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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE EFFECTS OF PRIMARY BILE ACIDS ON THE HEPATIC SYNTHESIS OF CHOLESTEROL IN THE FEMALE RAT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

DOCTOR OF PHILOSOPHY

BY

EUGENE EUCHUAN TAN

Oklahoma City, Oklahoma

1973

THE EFFECTS OF PRIMARY BILE ACIDS ON THE HEPATIC SYNTHESIS OF CHOLESTEROL IN THE FEMALE RAT

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TO

MY FAMILY

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THE EFFECTS OF PRIMARY BILE ACIDS ON THE HEPATIC SYNTHESIS OF CHOLESTEROL IN THE FEMALE RAT

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Chenodeoxycholic acid (CDCA) has recently been reported to be effective in decreasing the lithogenic potential of human bile (1) and in dissolving human cholesterol gallstones (2-3). However, potential side-effects that accompany the administration of this biological compound to gallstone patients have not yet been established (3-4). Currently, only a few clinical studies have been reported (2-3); evidence is insufficient to indicate positive therapeutic effects of this drug. Notably lacking are any studies that reveal whether CDCA might alter the regulation of various biological systems. In the investigation described in this thesis, the effects of CDCA and cholic acid (CA) on the hepatic synthesis of cholesterol and related neutral lipids in the female rat were studied.

In liver, smooth endoplasmic reticulum is the major site for de novo synthesis of cholesterol (5). All enzymes required for cholesterol synthesis are located in the microsomal fraction. In rat liver, the maximal rate of cholesterol synthesis in vitro requires the 105,000 x g supernatant fraction, which contains noncatalytic sterol carrier protein

(SCP) (6). Scallen et al. (6) claim SCP to be nondialyzable, heat-labile and destructible by trypsin; supposedly, it is derived from endoplasmic reticulum and yields a protein peak on Sephadex chromatography. Ritter and Dempsey (7-8) report that SCP is a heat-stable protein and that cholesterol precursors are metabolized markedly faster if they are bound with SCP than if they are initially unbound.

Cholesterol is synthesized from a simple precursor, acetyl-CoA (9). Acetyl-CoA undergoes successive condensations with other acetyl-CoA molecules to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is converted to HMG-S-Enzyme (Figure 1). Then 2 moles of NADPH are utilized for conversion of HMG-S-Enzyme to mevalonic acid by HMG-CoA reductase. Compounds formed prior to mevalonic acid may enter multiple metabolic pathways, while compounds subsequent to mevalonic acid are specific precursors for cholesterol synthesis. In the presence of magnesium ion and 3 moles of ATP, mevalonic acid is pyrophosphorylated at the 5-position and phosphorylated at the 3-position to form 3-phospho-5-pyrophosphomevalonate. This is then converted to isopentenyl-pyrophosphate after decarboxylation and removal of the phosphate group at the 3-position. The action of isomerase turns isopentenyl-pyrophosphate into dimethylallyl-pyrophosphate. Head-to-head condensation of isopentenyl-pyrophosphate and dimethylallyl-pyrophosphate forms geranyl-pyrophosphate. This condenses (head-to-tail) with isopentenyl-pyrophosphate to form farnesyl-pyrophosphate. Squalene is made by tail-to-tail condensation of two farnesyl-pyrophosphate molecules at the expense of NADPH (Figure 2). Molecular oxygen is used to convert squalene to squalene-2,3-oxide. Lanosterol is formed after cyclization of squalene-2,3-oxide and rearrangement of

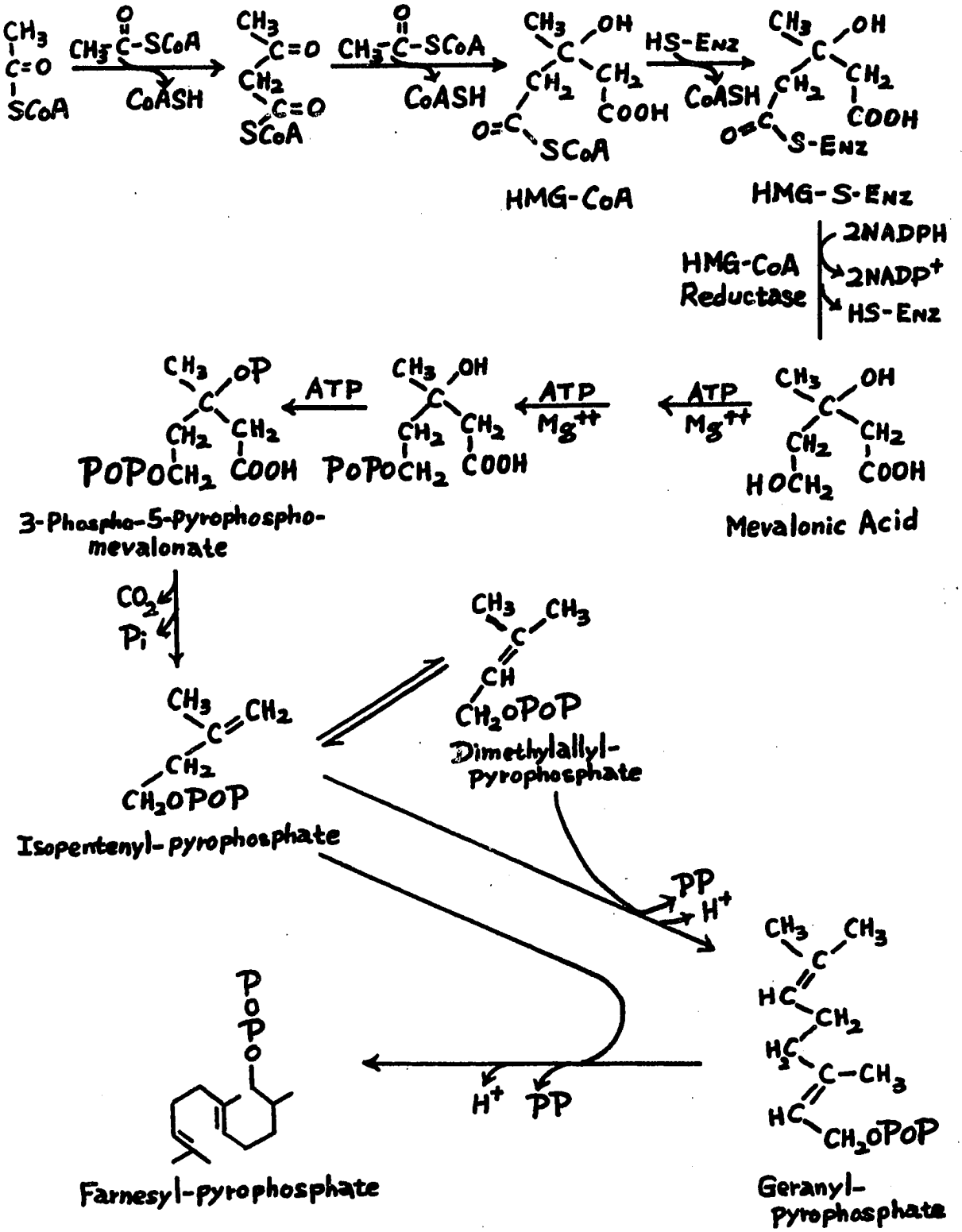


Figure 1. Conversion of acetyl-CoA to farnesyl-pyrophosphate.

