Bile Acid Synthesis in the Isolated, Perfused Rabbit Liver

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ABSTRACT These experiments were carried out to demonstrate the usefulness of the perfused rabbit liver for studies of bile acid metabolism, and to determine the rate-limiting enzyme of bile acid synthesis. Rabbits were fed a semisynthetic diet, with or without the addition of 1% cholestyramine, under controlled conditions. At the end of 2–5 wk, the livers were removed and perfused for 2.5 hr employing various ^{14}C-labeled precursors to measure *de novo* cholic acid synthesis. The livers were then analyzed for cholesterol, and the bile collected during the perfusion was analyzed for cholesterol and bile acids. Control bile contained, on the average, 0.34 mg of glycocholate, 7.4 mg of glycodeoxycholate, and 0.06 mg of cholesterol. After cholestyramine treatment of the donor rabbits, the bile contained 3.3 mg of glycocholate, 3.7 mg of glycodeoxycholate, and 0.05 mg of cholesterol. It was assumed that in cholestyramine-treated animals the enterohepatic circulation of the bile acids had been interrupted sufficiently to release the feedback inhibition of the rate-controlling enzyme of bile acid synthesis. Therefore, a given precursor should be incorporated into bile acids at a more rapid rate in livers of cholestyramine-treated animals, provided that the precursor was acted upon by the rate-controlling enzyme. It was found that the incorporation of acetate-^{14}C, mevalonolactone-^{14}C, and cholesterol-^{14}C into cholate was 5–20 times greater in the livers of cholestyramine-treated animals than in the controls. In contrast, there was no difference in the incorporation of 7α-hydroxycholesterol-^{14}C into cholate regardless of dietary pretreatment. It was concluded that given an adequate precursor pool, the 7α-hydroxylation of cholesterol is the rate-limiting step in bile acid formation.

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INTRODUCTION

The use of perfused livers in studies of biliary sterol and bile acid metabolism can be disappointing (1) because the bile obtained from livers of fasted animals, or from those fed ad lib., contains mainly "old" bile salt (glycodeoxycholic acid in the rabbit) which had returned to the liver via the enterohepatic circulation and was being "washed out" during the perfusion. Newly-synthesized bile salt (glycocholate in the rabbit) may be present in trace amounts only, or be absent altogether and thus such livers obtained from these animals are not very useful for studies of *de novo* bile acid formation. In contrast, the livers of rabbits maintained for 2 wk or longer on a diet containing 1% cholestyramine (a bile acid sequestering anion exchange resin) and maintained on an appropriate feeding schedule, should show active glycocholate synthesis during the perfusion, since bile acid production is stimulated by the action of the cholestyramine, which partially interrupts the enterohepatic circulation of the bile acids (2, 3).

It seems safe to assume that the livers of control rabbits (not pretreated with cholestyramine) exhibit the same low rates of bile acid synthesis usually observed in animals with intact enterohepatic circulation ("inhibited livers") (4, 5). Such livers then act as controls for comparison with preparations from cholestyramine-treated rabbits in which the enterohepatic circulation has been interrupted, resulting in high rates of bile acid synthesis ("noninhibited livers").

The pathway of bile acid biosynthesis from acetate may be represented in a greatly abbreviated form as follows:

Acetate → HMG-CoA → mevalonate → cholesterol → 7α-hydroxycholesterol → cholic acid.

If a given substrate, such as acetate, has to funnel through the rate-determining enzyme, the "noninhibited" livers will exhibit a faster incorporation rate of precursor to bile acid than the "inhibited" livers.

This concept is illustrated in Fig. 1, where precursor 1 is acted upon by a rate-limiting enzyme while precursor 2 is not. If the rate-limiting enzyme is not inhibited, it operates at an increased velocity, and precursors 1 and 2 will be converted to bile acids at similar rapid rates. This is the case in cholestyramine-fed rabbits where the amount of bile salts reaching the liver is reduced. In contrast, in livers from normal rabbits with intact enterohepatic circulation, the rate-limiting enzyme is inhibited. As a result, the rate of incorporation of precursor 1 into cholic acid will be a rate which is dependent upon the presence of the circulating bile-acid pool (4) while the rate of incorporation of precursor 2 will not be affected.

To determine the rate-limiting step, studies were done using acetate-1-^{14}C, mevalonate-2-^{14}C, cholesterol-4-^{14}C, and 7α-hydroxycholesterol-4-^{14}C as precursors. The bile samples from each experiment were assayed for *de novo* glycocholate synthesis in the manner described.

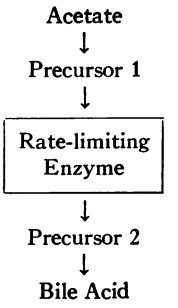

FIGURE 1 Channeling of bile acid precursors through the rate-limiting enzyme.

METHODS

Experimental animals. Male rabbits (New Zealand white), weighing between 1 and 2 kg, were maintained for 14–25 days on a pelleted, semisynthetic diet (General Biochemicals, 170920). The experimental animals received the same pellets containing cholestyramine (1% by weight).

Both groups were placed on a feeding schedule: food was removed from 9 a.m. to 5 p.m.; food was presented from 5 p.m. to 9 a.m.; water was available ad lib. The animals were operated upon between 10 and 11 a.m.; the perfusion was done immediately thereafter.

Liver perfusion. The technique for the rabbit liver perfusion has been described in detail (6). Briefly, under light ether anesthesia, the portal vein was cannulated and perfusion started immediately from a reservoir. The bile duct and inferior vena cava were cannulated, the inferior vena cava was ligated above the diaphragm, and the liver removed. The gall bladder was ligated at its neck and the liver removed and mounted on a platform in a 37°C humidified box. The outflow from the inferior vena cava was directed into a disc oxygenator and pumped back into the portal vein at a rate of 0.8–1.4 ml/g per min. The perfusate consisted of 2/3 rabbit blood with Krebs-Henseleit buffer containing 2–3 μmoles of amino acid per 100 ml. Bile was collected from the cannulated bile duct at 30- or 60-min intervals. Tracer amounts of precursors were injected directly into the inflow tube connected to the portal vein. The perfusion was continued for 2–6 hr. At the end of the perfusion period, a portion of liver tissues (2–5 g) was frozen for storage and subsequent sterol analysis. Bile samples were stored at 4°C before analysis.

