3-keto bile acids *in vitro*. *Ark. Kem.* **26**: 553.
42. Kallner, A. 1967. On the biosynthesis and metabolism of allodeoxycholic acid in the rat. Bile acids and steroids 175. *Acta Chem. Scand.* **21**: 315.
43. Ogura, M., and K. Yamasaki. 1967. Non-stereospecific reduction of 3-oxochol-4-enoic-[24-^{14}C] acid in the rat. *Steroids.* **9**: 607.
44. Björkhem, I., and J. Gustafsson. 1971. On the conversion of cholestanol into allocholic acid in rat liver. *Eur. J. Biochem.* **18**: 207.
45. Berséus, O. 1967. Conversion of cholesterol to bile acids in rat: purification and properties of a Δ^4-3-ketosteroid 5β-reductase and a 3α-hydroxysteroid dehydrogenase. Bile acids and steroids 187. *Eur. J. Biochem.* **2**: 493.
46. Norman, A., and J. Sjövall. 1958. On the transformation and enterohepatic circulation of cholic acid in the rat. Bile acids and steroids 68. *J. Biol. Chem.* **233**: 872.
47. Kikuchi, H. 1972. The metabolic sequence for the occurrence of an anomalous bile acid, 12-ketochenodeoxycholic acid, found in the bile of hepatobiliary diseased patients. *J. Biochem. (Tokyo).* **72**: 165.
48. Young, D. L., and K. C. Hanson. 1972. Effect of bile salts on hepatic phosphatidylcholine synthesis and transport into rat bile. *J. Lipid Res.* **13**: 244.
49. Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall. 1966. Identification of mono- and dihydroxy bile acids in human feces by gas-liquid chromatography and mass spectrometry. *J. Lipid Res.* **7**: 511.
50. Sjövall, J. 1960. Bile acids in man under normal and pathological conditions. Bile acids and steroids 73. *Clin. Chim. Acta.* **5**: 33.
51. Nakayama, F. 1967. Quantitative microanalysis of bile. *J. Lab. Clin. Med.* **69**: 594.
52. Matschiner, J. T. 1971. Naturally occurring bile acids and alcohols and their origins. *In* The Bile Acids: Chemistry, Physiology, and Metabolism. Vol. 1: Chemistry. P. P. Nair and D. Kritchevsky, editors. Plenum Publishing Corporation, New York. 11.
53. Forker, E. L. 1966. Two sites of bile formation in the biliary tree of the guinea pig. *J. Lab. Clin. Med.* **68**: 875. (Abstr.)
54. Bargeton, D., J. Salesse, J. Barber, and C. Delavierre. 1952. Influence du nombre et de la position des fonctions hydroxylées et cétoniques sur l'ativité cholérétique et la toxicité de quelques acides biliares. *Arch. Int. Pharmacodyn. Ther.* **90**: 18.
55. Mekhjian, H. S., S. F. Phillips, and A. F. Hofmann. 1971. Colonic secretion of water and electrolytes induced by bile acids: perfusion studies in man. *J. Clin. Invest.* **50**: 1569.
56. Wingate, D. L., S. F. Phillips, and A. F. Hofmann. 1973. Effect of glycine-conjugated bile acids with and without lecithin on water and glucose absorption in perfused human jejunum. *J. Clin. Invest.* In press.
57. Hepner, G. W., and A. F. Hofmann. 1973. Different effects of free and conjugated bile acids and their keto derivatives on (Na^+,K^+)-stimulated and Mg^{2+}-ATPase of rat intestinal mucosa. *Biochim. Biophys. Acta.* **291**: 237.
58. Hofmann, A. F., and E. H. Mosbach. 1964. Identification of allodeoxycholic acid as the major component of gallstones induced in the rabbit by 5α-cholestan-3β-ol. *J. Biol. Chem.* **239**: 2813.
59. Hofmann, A. F., V. Bokkenheuser, R. L. Hirsch, and E. H. Mosbach. 1968. Experimental cholelithiasis in the rabbit induced by cholestanol feeding: effect of neomycin treatment on bile composition and gallstone formation. *J. Lipid Res.* **9**: 244.
60. Hofmann, A. F. 1969. Relationship between the molecular structure of bile salts and their physiologic functions. *In* Bile Salt Metabolism. L. Schiff, J. B. Carey, Jr., and J. Dietschy, editors. Charles C Thomas, Publisher, Springfield, Ill. 160.