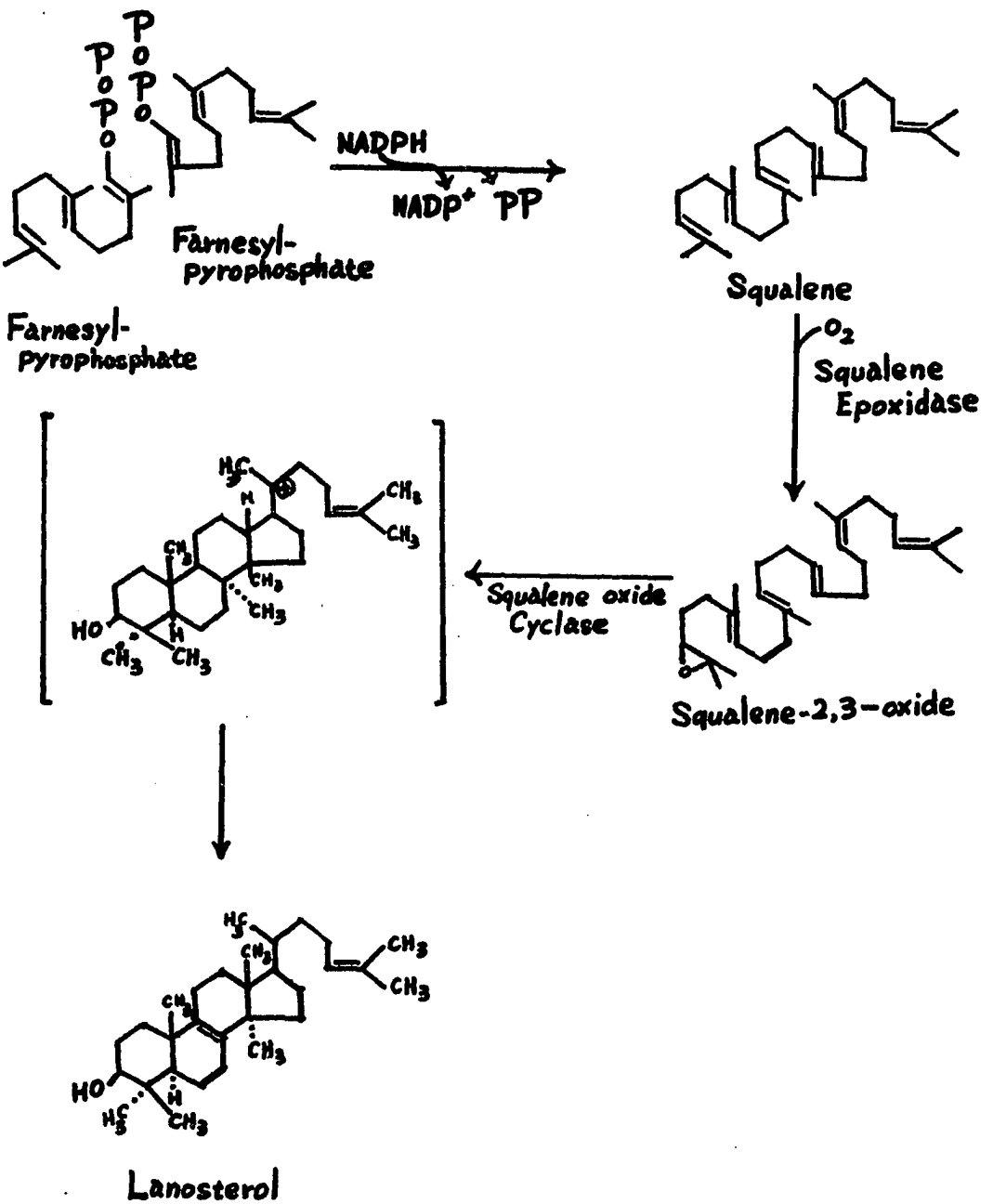


Figure 2. Conversion of farnesyl-pyrophosphate to lanosterol.

methyl groups attached to the C and D rings. It appears that there are more than two pathways for the formation of cholesterol from lanosterol. However, the two pathways shown in Figure 3 are supported by most of the evidence (10).

There are two rate-limiting steps in the cholesterol biosynthetic pathway. One of them is HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonic acid. Although the site of the second rate-limiting step has not been defined exactly, there is general agreement that it appears to be between lanosterol and cholesterol (10-12). According to a recent report, this second rate-limiting step is inhibited by various steroids, including steroid hormones (11). Also, 3-hydroxy-3-methylglutaric acid has recently been reported to be a potential inhibitor for cholesterol synthesis in the liver and in the intestine in some species, such as the rat (13).

Dietary cholesterol is absorbed from the intestinal lumen with the aid of bile salts. Intestinal absorption of cholesterol is enhanced more efficiently by taurine-conjugated bile salts than by the corresponding glycine-conjugated bile salts (14). Yet, the intestinal reabsorption of taurine-conjugated bile salts is much poorer than that of glycine-conjugated bile salts or unconjugated bile acids (15-16). Interestingly, the taurine or glycine conjugates of dihydroxy bile acids play a special role in the esterification of the sterol during intestinal absorption (14).

Four-fifths of hepatic cholesterol and two-thirds of plasma cholesterol are esterified with fatty acid at the 3-hydroxyl group (17), whereas more than 95% of biliary cholesterol is free (18). Biliary secre-