Due to the large size of rabbit liver (30–70 g), the volume of perfusate pumped into the portal vein is high and characterization of hepatic function is necessary. The following criteria were used: (*a*) bile flow, (*b*) urea production, (*c*) histological examination, (*d*) O_2 consumption, and (*e*) lactate and pyruvate levels. In a given liver, bile flow remains constant during the perfusion, but different livers may vary from 1 to 3 ml/hr in bile volume.

Labeled compounds. Sodium acetate-1-^{14}C, D,L-mevalonolactone-2-^{14}C, and cholesterol-4-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. 7α-hydroxycholesterol-4-^{14}C was prepared by modifying the synthesis of Fieser, Herz, Klohs, Romero, and Utne (7) to a semimicro scale. The acetate and mevalonate were dissolved in isotonic saline for either pulse or infusion labeling during the liver perfusion. The water-insoluble precursors, cholesterol, and 7α-hydroxycholesterol were "solubilized" in saline with Tween 20 (8).

Synthesis of 7α-hydroxycholesterol-4-^{14}C. 0.1 mCi (0.365 mg) of cholesterol-4-^{14}C (New England Nuclear Corp.) was added to 15.0 mg of unlabeled cholesterol and the mixture was refluxed with 2.5 ml of acetic anhydride for 1 hr. After cooling, 5 ml of water and 2.5 ml of methanol were added, and the mixture was extracted three times with 12 ml of *n*-hexane. The pooled hexane extracts were washed once with saturated $NaHCO_3$ solution, three times with water, and then dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness at 50°C under nitrogen gas. The residue was dissolved in 3 ml of acetic acid (glacial) at 50°C, and 19 mg of CrO_3 were added slowly with constant stirring. After all of the CrO_3 was added, stirring was continued for 30 min. 5 ml of water and 3.5 ml of methanol were added, and the reaction mixture was extracted three times with 10 ml of *n*-hexane. The hexane was washed with saturated $NaHCO_3$ solution, then with water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness at 40°C under nitrogen gas. The product was purified by preparative TLC[1] on Silica Gel G (Brinkmann Instruments, Westbury, N. Y.) using ethyl acetate: benzene, 1:3 (v/v), as solvent system (7-ketocholesterol acetate: R_f 0.75). The 7-ketocholesterol acetate band was scraped off the plate, the acetate eluted with chloroform, and the solvent evaporated to dryness at room temperature under nitrogen gas. The 7-ketocholesterol acetate was suspended in 6 ml of methanol at 14°C, and $NaBH_4$ (2 moles/mole of ketone) was added slowly with stirring until the reaction mixture was clear. The reaction mixture was allowed to stand at room temperature overnight. This mixture contained both 7α- and 7β-hydroxycholesterol-4-^{14}C acetates. These products were extracted with two portions of *n*-hexane: benzene (v/v), after the addition of an equal volume of water (6 ml). The extracts were pooled, washed with water, dried

[1] *Abbreviations used in this paper:* GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

over Na_2SO_4, filtered, and evaporated to dryness at room temperature under nitrogen gas.

The 7α- and 7β-hydroxycholesterol acetates were separated by preparative TLC using Silica Gel G plates and ethyl acetate: benzene, 1:4 (v/v) as solvent system. The compounds were made visible by spraying the edges of the developed plate with 50% H_2SO_4. A blue color is exhibited by the 7α-hydroxycholesterol acetate (R_f 0.13), and a green-blue color by the 7β-compound (R_f 0.64). The band containing the 7α-compound was scraped off the plate and eluted with chloroform, and the solvent was evaporated at room temperature under nitrogen gas. 7α-Hydroxycholesterol-4-^{14}C was obtained by hydrolyzing the acetate for 30 min at 37°C in alcoholic KOH (6% KOH in 95% ethanol), adding an equal volume of water, extracting three times with *n*-hexane: benzene, 1:1 (v/v), washing the extracts with water, drying over Na_2SO_4, filtering, and evaporating at room temperature under nitrogen gas. Purity was determined by TLC (single spot after spraying with 50% H_2SO_4), and by monitoring the distribution of ^{14}C counts on an unsprayed plate. The synthesis yielded 0.44 mg of 7α-hydroxycholesterol-4-^{14}C (29×10^6 dpm) with a purity of 98–99%. The yield based on radioactivity was 11%. The yield based on weight of starting material was lower, due to the difficulty of obtaining an accurate weight of the final product.

Analytical methods. A portion of bile from each sample was deproteinized by the addition of 20 volumes of hot methanol. The tubes were cooled and centrifuged for 5 min, and the methanol was decanted from the protein precipitate and evaporated to dryness at 60° under air. The residues, containing the glycocholanoic acids, were hydrolyzed by autoclaving for 3 hr at 15 psi, 120°C, in 2 ml of water containing 10% KOH. The tubes were placed in ice and to each was added 1 ml methanol, 2 ml 6N HCl, and 5 ml H_2O. The free cholanoic acids were then extracted twice with 5 ml of fresh ethyl ether (9). A portion of the free cholanoic acids was subjected to TLC and the remainder was dissolved in 1 ml of methanol, 1 drop of conc. H_2SO_4 was added, and esterification was allowed to proceed at room temperature overnight. The methyl esters of the bile acids were extracted with two volumes of an ethyl ether: benzene mixture (2:3, v/v) (10), their radioactivity was determined, and portions were taken for quantitation by GLC.

Gas-liquid chromatography (GLC). GLC was used to determine the concentrations of biliary cholesterol, chenodeoxycholic acid, cholic acid, and deoxycholic acid. The instrument used was a Barber-Colman Selecta 5000 gas chromatograph (division of Nuclear Chicago Corp.) with hydrogen flame detectors and disc integrators. The methyl ester mixture was dissolved in chloroform and injected into a 180 cm × 4 mm glass column packed with 3% QF-1 on 80–100 mesh gas-chrom Q (Applied Science Laboratories Inc.). The column temperature was 262°C. The bile acids were quantitated by comparison with standards of known concentrations which were measured with each series of bile samples worked up.

Thin-layer chromatography (TLC). The individual bile acids were separated on TLC as free acids, and the spots were removed from the plate for radioactivity assay. A suitable portion from each sample was spotted on Silica Gel G (Brinkmann Instruments, Inc.) and developed in acetic acid: isopropyl ether: isooctane [1:1:2] (11) as solvent system, which gave satisfactory separation of the following constituents: cholic acid (R_f 0.18), chenodeoxycholic acid (R_f 0.41), deoxycholic acid (R_f 0.49), and cholesterol (R_f 0.79).

The bile acid spots were removed with suction (12) and transferred to scintillation vials. The bile acids were eluted from the silica gel by adding 4 ml of ethanol (absolute) and heating the closed vial for 30 min at 60°C. The radioactivity of the samples was determined after the addition of 11 ml of 2,5-bis[2-(5-*tert*-butyl benzoxazolyl)]-thiophene solution (4 g/liter in toluene) in a liquid scintillation counter (Beckman LS 200 B) and corrected for background and quenching.

Hepatic cholesterol determination. 2 g of liver were hydrolyzed in 25 ml of 95% ethanol containing 25% KOH by refluxing for 3 hr. Hepatic cholesterol was determined as the digitonide (13). Radioactivity of the digitonide was determined by counting the precipitate on the filter paper in a Nuclear-Chicago low background planchet counter (Nuclear-Chicago Corp.), corrections being made for background and self-absorption.

Radiochemical purity of the biosynthesized bile acids. Biles from livers perfused with labeled precursors were pooled, deproteinized, hydrolyzed, and the extracted free cholanoic acids were converted to their methyl esters. Cholic acid was isolated by preparative TLC, eluted with chloroform, and the solvent evaporated. To the residue containing the biosynthetic bile acid, 100 mg of authentic, unlabeled methyl cholate was added, and the mixture was recrystallized three times, yielding crystals of methyl cholate with a constant specific activity within the precision of measurement (Table I). Free cholate and chenodeoxycholate were isolated from livers perfused with 7α-hydrocholesterol-4-^{14}C in like manner. Chenodeoxycholate was crystallized as the free acid, and cholic acid as the methyl ester (Table I). Again, the specific radioactivities remained constant during repeated recrystallizations.

RESULTS

It was first necessary to establish that the isolated, perfused rabbit liver was suitable for studies of bile acid biosynthesis. The experiments summarized in Table II show that in bile collected from the livers of fasted animals, glycodeoxycholate predominated, but some glycocholate was also present. When sodium acetate-1-^{14}C was added to the perfusion fluid it was not incorporated into biliary bile acids to a measurable extent. This suggests that bile secreted by such livers contained largely "old" bile salts which had returned to the liver via the enterohepatic circulation and were being "washed out" during the perfusion. In contrast, the livers of rabbits maintained on the stock diet, or stock diet plus 1% cholestyramine, and on a controlled feeding schedule incorporated acetate-1-^{14}C into biliary glycocholate (see Table IV). Therefore, if it is assumed that the incorporation of sodium acetate-1-^{14}C is a measure of *de novo* bile acid synthesis some of this glycocholate must have been synthesized during the perfusion period. It is apparent from Table IV that glycocholate was synthesized by the livers obtained both from the nonfasted controls and from cholestyramine-treated rabbits; however, synthesis was much greater when the liver donors had been pretreated with cholestyramine.

TABLE I

Confirmation of Identity of Biosynthetic Bile Acids by Reverse Isotope Dilution

Substance	Source	Recrystallization	Solvent	Weight	Radioactivity	Specific radioactivity
				mg	*dpm*	*dpm*
Methyl cholate	Pooled from experiments with acetate-1-^{14}C, mevalonate-2-^{14}C and cholesterol-4-^{14}C	1	Methanol-H_2O	104.5	152,200	1456
		2	Methanol-H_2O	70.4	104,200	1480
		3	Ethyl acetate-n-hexane	49.1	76,600	1560
Methyl cholate	Pooled from experiments with 7α-hydroxy-cholesterol-4-^{14}C	1	Methanol-H_2O	62.6	32,500	519
		2	Methanol-H_2O	54.0	27,100	502
		3	Ethyl acetate-n-hexane	36.8	18,700	508
Chenodeoxycholic acid	Pooled from experiments with 7α-hydroxy-cholesterol-4-^{14}C	1	Ethyl acetate-n-hexane	30.6	7,700	252
		2	Methanol-H_2O	6.8	1,800	265

Although most perfusions were carried out for 2½ hr, it was found that bile secretion and glycocholate synthesis proceeded for periods of 6 hr or more. An example is shown in Table III which gives bile volume, biliary cholesterol, biliary glycocholate and glycodeoxycholate, and ^{14}C incorporation for this long term experiment, indicating that the preparation continues to secrete bile and bile acid at a relatively constant rate for more than twice the standard perfusion period.

The data summarized in Table III also illustrate certain limitations of the perfused rabbit liver in studies of bile acid synthesis. Thus, the specific radioactivities of biliary cholesterol were higher than those of biliary glycocholate throughout most of the experiment. This can be ascribed to a number of factors; an isotopic steady state may not have been achieved during the perfusion period; biliary cholesterol and biliary glycocholate may originate from different hepatic pools; or biliary glycocholate was diluted with preexisting unlabeled glycocholate which was washed out of the liver during the perfusion.

As can be seen in Table II a total of 31 rabbits was studied. In addition to 10 rabbits maintained on a controlled feeding schedule on the stock diet (group B), and 15 rabbits on a controlled feeding schedule with 1% cholestyramine incorporated into the stock diet (group C), 6 rabbits were studied which were fed the stock diet and then fasted for 24 hr before perfusion (group A).

The six livers obtained from the rabbits in group A secreted from 0.0 to 1.58 mg of glycocholate during the perfusion period; however, there was no incorporation of ^{14}C into this bile salt. This suggests that in the fasted rabbit (as in the rat), hepatic cholesterol biosynthesis is strongly inhibited so that insufficient cholesterol was available for bile acid production. At present it is not known whether in the rabbit fasting inhibits one or several steps on the pathway leading from acetate to cholic acid. The livers from the animals of groups B and C, maintained on the controlled feeding schedule, synthesized glycocholate during the perfusion, but the new synthesis was far greater in the livers from the cholestyramine-treated animals. The latter (group C), synthesized approximately 10 times the amount of glycocholate and incorporated considerably more radioactivity.