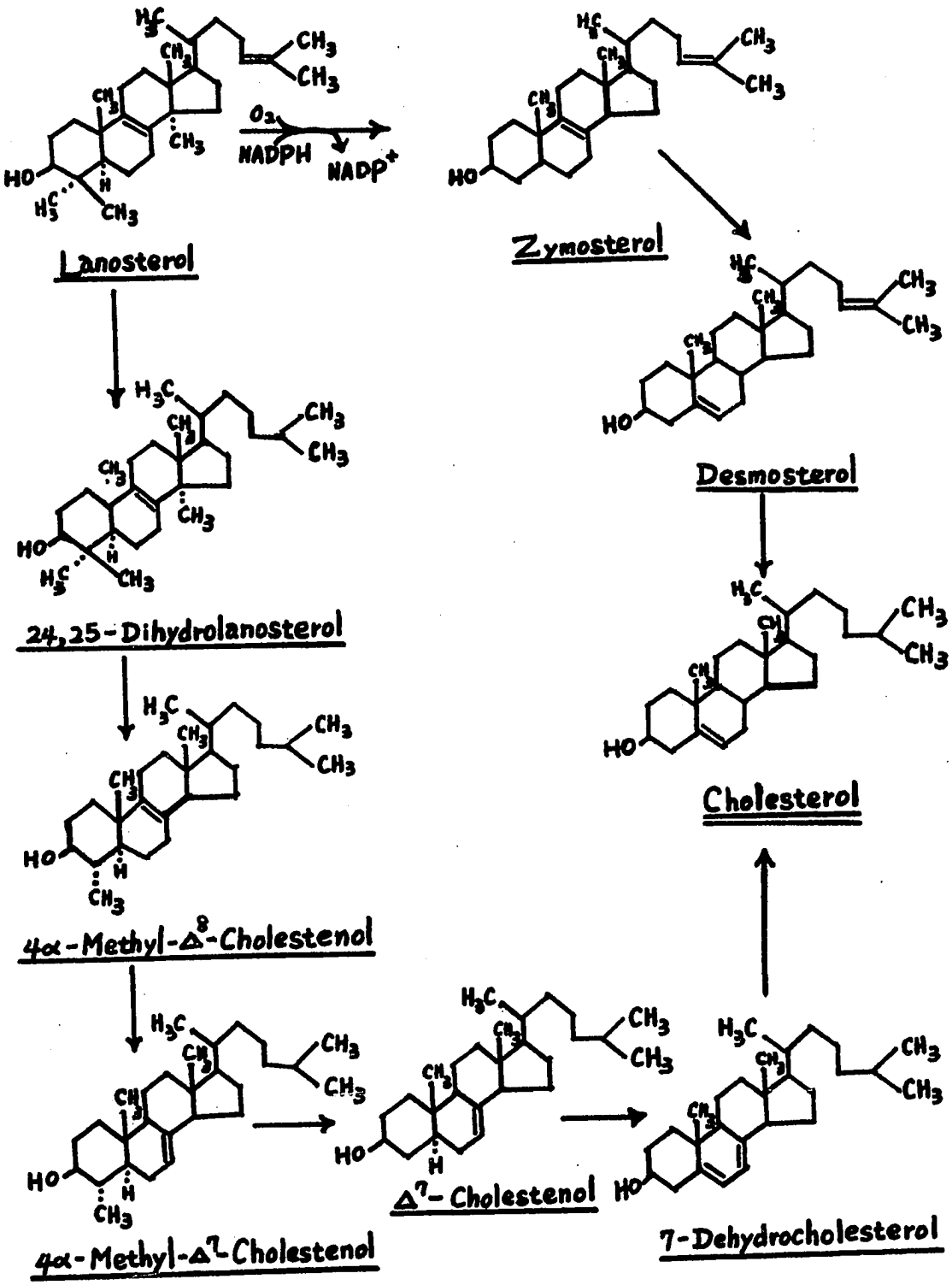


Figure 3. Conversion of lanosterol to cholesterol.

tion of free cholesterol, excluding cholesterol ester, may relate to differences in micellar solubility. Alternatively, the explanation may be that most of the cholesterol in liver cell membranes is unesterified (19). However, there is a possibility that esterified cholesterol is secreted into bile and is rapidly reabsorbed (20).

Hepatic, plasma, and biliary cholesterol are in rapid isotopic equilibrium (21). They constitute the major portion of the rapidly exchanging cholesterol pool, part of which is also contributed by the small intestine in the human (22), the baboon (23), and the squirrel monkey (24). In the human, the mean size of this rapidly exchanging cholesterol pool is about 28 grams (25). However, there is no relationship between the cholesterol concentrations in bile and in serum (26).

Dietary fats have an effect on the serum cholesterol concentration. In man, the serum-cholesterol-raising effect of saturated fatty acids with 12 to 16 carbon atoms is twice as great as the serum-cholesterol-depressing effect of the polyunsaturated fatty acids (27). Saturated fatty acids with fewer than 12 carbon atoms and stearic acid, which do not affect serum cholesterol concentration, elevate serum triglyceride concentration (27).

Bile acids are the major catabolic and excretory products of cholesterol (28-29). Hepatic synthesis of bile acids from cholesterol contributes more than one half of the total cholesterol catabolism (30). The major bile acids found in human bile are the glycine:taurine (=3:1) conjugated derivatives of cholic, chenodeoxycholic, and deoxycholic acids (30) in the approximate proportions of 40:40:20 with only trace amounts of lithocholic acid (31-32). Bile acid composition differs in different

species; it also varies at different developmental stages within species (33).

In the hepatic synthesis of primary bile acids (cholic acid and chenodeoxycholic acid) from cholesterol, the initial step involves hydroxylation of the steroid nucleus at the 7-position. There are two alternative pathways in the smooth endoplasmic reticulum. The 12-position of the steroid nucleus can be further hydroxylated in the cholic acid pathway to form 5β-cholestane-3α,7α,12α-triol (Figure 4). Alternatively, the steroid nucleus can directly undergo change to form 5β-cholestane-3α,7α-diol via the chenodeoxycholic acid pathway (Figure 5). Oxidation of the side-chain begins with hydroxylation at the 26-position in mitochondria and cytosol. However, in the human (Figure 5), chenodeoxycholic acid can also be synthesized from 26-hydroxycholesterol (34), whereas this is not true for cholic acid synthesis (34). Microsomal enzymes convert bile acids to their CoA derivatives; then the carboxyl groups of bile acids undergo conjugation with the amino groups of either glycine or taurine to form a peptide linkage. This conjugating reaction takes place in microsome and is facilitated by lysosomal conjugating enzymes (35).

Bile salts are necessary for solubilization of lecithin (36), and lecithin, in turn, is required for dissolving the quantities of cholesterol found in bile (37). Cholesterol is really solubilized in mixed micelles formed by bile salts and lecithin (38). The type of fatty acid in the lecithin and the type of bile acid found in the micelle can affect the cholesterol-solubilizing capacity of the micelle. The cholesterol-holding capacity is higher if the lecithin contains polyunsaturated fatty acid than if the lecithin contains saturated fatty acid (39).

NAMES OF COMPOUNDS DESIGNATED BY ROMAN NUMERALS IN FIGURE 4 (OPPOSITE)