Data summarized in Table II for groups B and C are shown in greater detail in Figs. 2–9. Bile volume was similar for both groups and remained linear with respect to time during the perfusion period of 2½ hr (Fig. 2). Glycocholate secretion is shown in Fig. 3. The livers of cholestyramine-treated rabbits synthesized glycocholate at a far greater rate than the controls. The rate of radioactivity from acetate-1-^{14}C into biliary and hepatic *secretion* of glycodeoxycholate (Fig. 4), on the other hand, was significantly greater in the livers from con-

TABLE II

Effect of Pretreatment of Donor Rabbits on Bile Composition

Group	Pretreatment	No. of Rabbits	Bile volume	Cholesterol	NaGC*	NaGDC‡
			ml	*mg*	*mg*	*mg*
A	Stock diet, 24 hr fast	6	7.9	0.05 (0.03–0.07)‖	0.75 (0.0–1.58)	13.75 (2.8–24.0)
B	Stock diet, feeding schedule§	10	6.4	0.06 (0.01–0.09)	0.34 (0.0–0.84)	7.41 (1.5–13.01)
C	Stock diet + 1% cholestyramine, feeding schedule	15	6.3	0.05 (0.01–0.10)	3.27 (0.75–6.22)	3.72 (0.98–6.04)

* Sodium glycocholate.
‡ Sodium glycodeoxycholate.
§ Food removed 9 a.m.–5 p.m.; food presented 5 p.m.–9 a.m.
‖ Range.

trol rabbits than in the livers from cholestyramine-treated rabbits. Secretion of biliary cholesterol was not affected by cholestyramine treatment (Fig. 5).

Typical experiments dealing with *de novo* synthesis of glycocholate as measured by incorporation of ^{14}C precursors are shown in Figs. 6–9. Sodium acetate-1-^{14}C (Fig. 6), D,L-mevalonolactone-2-^{14}C (Fig. 7), and cholesterol-4-^{14}C (Fig. 8) were incorporated at significantly greater rates into glycocholate by livers of cholestyramine-treated rabbits. With 7α-hydroxycholesterol-4-^{14}C as precursor, cholestyramine pretreatment had no effect on the incorporation of ^{14}C into glycocholate (Fig. 9).

The radioactivity data obtained in these experiments are summarized in Tables IV–VII. When acetate-1-^{14}C, mevalonate-2-^{14}C, or cholesterol-4-^{14}C (Tables IV–VI) were used as precursors, the *de novo* synthesis of glycocholate, measured either by weight or by radioactivity incorporation, was significantly increased in the livers of cholestyramine-treated rabbits. The incorporation of cholesterol was also increased by cholestyramine pretreatment, but no consistent effect ascribed to cholestyramine was observed in the mevalonate-2-^{14}C and cholesterol-4-^{14}C experiments.

However, when 7α-hydroxycholesterol-4-^{14}C was used as the precursor (Table VII), livers from both control and cholestyramine-pretreated rabbits showed almost

TABLE III

*Bile Acid Production during 6 Hr Perfusion of Isolated Rabbit Liver**

Time	Bile		Biliary cholesterol			Biliary NaGC‡			Biliary NaGD§
min	*ml*	*dpm*	*μg*	*dpm*	*dpm/μg*	*mg*	*dpm*	*dpm/μg*	*mg*
0–30	1.4	11,200	13	40	3.1	0.15	50	0.3	0.37
30–60	1.0	14,900	4	40	10.0	0.28	1500	5.4	0.32
60–90	0.8	11,700	8	50	6.3	0.20	2500	12.5	0.15
90–120	0.7	10,400	5	70	14.0	0.20	1900	9.5	0.22
120–150	1.0	22,100	4	80	20.0	0.30	3100	10.3	0.61
150–180	1.0	23,300	3	160	53.3	0.24	2900	12.1	0.67
180–210	1.0	32,800	8	260	32.5	0.39	3500	8.9	1.10
210–240	1.0	33,400	6	230	38.3	0.21	2700	12.9	1.15
240–270	1.1	42,500	5	290	58.0	0.27	2300	8.5	1.10
270–300	1.0	35,400	6	290	48.3	0.19	2000	10.5	0.80
300–330	1.2	49,900	10	180	18.0	0.22	2600	11.8	0.90
330–360	1.2	50,800	12	300	25.0	0.16	2500	15.6	0.92

* Na acetate-1-^{14}C, 4.4 × 10^7 dpm, 20 μg, dissolved in 30 ml of 0.9% saline and infused at rate of 3.3 ml per hr from 0 to 360 min. Rabbit 34, stock + 1% cholestyramine diet for 20 days. Wet weight of liver 41 g.
‡ Sodium glycocholate.
§ Sodium glycodeoxycholate.

identical rates of incorporation of radioactivity into glycocholate (Fig. 9). This indicates that 7α-hydroxycholesterol does not channel through the rate-controlling enzyme (Fig. 1). 7α-Hydroxycholesterol-4-^{14}C does not serve as a precursor of hepatic or biliary cholesterol.

DISCUSSION

To our knowledge, this is the first time that the isolated, perfused rabbit liver has been employed in studies of bile acid synthesis. An identical preparation had been employed previously in studies of albumin synthesis and had been considered suitable for this purpose by currently accepted standards (6). In the present experiments, a number of additional criteria (glycocholate production and incorporation of ^{14}C from labeled precursors) were used to establish that this preparation is suitable for studies of *de novo* bile-acid synthesis. In previous liver perfusion studies—mainly carried out in rats (1, 14, 15), fasted or fed ad lib.—"old" bile salts, which had returned to the liver via the enterohepatic circulation before perfusion of the liver, were the major bile-salt components of the bile.

In the present study, two procedures were employed to make sure that the isolated, perfused rabbit liver produced bile acids *de novo*. The control animals were fed a semisynthetic diet for a limited period (from 5 p. m. to 9 a. m.) each day. A diurnal cycle of cholesterol and bile acid synthesis (16) in rabbits has not been reported, but the animals were observed to eat more avidly under the conditions of a controlled feeding schedule. It was hoped that the rabbits would be in a nutritional state adequate to assure active hepatic steroid synthesis. Livers of these animals were considered to be

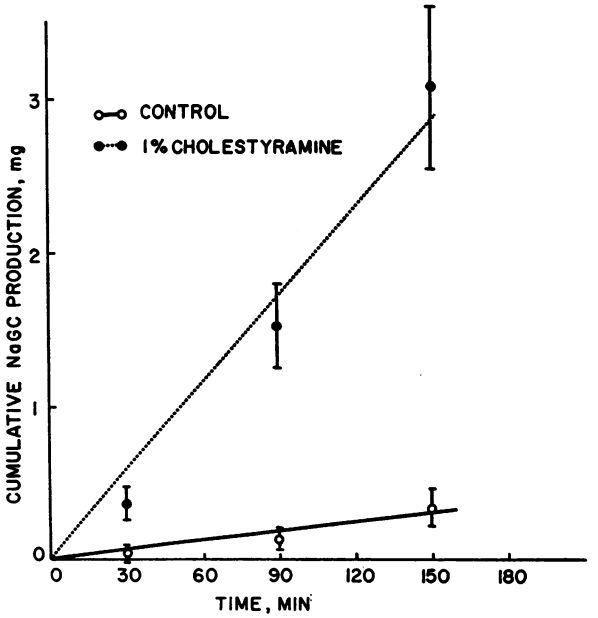

FIGURE 3 Production of sodium glycocholate by perfused rabbit liver. Average values with standard deviation for 10 control rabbits (fed stock diet) and 15 experimental rabbits (fed stock diet + 1% cholestyramine). NaGC = sodium glycocholate.

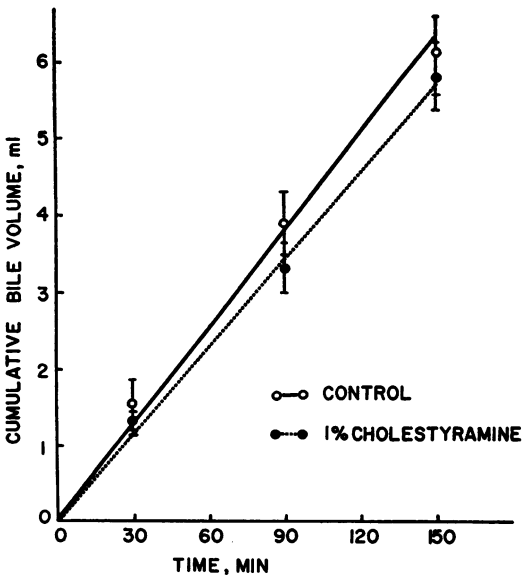

FIGURE 2 Bile secretion by perfused rabbit liver. Average values (and standard deviation) for 10 control rabbits (fed stock diet) and 15 experimental rabbits (fed stock diet + 1% cholestyramine).

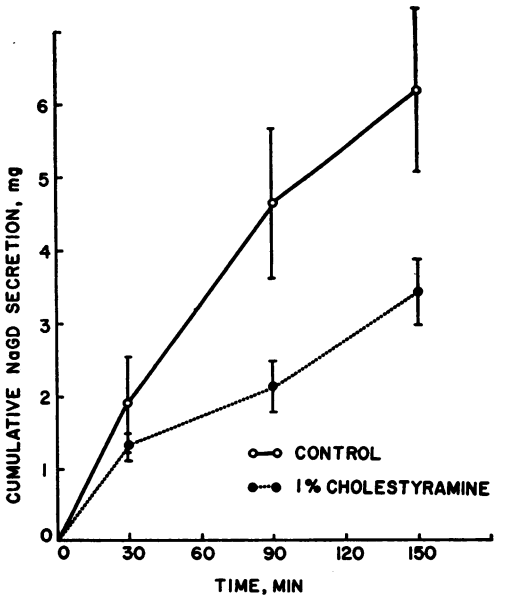

FIGURE 4 Secretion of sodium glycodeoxycholate by perfused rabbit liver. Average values with standard deviation for 10 control rabbits (fed stock diet) and 15 experimental rabbits (fed stock diet + 1% cholestyramine). NaGD = sodium glycodeoxycholate.

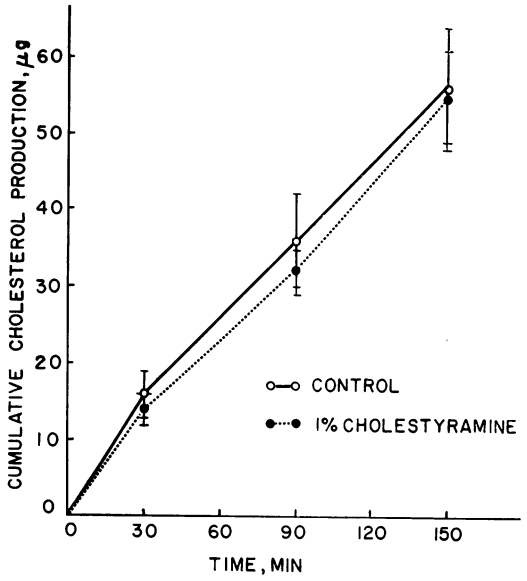

FIGURE 5 Production of biliary cholesterol by perfused rabbit liver. Average values with standard deviation for 10 control rabbits (fed stock diet) and 15 experimental rabbits (fed stock diet + 1% cholestyramine).

representative of animals with an intact enterohepatic circulation. To simulate interruption of the enterohepatic circulation, feeding of the stock diet with added 1% cholestyramine was employed. The binding of bile salts in the gut by cholestyramine has the same action as biliary diversion (bile fistula): interruption of the enterohepatic circulation resulting in high rates of bile acid synthesis.

In this connection, it is of interest to compare the bile output and bile salt secretion of the isolated, perfused rabbit liver with a recent study on bile fistula rabbits by Gregg and Poley (17). These investigators found that during the 2nd day after surgery (the 1st day's secretion includes the bile acid pool), the bile volume was 48 ml/day (2 ml/hr), glycocholate secretion was 27.8 mg/day (1.2 mg/hr), and glycodeoxycholate output was 24.0 mg/day (1 mg/hr). This compares quite closely with the data obtained in the present study from livers of cholestyramine-treated rabbits: bile volume of 2.5 ml/hr; glycocholate production of 1.2 mg/hr; and glycodeoxycholate output of 1.7 mg/hr, respectively.