I ---- Cholesterol

II ---- 7α-Hydroxycholesterol

III ---- 7α-Hydroxycholest-4-ene-3-one

IV ---- 7α,12α-Dihydroxycholest-4-ene-3-one

V ---- 7α,12α-Dihydroxy-5β-cholestan-3-one

VI ---- 3α,7α,12α-Trihydroxy-5β-cholestane

VII ---- 3α,7α,12α,26-Tetrahydroxy-5β-cholestane

VIII ---- 3α,7α,12α-Trihydroxy-5β-cholestanoic acid

IX ---- 3α,7α,12α-Trihydroxy-5β-cholestanoyl-CoA

X ---- Cholyl-CoA

XIa ---- Glycocholate

XIb ---- Taurocholate

XII ---- 7α,12α-Dihydroxycholesterol

XIII ---- 3α,7α-Dihydroxy-5β-cholestane

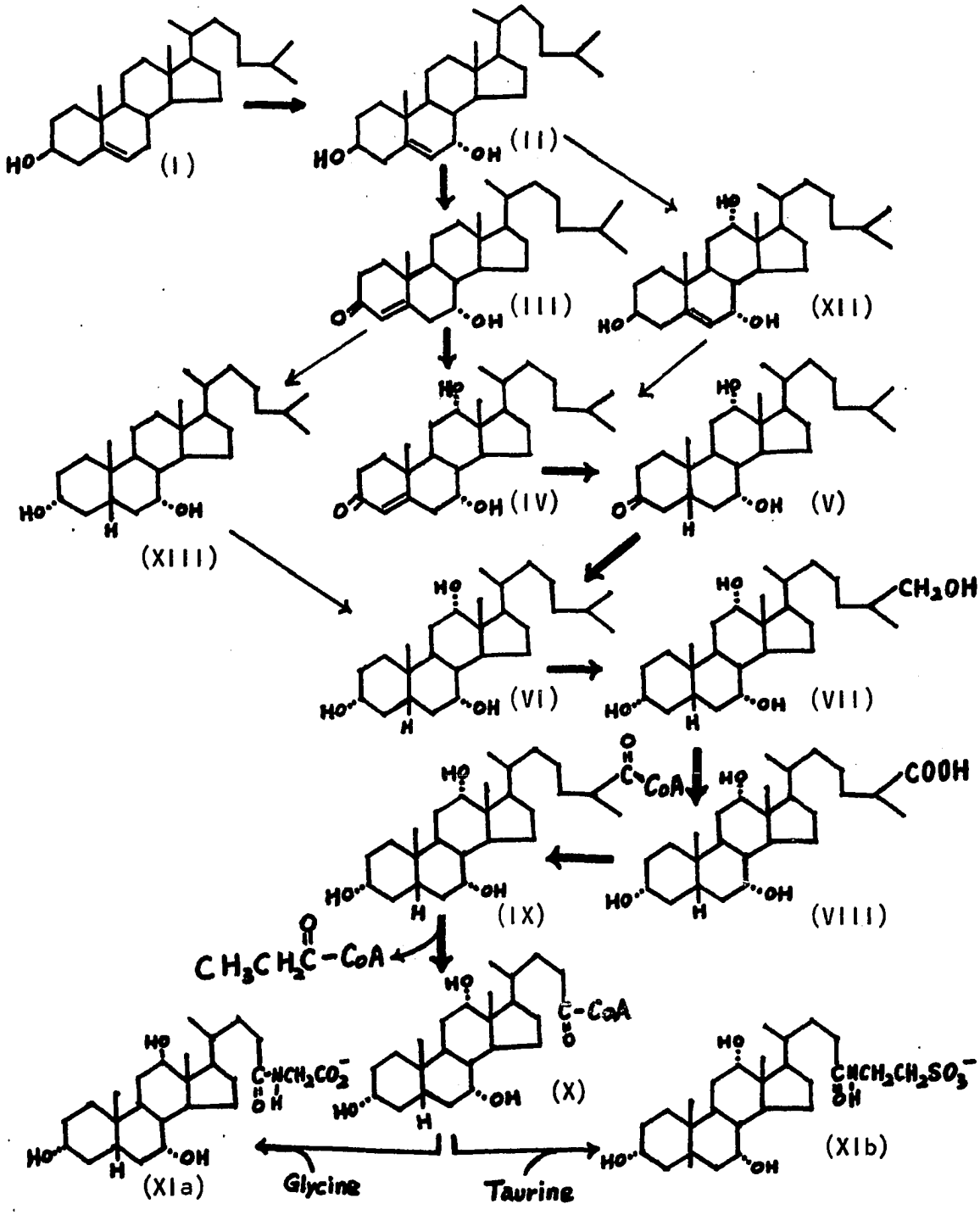


Figure 4. Conversion of cholesterol to glycocholate and taurocholate.

NAMES OF COMPOUNDS DESIGNATED IN ROMAN NUMERALS IN FIGURE 5 (OPPOSITE)

I ---- Cholesterol

II ---- 7α-Hydroxycholesterol

III ---- 7α-Hydroxycholest-4-ene-3-one

IV ---- 3α,7α-Dihydroxy-5β-cholestane

V ---- 3α,7α,26-Trihydroxy-5β-cholestane

VI ---- 3α,7α-Dihydroxy-5β-cholestanoic acid

VII ---- Chenodeoxycholic acid

VIII ---- 26-Hydroxycholesterol

IX ---- 7α,26-Dihydroxycholesterol

X ---- 7α,26-Dihydroxycholest-4-ene-3-one

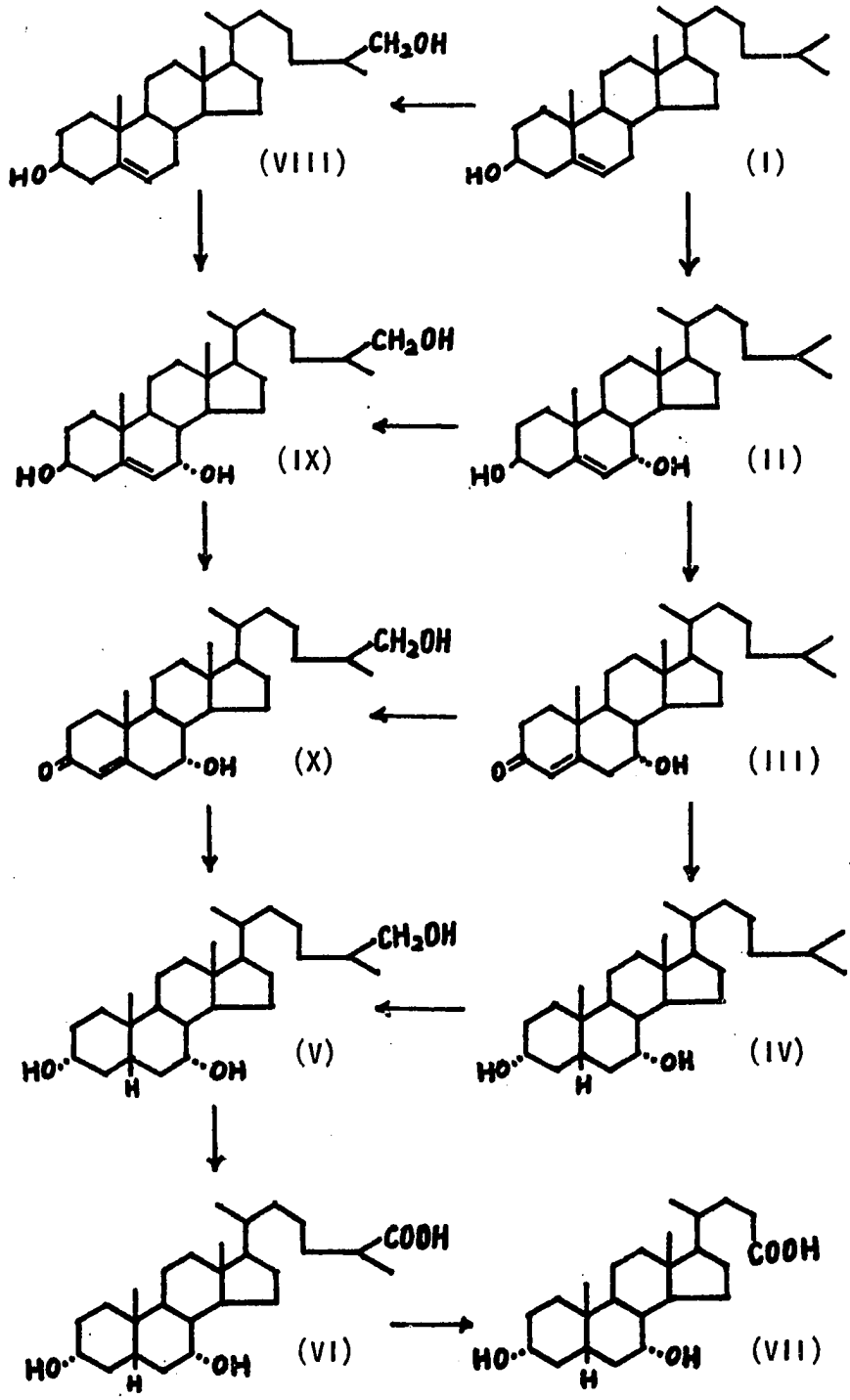


Figure 5. Conversion of cholesterol to chenodeoxycholic acid.

There is a higher cholesterol-solubilizing capacity for the micelle formed by dihydroxylated bile acids (chenodeoxycholic acid and deoxycholic acid) than for the micelle formed by trihydroxylated bile acids (cholic acid) (16). Cholesterol saturation of 75-90% is normal in human bile (40). A gallstone may be formed by the precipitation of cholesterol in bile. Cholesterol precipitation may result from changes in relative concentrations of cholesterol, bile salts, and lecithin.