That glycocholate is actively synthesized in these preparations is shown by the incorporation of ^{14}C-labeled bile acid precursors. The bile acids were identified by TLC, GLC, and reverse isotope dilution [corecrystallization of the methyl ester or free acid with an authentic sample to a constant specific activity (see Methods and Table I)]. Chenodeoxycholate identification was not attempted in the experiments utilizing acetate-1-^{14}C, D,L-mevalonolactone-2-^{14}C, and cholesterol-4-^{14}C as precursors because of the low incorporation of radioactivity.

In the experiments utilizing 7α-hydroxycholesterol-4-^{14}C, relatively larger amounts of ^{14}C-label were incorporated into chenodeoxycholate, and it was considered desirable to confirm the identification of both cholate and chenodeoxycholate by reverse isotope dilution (Table I). The reason for increased incorporation of

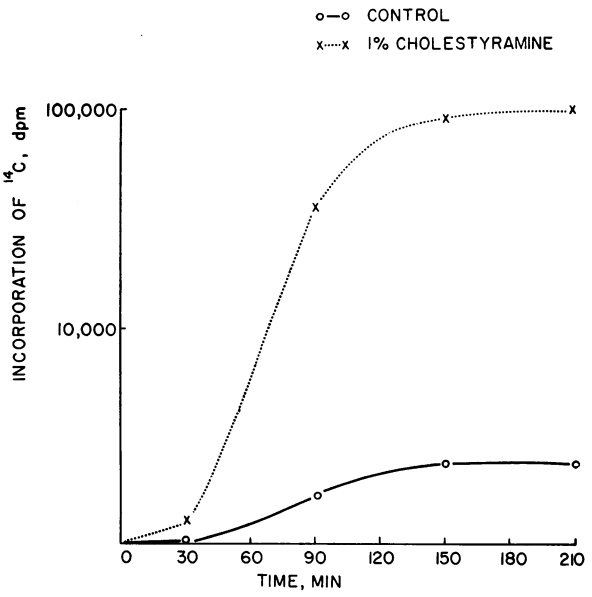

FIGURE 6 Cumulative incorporation of acetate-1-^{14}C into biliary glycocholate (5×10^6 dpm infused, 0–120 min). O——O Rabbit 22; weight 1.53 kg; liver weight 113 g; fed 20 days on stock diet. × · · · × Rabbit 23; weight 1.73 kg; liver weight 76 g; fed 22 days on stock diet + 1% cholestyramine.

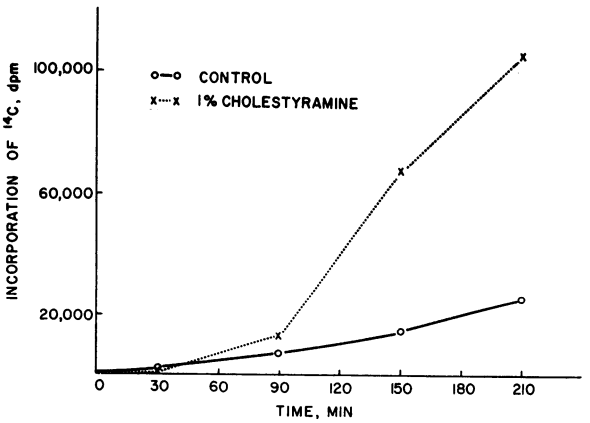

FIGURE 7 Cumulative incorporation of mevalonolactone-2-^{14}C into biliary glycocholate (5.6×10^6 dpm infused, 0–120 min). O——O Rabbit 24; weight 1.25 kg; liver weight 80 g; fed 18 days on stock diet. × · · · × Rabbit 25; weight 1.80 kg; liver weight 84 g; fed 24 days on stock diet + 1% cholestyramine.

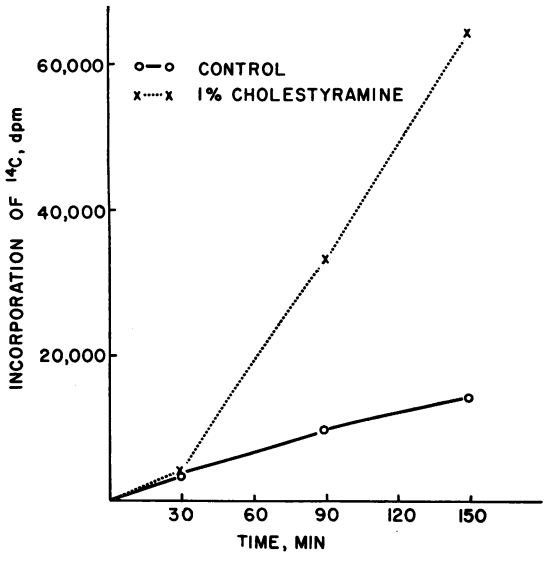

FIGURE 8 Cumulative incorporation of cholesterol-4-^{14}C into biliary glycocholate (2.3×10^6 dpm, pulse label). O——O Rabbit 14; weight 1.20 kg; liver weight 78 g; fed 16 days on stock diet. × · · · × Rabbit 16; weight 1.30 kg; liver weight 38 g; fed 26 days on stock diet + 1% cholestyramine.