Hepatic synthesis and secretion of bile acid and lecithin into bile is determined by the quantities of bile salts in the enterohepatic circulation (41). These quantities, in turn, depend on the intestinal reabsorption of bile salts. Glycine (pK = 4) and taurine-conjugates (pK = 2), which are stronger acids than free bile acids (pK = 6), are fully ionized at jejunal pH. Virtually, no jejunal absorption of conjugated bile salts occurs except for glycine-conjugated dihydroxy bile acids (16). Free bile acids, which are present only in disease states, can be absorbed passively throughout the intestines (20). In a normal human colon, intestinal bacteria can deconjugate and remove the 7α-hydroxyl group, converting primary bile salts into the secondary bile salts, deoxycholate and lithocholate (42) (Figure 6). Deoxycholate is absorbed in part by passive diffusion as well as by active ileal transport; lithocholate, which is the least soluble in water, is excreted in the feces (16). The reabsorbed bile acids are returned via the portal circulation to the liver, where they are reconjugated with glycine or taurine and are incorporated into the circulating bile acid pool (43-44). The liver is able to remove bile acids completely from portal blood in one hepatic passage (45). The daily loss in the feces of a small fraction

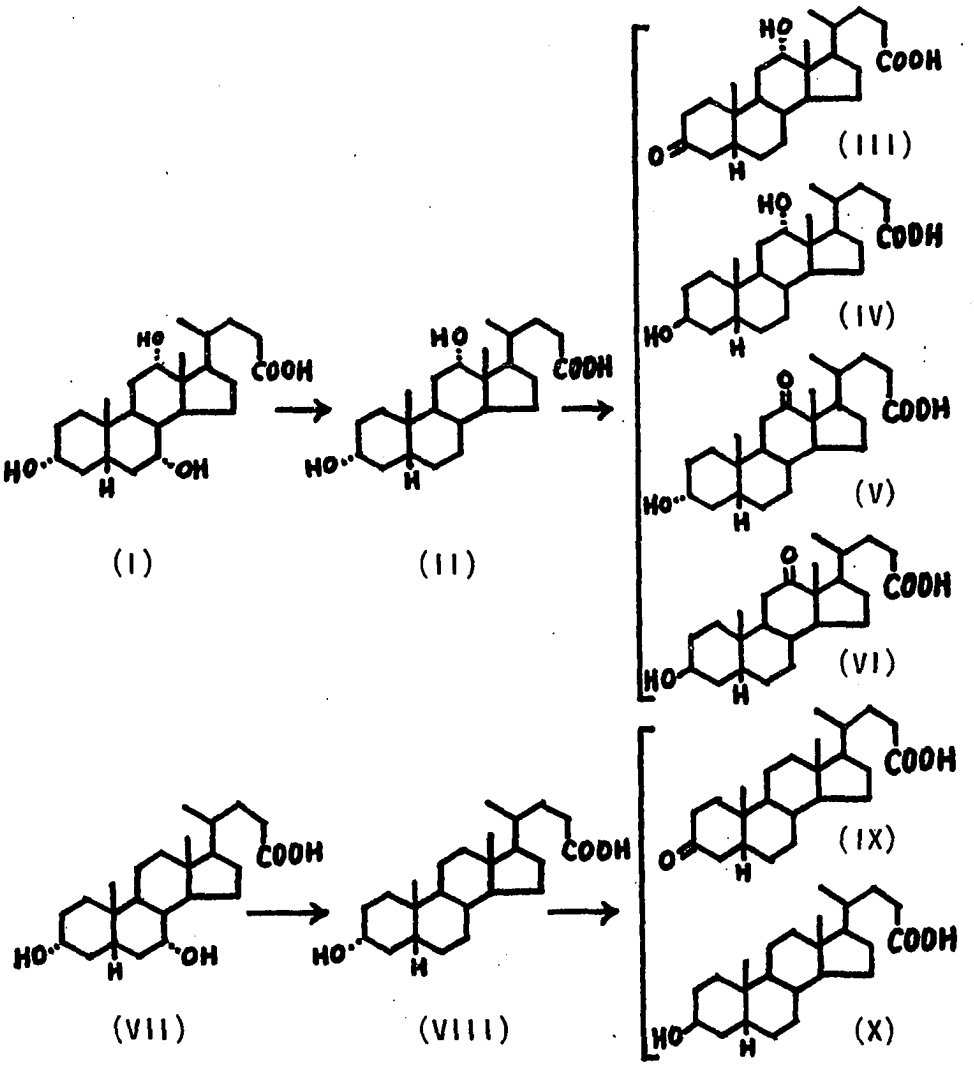


Figure 6. The main metabolites of the primary bile acids formed by the action of intestinal microorganisms. I- Cholic Acid; II--Deoxycholic Acid; III- 12α-Hydroxy-5β-cholan-3-one-24-oic Acid; IV- 3β,12α-Dihydroxy-5β-cholanoic Acid; V- 3α-Hydroxy-5β-cholan-12-one-24-oic Acid; VI- 3β-Hydroxy-5β-cholan-12-one-24-oic Acid; VII- Chenodeoxycholic Acid; VIII- Lithocholic Acid; IX- 5β-Cholan-3-one-24-oic Acid; X- 3β-Hydroxy-5β-cholanoic Acid.

of the total bile acid pool is replaced by the hepatic synthesis. This synthesis of bile acids from cholesterol causes the bile acid pool to remain constant (30).

Coronary heart disease is related to atherosclerosis, which, in turn, is associated with an elevated level of serum cholesterol. Cholelithiasis is a disease with a relative excess of biliary cholesterol. In the human, hypercholesterolemia is associated with decreased bile acid secretion, while a combination of hypercholesterolemia and a hypertriglyceridemia is associated with increased bile acid secretion (46).

Therefore, if a patient or an animal is treated with either chenodeoxycholic acid or cholic acid, and if either bile acid could augment hepatic synthesis of cholesterol and other neutral lipids, what will be the fate of the increased quantity of cholesterol that might be synthesized during bile acid treatment? Will it be excreted in the feces or deposited on the arterial walls to form atherosclerotic plaques and eventually lead to coronary heart disease?

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Adult female rats of the Stanley-Gumbreck strain of King-Holtzman hybrid species were purchased from the International Foundation for the Study of Rat Genetics and Rodent Pest Control, Oklahoma City, Oklahoma.

Chemicals

[Carboxyl-^{14}C]-cholic acid [specific activity: 3.3 mCi/mmole], iodine, KCl, KH_2PO_4, and $NaHCO_3$ were products of Mallinckrodt Chemical Works, St. Louis, Missouri.

Na_2HPO_4, diethyl ether, and petroleum ether (bp 30-60°) were bought from Baker Chemical Company, Phillipsburg, New Jersey.

Silica gel H was purchased from E. Merck A. G., Darmstadt, Germany.

Chenodeoxycholic acid (A grade) was bought from Calbiochem, San Diego, California.

Toluene was obtained from Eastman Kodak Company, Rochester, New York.

PPO (2,5-diphenyloxazole), POPOP (1,4-bis-[2-(4-methyl-5-phenyl-oxazolyl)]-benzene), and Triton X-100 (scintillation grade) were purchased from Packard Instrument Company, Downers Grove, Illinois.

$MgCl_2$, $MgSO_4$, GSH (reduced form), D(+)-glucose, G-6-P (D-glucose-6-phosphate), ATP (adenosine-5'-triphosphate), NADH (nicotinamide adenine dinucleotide, reduced form, dipotassium salt), NADPH (nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt), sodium cholate, and lipid standard (containing 20 mg each of cholesterol, cholesteryl oleate, oleic acid, oleic acid methyl ester, and triolein) were bought from Sigma Chemical Company, St. Louis, Missouri.