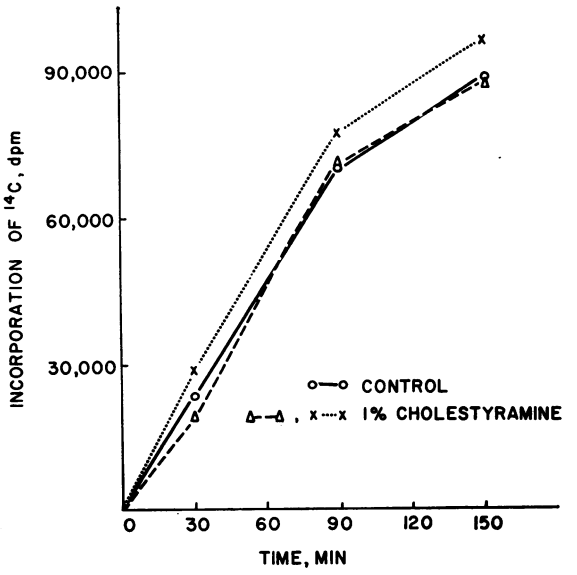

FIGURE 9 Cumulative incorporation of 7α-hydroxycholesterol-4-^{14}C into biliary glycocholate (5.4×10^5 dpm, pulse label). O——O Rabbit 11; weight 1.10 kg; liver weight 64 g; fed 14 days on stock diet. △ – – △ Rabbit 12; weight 1.25 kg; liver weight 36 g; fed 17 days on stock diet + 1% cholestyramine. × · · · × Rabbit 13; weight 1.48 kg; liver weight 82 g; fed 22 days on stock diet + 1% cholestyramine.

label into chenodeoxycholate when 7α-hydroxycholesterol was the precursor is not understood, but it may be related to the fact that the intermediate common to both cholic and chenodeoxycholic acid, namely 3α,7α-dihydroxycholest-4-en-3-one, is not readily available to the 12α-hydroxylase for further metabolism to cholic acid. Thus, there would be greater channeling into the chenodeoxycholate pathway.

By use of the various ^{14}C-labeled precursors and comparison of the rates of incorporation into bile acids in the livers from control animals (those fed the stock diet) and livers from experimental animals (those fed the stock diet with 1% cholestyramine), it is possible to deduce that the rate-limiting step of bile acid biosynthesis is the 7α-hydroxylation of cholesterol. The livers from the control rabbits are representative of an intact enterohepatic circulation and are considered to be inhibited. This is shown by their relatively low rates of synthesis as measured by the incorporation of ^{14}C-labeled acetate, mevalonate, and cholesterol into bile acid (Tables IV–VI). In contrast, 7α-hydroxycholesterol-4-^{14}C was incorporated at similar rates in livers from both control and experimental rabbits (Table VII), indicating that 7α-hydroxycholesterol is not acted upon by the rate-limiting enzyme. This is in agreement with

TABLE IV

Bile Acid Synthesis from Acetate-1-^{14}C in Perfused Rabbit Liver

Rabbit	Dietary Pretreatment	Administered	Duration of experiment	Wet weight of liver	Bile volume	Biliary cholesterol			Biliary glycocholate			Hepatic cholesterol		
						total	total	final spec. act.	total	total	final spec. act.			final spec. act.
		dpm	*min*	*g*	*ml*	*mg*	*dpm*	*dpm/mg*‡	*mg*	*dpm*	*dpm/mg*‡	*mg/100g*	*dpm/100g*	*dpm/mg*
1	Stock	3×10^6	150	78	3.1	0.07	4,500	20,700	0.63	23,800	51,700	197.4	131,400	666
2	Stock + 1% cholestyramine	3×10^6*	150	35	7.1	0.09	8,400	32,600	4.94	965,000	66,400	137.1	911,000	6640
22	Stock	5×10^6§	210	113	10.6	0.05	9,400	239,000	0.10	2,800	19,130	212	83,900	396
23	Stock + 1% cholestyramine	5×10^6§	210	76	11.2	0.06	11,200	256,000	2.95	99,800	12,810	204	1,139,000	5580

μg dissolved in 100 μl of 0.9% saline and injected at 0 min.
During final hour of perfusion period (not calculated from totals).
μg dissolved in 8 ml of 0.9% saline and infused at rate of 3 ml per hr for 2 hr from 0 to 120 min.

TABLE V

Bile Acid Synthesis from Mevalonate-2-^{14}C in Perfused Rabbit Liver

Rabbit	Dietary pretreatment	Administered	Duration of experiment	Wet weight of liver	Bile volume	Biliary cholesterol			Biliary glycocholate			Hepatic cholesterol		
						total	total	final spec. act.	total	total	final spec. act.			final spec. act.
		dpm	*min*	*g*	*ml*	*mg*	*dpm*	*dpm/mg*‡	*mg*	*dpm*	*dpm/mg*‡	*mg/100g*	*dpm/100g*	*dpm/n*
3	Stock	9 × 10^5*	150	49	5.0	0.01	800	—	0.0	500	—	317	420,000	1330
4	Stock + 1% cholestyramine	9 × 10^5*	150	51	4.2	0.03	500	36,800	3.04	13,500	4230	173.9	338,000	1944
5	Stock + 1% cholestyramine	9 × 10^5*	150	52	4.3	0.01	200	—	0.75	4,400	6970	316	439,000	1389
17	Stock	3.3 × 10^6§	150	81	5.6	0.06	1500	68,900	0.83	27,900	72,300	188	683,000	3630
18	Stock + 1% cholestyramine	3.3 × 10^6§	150	46	7.9	0.07	9300	119,100	6.17	141,900	25,400	238	1,782,000	7490
24	Stock	5.6 × 10^6‖	210	69	9.8	0.08	27,300	454,000	0.57	27,500	245,000	244	954,000	3910
25	Stock + 1% cholestyramine	5.6 × 10^6‖	210	50	10.3	0.04	19,300	308,500	1.22	105,100	92,100	309	1,815,000	5870

* 1 μg dissolved in 1 ml saline and given as single injection at 0 min.
‡ During last hour of perfusion period (not calculated from totals).
§ 3 μg dissolved in 1 ml saline and given as single injection at 0 min.
‖ 6 μg dissolved in 8 ml of saline and infused at rate of 3 ml per hr from 0 to 120 min.

similar studies in the bile fistula rat (18), where it was shown that the steps on the pathway beyond 7α-hydroxycholesterol were not longer under the control of the circulating bile acid pool.

In the experiments with cholesterol-4-^{14}C and 7α-hydroxycholesterol-4-^{14}C, these water-insoluble tracers were "solubilized" with Tween 20. As a result these non-physiological "solutions" were only partially utilized for bile acid synthesis. This is presumably due to the fact that only a certain fraction of the "solubilized" sterol reaches the intracellular site of bile acid synthesis; from 13 to 60% of the administered tracer was actually recovered from the perfusion fluid. It can be seen from experiment 21 (Table VII), however, that this does not represent an inherent limitation of the livers to metabolize larger amounts of 7α-hydroxycholesterol. During the first 2 hr of a 5½ hr perfusion, 6.12 mg of 7α-hydroxycholesterol-4-^{14}C was added to the perfusate at a rate of 3.1 mg per hr, and 2.1 mg was converted to glycocholate. The per cent utilization of a given emulsion may depend largely on the physical state of the dispersed sterol, and this is likely to vary for any given emulsion. Even with a water-soluble substrate (e.g. mevalonolactone-2-^{14}C) there may exist a problem of substrate penetration, and this may explain the pronounced lag-period seen with this tracer (Fig. 7).