[2-^{14}C]-Sodium acetate [specific activity: 55.5 mCi/mmole] was obtained from International Chemical and Nuclear Corporation, Irvine, California.

[2-^{14}C]-DL-Mevalonic acid (N,N'-dibenzylethylenediamine salt) [specific activity: acid, 6.33 mCi/mmole; salt, 12.66 mCi/mmole], Aquasol, and Protosol were purchased from New England Nuclear Corporation, Boston, Massachusetts.

NaCl, methanol, chloroform, glacial acetic acid, Celite, KOH, and NaOH were bought from Fisher Chemical Company, Fair Lawn, New Jersey.

Penicillin-streptomycin solution (5000 units penicillin and 5000 µg streptomycin per ml) was obtained from Grand Island Biological Company, Grand Island, New York.

Methods

Celite was suspended in distilled water to make 20 mg Celite per ml water suspension.

Chenodeoxycholic acid solution was prepared by dissolving chenodeoxycholic acid in an equivalent quantity of sodium hydroxide solution. The solution was then adjusted to pH 7.5 and diluted with distilled water (pH 7.5). Eight different concentrations were prepared, as follows: 0.02 M,

0.04 M, 0.08 M, 0.12 M, 0.16 M, 0.20 M, 0.40 M, 0.60 M.

Cholic acid solution was prepared by dissolving sodium cholate in distilled water. The solution was then adjusted to pH 7.5 and diluted with distilled water (pH 7.5). Eight different concentrations were prepared, corresponding to those in the preparation of chenodeoxycholic acid solution.

[2-^{14}C]-Sodium acetate was diluted with distilled water to make a 45 μCi/ml water solution (8.11×10^{-4} M). [2-^{14}C]-DL-Mevalonic acid DBED salt was diluted with distilled water to make a 10 μCi/ml water solution (15.8×10^{-4} M). These were then used for injection in the experiments in vivo.

Sodium cholate solutions at four different concentrations (5×10^{-3} M, 15×10^{-3} M, 50×10^{-3} M, and 150×10^{-3} M, one ml of each solution containing approximately 5-8 μCi [carboxyl-^{14}C]-cholic acid) were prepared by mixing sodium cholate and [carboxyl-^{14}C]-cholate in distilled water. The specific activity of each prepared solution was determined, as follows: 2,288,000 dpm/μmole, 826,400 dpm/μmole, 228,680 dpm/μmole, 101,787 dpm/μmole for 5×10^{-3} M, 15×10^{-3} M, 50×10^{-3} M, 150×10^{-3} M cholate solutions, respectively. These solutions were then used for intraperitoneal injection in the experiments for determining the effects of different doses on rate of incorporation of injected cholate into liver and intestinal contents.

All hypotonic solutions used for injection had been adjusted to isotonic level (0.14 M) with 0.56 M saline before they were used for injection in the experiments in vivo.

The modified Hank's solution was prepared by dissolving 8000 mg

NaCl, 400 mg KCl, 100 mg $MgSO_4.7H_2O$, 100 mg $MgCl_2.6H_2O$, 60 mg KH_2PO_4, 60 mg $Na_2HPO_4.2H_2O$, 1000 mg D(+)-glucose, and 350 mg $NaHCO_3$ in distilled water to make 1 liter. The solution was adjusted to approximately pH 10-11 with potassium hydroxide solution. This yielded a pH of 7.5 to 7.6 in the 7,000 x g liver supernatant fraction at 0^o. The solution was kept ice-cold for later use.

The lipid standard was diluted with toluene/methanol (9/1) to make 10 ml stock solution.

The composition of the scintillation fluid was as follows: 7 g PPO, 0.35 g POPOP, 333 ml Triton X-100, and 667 ml toluene.

Experiments In Vitro

Preparation of 7,000 x g liver supernatant fraction. A rat, weighing about 150 g, was decapitated with one stroke. The liver was quickly excised, weighed, and rinsed two to three times in ice-cold modified Hank's solution. The liver tissue was then minced and homogenized in the ice-cold modified Hank's solution, at a ratio of one part of fresh liver tissue (by weight) to three parts of modified Hank's solution (by volume), with a Ten-Broeck homogenizer at medium speed for three to four complete strokes. The homogenate was then subjected to centrifugation at 9,000 rpm (7,000 x g) for 20 minutes in the Spinco rotor #30. The resulting 7,000 x g liver supernatant fraction, which contained both microsomal and soluble fractions, was decanted and refrigerated for later use. The processing yielded 3 ml of 7,000 x g liver supernatant fraction per gram of fresh liver tissue.

Incubation. Bacterial action during incubation was prevented by adding penicillin-streptomycin solution to the incubation mixture.

The volume of penicillin-streptomycin solution used was approximately 1% of the final incubation volume (20 units penicillin and 20 μg streptomycin per incubation).

In experiments with ^{14}C-acetate as a precursor, each incubation mixture contained 2.8×10^{-7} moles NADH, 2.4×10^{-7} moles NADPH, 11.7×10^{-7} moles ATP, 7.1×10^{-7} moles G-6-P, 0.45 μCi of [2-^{14}C]-sodium acetate (55.5 mCi/mmole), 0.3 ml 7,000 x g liver supernatant fraction, and 5 μl of either sodium chenodeoxycholate or sodium cholate solution at one of the eight different concentrations listed in the earlier part of the methods section. In control preparations, 5 μl bile acid solution was omitted, and instead, 5 μl distilled water was added to each incubation mixture. Final incubation volume was 360 μl, and final incubation pH was 7.5.

In experiments with ^{14}C-mevalonate as a precursor, each incubation mixture contained 7×10^{-7} moles NADH, 6×10^{-7} moles NADPH, 4.5×10^{-7} moles ATP, 17.8×10^{-7} moles G-6-P, 35.2×10^{-7} GSH, 0.045 μCi of [2-^{14}C]-DL-mevalonic acid (6.33 mCi/mmole), 0.3 ml 7,000 x g liver supernatant fraction, and 5 μl of either sodium chenodeoxycholate or sodium cholate solution at one of the eight different concentrations listed in the earlier part of the methods section. In control Preparations, 5 μl bile acid solution was omitted, and instead, 5 μl distilled water was added to each incubation mixture. Final incubation volume was 360 μl, and final incubation pH was 7.5

Each incubation mixture was contained in a 20 ml test tube, covered with aluminum foil. All incubations were carried out in a water bath (made by Precision Scientific, Subsidiary of GLA Corporation,

Chicago, Illinois) at 37°C for 20 hours, shaken at a speed of 100 rpm.

Extraction. At the end of the incubation period, 12 ml chloroform: methanol (2:1) was added to each incubation mixture for extracting total lipids. Each tube was vigorously mixed for 1 minute at medium speed with a Polytron homogenizer (made by Kinematica GMBH, Luzerne, Switzerland; distributed by Brinkmann Instruments, Westbury, New York). The mixture was then filtered through Whatman #41 filter paper, and the filtrate, which contained total lipids, was completely dried at reduced presure at room temperature.

Analysis. The dried residue was dissolved in 80 µl redistilled chloroform and applied to a thin-layer plate coated with silica gel H. The lipid standard was also applied to the same thin-layer plate for identifying the positions of corresponding spots. The thin-layer plate was developed in a solvent system consisting of petroleum ether: diethyl ether: acetic acid (80:20:2) for 25 to 30 minutes. The plate was then dried and exposed in an iodine vapor chamber for detection of the spots of cholesterol, free fatty acid, triglyceride, and cholesteryl ester. These were scraped into separate scintillation vials, then 10 ml Aquasol were added. The samples were vigorously shaken and then counted in a Packard Tri Carb Liquid Scintillation Spectrometer. The data obtained were converted to dpm and then plotted against molar concentration of CDCA or CA.

Uptake of Injected Bile Acid into Liver and Excretion into Intestine

Each animal received an intraperitoneal injection of labeled sodium cholate solution at one of the four different concentrations for

determining the effects of different doses on rate of incorporation of injected cholate into liver and intestinal contents listed in the earlier part of the methods section. Each solution was administered according to the body weight of the individual animal, namely 200 µl per 100 g body weight. One hour after injection, the rat was killed. Liver, small and large intestines were removed, rinsed in running water, and kept in a freezer for later processing. The liver tissue was weighed, homogenized in distilled water with a Polytron homogenizer at a ratio of approximately one part fresh liver tissue (by weight) to three parts distilled water (by volume). An aliquot of homogenate (equivalent to 0.1 g fresh liver weight) was digested in 1 ml Protosol at 55°C overnight in a glass scintillation vial covered with a plastic cap. At the end of digestion, 10 ml scintillation fluid (containing Triton X-100) was added. The sample was then mixed well and counted in a liquid scintillation counter. The quantity (µmoles), Q, of injected bile acid incorporated into liver tissue per 100 g body weight, was calculated according to the following formula:

$$Q = \frac{CPM \times 100}{SA \times Eff.} \times \frac{L.W. \times 10 \times 100}{B.W.} \qquad \text{Eq. 1}$$

where CPM is the observed radioactivity (counts per minute) obtained from 0.1 g liver; SA is the specific activity (dpm per µmole) of the cholate solution used for injection; Eff. is the counting efficiency (%) for that particular sample, as determined from a quenching curve; L.W. is the total weight (g) of liver; B.W. is the body weight. The first part of equation 1 converts observed CPM to µmoles per 0.1 g liver tissue; the second part normalizes the result to a basis of 100 g body weight.

A quenching curve was made by plotting the observed counting efficiency (%) against the channel ratio. This experiment was done by preparing a set of 14 samples, each contained 2 ml of Protosol and a quantity of liver homogenate (or homogenate of intestinal contents) equivalent to 300, 275, 250, 225, 200, 175, 150, 125, 100, 75, 50, 30, 10, and 0 mg of wet liver tissue (or wet intestinal contents) from a normal, untreated adult rat. These samples were contained in glass scintillation vials covered with plastic caps and digested at 55°C overnight. At the end of digestion, each sample was brought to a volume of 2.3 ml with distilled water. Then, 4.17×10^4 dpm of ^{14}C-toluene and 10 ml scintillation fluid were added to each sample. The samples were mixed well before the radioactivity was counted.

The small intestinal contents were squeezed into tared 25 ml graduated cylinders, weighed, and homogenized in distilled water with a Polytron homogenizer, at a ratio of approximately one part intestinal contents to four parts distilled water. Then, an aliquot of the homogenate (equivalent to 0.1 g intestinal contents) was removed and processed in the same manner as in the experiment for determining the uptake of injected bile acid into liver tissue.

The contents of the large intestine between cecum and anus were treated in the same way as the small intestinal contents. The following formula was used to calculate the quantity (μmoles), Q, of injected bile acid excreted into intestine per 100 g body weight.

$$Q = \frac{CPM \times 100}{SA \times Eff.} \times \frac{I.C.W. \times 10 \times 100}{B.W.} \qquad \text{Eq. 2}$$

where I.C.W. is the total weight (g) of intestinal contents; other terms are defined as in equation 1.

In the experiments to determine the effect of time on uptake of injected bile acid into liver and intestinal contents, each rat received an intraperitoneal injection of labeled sodium cholate solution (15×10^{-3} M) at a dose of 200 µl cholate solution per 100 g body weight. Animals were killed at time intervals after injection of 30, 60, 90, and 120 minutes. The livers, small and large intestinal contents were treated as described above.

Cholesterol Synthesis In Vivo

Preparation of animals. Each animal simultaneously received intraperitoneal injection of either sodium chenodeoxycholate or sodium cholate solution (at a dose of either 3×10^{-6} moles or 10×10^{-6} moles per 100 g body weight) and one of the radioactive precursors. The labeled precursors were administered as follows: approximately 9 µCi [2-^{14}C]-sodium acetate (55.5 mCi/mmole) per 100 g body weight, and 2 µCi [2-^{14}C]-DL-mevalonic acid (6.33 mCi/mmole) per 100 g body weight. Control animals received only an intraperitoneal injection of one of the labeled precursors. One hour after injection, animals were killed. Their livers were quickly excised, weighed, rinsed in distilled water, and kept in a freezer at -10°C.

Analysis. The liver tissue was homogenized in distilled water, at a ratio of one part fresh liver tissue to two parts distilled water, with a Polytron homogenizer at medium speed. Three-tenths milliliter of homogenate (equivalent to 0.1 g fresh liver) and 12 ml chloroform: me-

thanol (2:1) were vigorously mixed together for one minute at medium speed on the homogenizer. The mixture was filtered through Whatman #41 filter paper. The filtrate was completely dried at reduced pressure at room temperature. The dried residue was dissolved in 0.1 ml redistilled chloroform and then processed in the same manner as in the experiment *in vitro*. The following equation was used to convert the data obtained to dpm, Q, of ^{14}C-cholesterol, ^{14}C-free fatty acid, ^{14}C-triglyceride, and ^{14}C-cholesteryl ester synthesized per 100 g body weight.

$$Q = \frac{CPM \times 100}{Eff.} \times \frac{L.W. \times 10 \times 100}{B.W.} \qquad \text{Eq. 3}$$

where the terms are defined as in equation 1.

Effects of irritation and stress. Each animal simultaneously received intraperitoneal injections of either Celite at a dose of 5 mg per 100 g body weight (47) or 0.42 M saline at a dose of 200 µl per 100 g body weight, and one of the radioactive precursors at the same doses that were used in the experiments relating injected bile acids with cholesterol synthesis. The liver tissue was then subjected to the procedure described above.

CHAPTER III

RESULTS

Uptake of Injected Bile Acid into Liver and Excretion into Intestine

When the quantity of administered cholate was increased, the uptake of injected ^{14}C-cholate into liver was also increased at the end of one hour, with the greatest change in the uptake occuring below 10 μmoles per 100 g body weight ($p = 0.002$ for comparing the doses of 3 and 10 μmoles per 100 g body weight)(Figure 7). The excretion of injected ^{14}C-cholate into the large intestine rapidly reached a plateau at 3 μmoles per 100 g body weight ($p = 0.025$ for comparing the doses of 1 and 3 μmoles per 100 g body weight). The excretion of injected ^{14}C-cholate into the small intestine and the total recovery of labeled cholate from liver and from the small and large intestinal contents increased linearly below 10 μmoles per 100 g body weight.