The data summarized in Tables III–VII illustrate a problem common to all tracer studies in which the pool size and specific activity of a precursor are not available for assay. In Tables III and IV when acetate-1-^{14}C was used as the precursor, the final specific activity of biliary cholesterol was usually considerably

TABLE VI

Bile Acid Synthesis from Cholesterol-4-^{14}C in Perfused Rabbit Liver

Rabbit	Dietary pretreatment	Administered	Duration of experiment	Wet weight of liver	Bile volume	Biliary cholesterol			Biliary glycocholate			Hepatic cholesterol		
						total	total	final spec. act.	total	total	final spec. act.			fina spec act
		dpm	*min*	*g*	*ml*	*mg*	*dpm*	*dpm/mg*‡	*mg*	*dpm*	*dpm/mg*‡	*mg/100g*	*dpm/100g*	*dpm*
6	Stock	5 × 10^5*	150	75	5.7	0.10	3100	13,690	0.62	8700	14,340	185.1	206,000	111
7	Stock + 1% cholestyramine	5 × 10^5*	150	64	5.6	0.05	400	6,080	2.17	18,900	9,290	177.0	317,000	179
14	Stock	2.3 × 10^6§	150	79	5.4	0.07	5100	166,400	0.07	14,400	65,600	239	1,119,000	468
15	Stock + 1% cholestyramine	2.3 × 10^6§	150	59	5.0	0.05	1600	73,400	4.57	60,600	17,070	165.9	1,305,000	787
16	Stock + 1% cholestyramine	2.3 × 10^6§	150	38	4.9	0.07	2400	79,200	4.55	64,800	16,120	237	2,230,000	944

* 1 μg solubilized in 20% Tween (0.1 ml) and 0.9 ml of 0.9% saline and given as single injection at 0 min.
‡ Specific activity during final hour of perfusion (not calculated from totals).
§ 5 μg solubilized in 20% Tween (0.1 ml) and 0.9 ml of 0.9% saline and given as single injection at 0 min.

TABLE VII

Bile Acid Synthesis from 7α-Hydroxycholesterol-4-^{14}C in Perfused Rabbit Liver

Rabbit	Dietary pretreatment	Administered	Duration of experiment	Wet weight of liver	Bile volume	Biliary cholesterol	Biliary glycocholate			^{14}C recovered from		Hepatic cholesterol
							total	total	final spec. act.	perfusate	liver	
		dpm	*min*	*g*	*ml*	*mg*	*mg*	*dpm*	*dpm/mg*	%		*mg/100g*
8	Stock	6.6 × 10^4*	150	52	7.9	0.03	0.09	6,500	15,140	34.7	22.7	196.9
9	Stock + 1% cholestyramine	6.6 × 10^4*	150	58	3.6	0.03	2.45	6,600	1,636	60.3	14.4	166.0
10	Stock + 1% cholestyramine	6.6 × 10^4*	150	55	4.2	0.09	2.59	6,200	1,432	19.4	25.6	209
11	Stock	5.4 × 10^5§	150	64	6.7	0.08	0.54	89,600	60,300	17.0	15.3	215
12	Stock + 1% cholestyramine	5.4 × 10^5§	150	36	4.9	0.06	3.40	96,600	14,210	12.8	13.8	298
13	Stock + 1% cholestyramine	5.4 × 10^5§	150	82	6.1	0.10	6.22	88,200	6,190	22.5	15.0	169.0
21	Stock	1.07 × 10^6‖	330	23	13.5	0.12	2.07	349,200	50,350	38.7	16.9	358

* 6 μg solubilized in 20% Tween (0.1 ml) and 0.9 ml of 0.9% saline and given as single injection at 0 min.
‡ During last hour of perfusion period (not calculated from totals).
§ 3 μg solubilized in 20% Tween (0.1 ml) and 0.9 ml of 0.9% saline and given as single injection at 0 min.
‖ 6.12 mg solubilized with 20% Tween in 0.9% saline to give a volume of 6.0 ml and infused into perfusate at a rate of 3 ml/hr during first 2 hr of experiment.

greater than that of hepatic cholesterol but either smaller or only a few times greater than that of biliary glycocholate. Similar degrees of variation are present with the other precursors (mevalonate-2-^{14}C, cholesterol-4-^{14}C). The data suggest that the hepatic precursor pools for biliary cholesterol and glycocholate vary not only from animal to animal but are in addition altered by cholestyramine administration. For example, when cholesterol-4-^{14}C was used as the precursor, the specific activity of biliary glycocholate was higher in the control livers than in those of donor rabbits pretreated with cholestyramine (Table VI). However, the amounts of glycocholate formed were many times greater in the latter than in the controls. This would suggest that the specific hepatic cholesterol pool for bile acid synthesis was increased in the cholestyramine experiments, thus diluting the available cholesterol-^{14}C at the site of synthesis.

While the data reported here reflect quantitative changes in the rates of *de novo* bile acid synthesis they cannot be used to quantitate the magnitude of the precursor pools. In the experiments with 7α-hydroxycholesterol it can be deduced that the pool size must be small or that the pool turns over very rapidly, but more accurate estimates must await the development of improved techniques.

It was observed that incorporation of acetate-1-^{14}C into hepatic cholesterol was increased by cholestyramine pretreatment (Table IV). This suggests, but does not prove, that bile acids inhibit hepatic HMG-CoA reductase. Thus, Weis and Dietschy (19) have proposed that the effect may be indirect and that it is exerted via the enterolymphatic circulation of cholesterol. Presumably, greater quantities of cholesterol reach the liver of control animals where more bile acid is present in the intestinal tract to favor cholesterol absorption. Therefore, the inhibitory substance may be cholesterol rather than bile acid.

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