The uptake of injected ^{14}C-cholate into liver was nearly constant at different time intervals, 30, 60, 90, and 120 minutes (Figure 8). The excretion of injected ^{14}C-cholate into small intestine markedly increased 90 minutes after injection. The recovery peak for excretion of labeled cholate into the large intestine observed at 60 minutes after

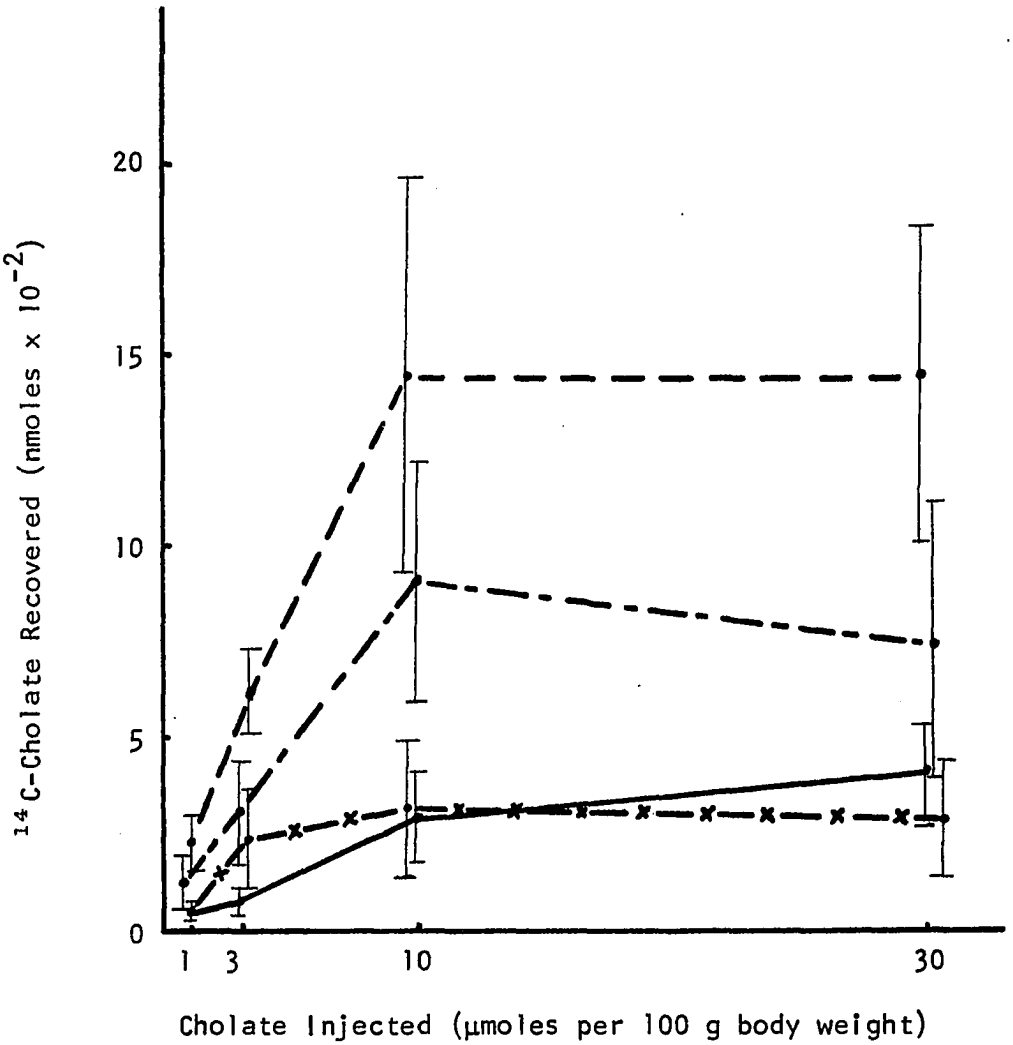


Figure 7. Effect of cholate dose on uptake of ^{14}C-cholate into liver and excretion into small and large intestines in one hour after intraperitoneal injection.

●——●, ●- - -●, ●—x—●, and ●– – –● represent recoveries from liver, small and large intestinal contents, and total recovery, respectively.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

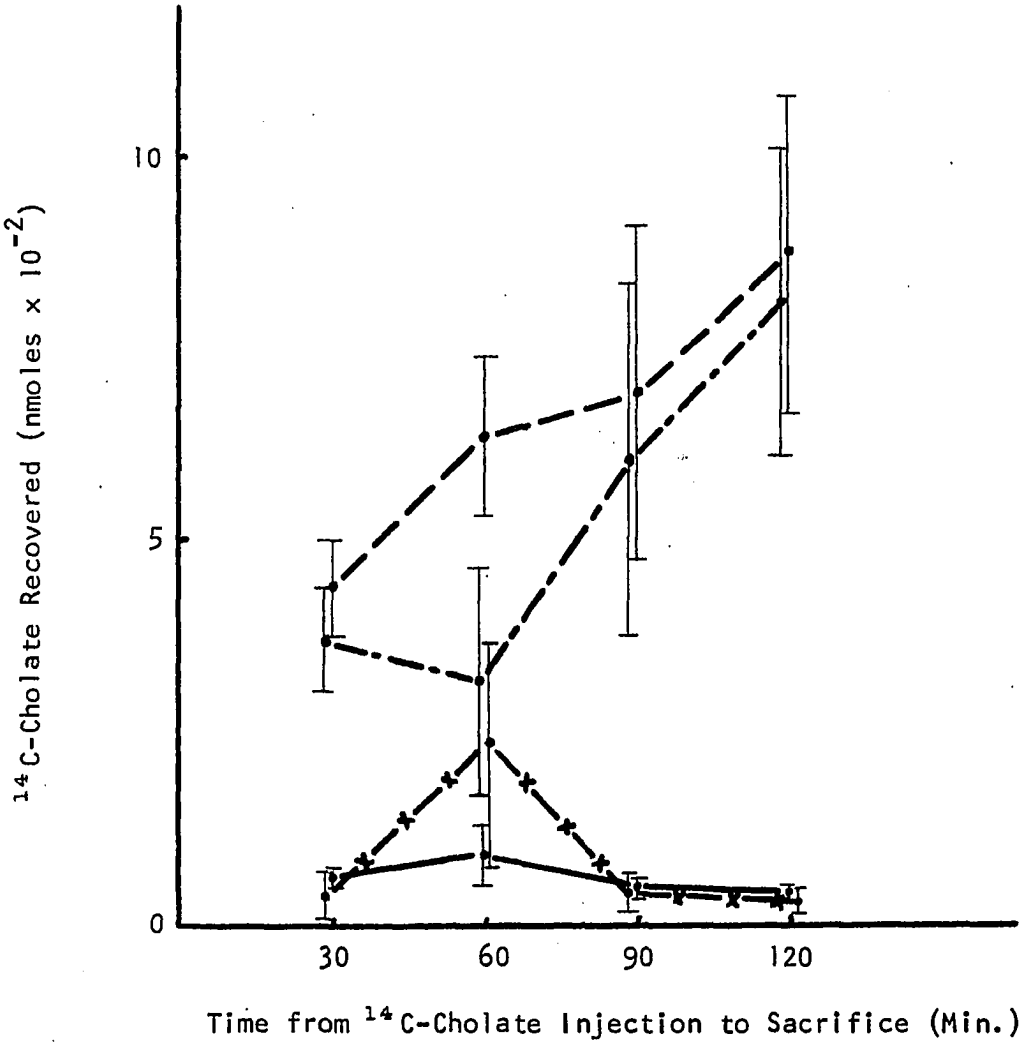


Figure 8. Uptake of ^{14}C-cholate into liver and excretion into intestines as a function of time. ^{14}C-Cholate was injected intraperitoneally at a dose of 3 μmoles per 100 g body weight.

●——●, ●—·—●, ●—x—●, and ●– – –● represent recoveries from liver, small and large intestinal contents, and total recovery, respectively.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

injection was probably not significant. The total recovery of ^{14}C-cholate from liver and from the small and large intestinal contents increased steadily with time.

Cholesterol

Experiments In Vitro

Increasing concentrations of either chenodeoxycholic acid (CDCA) or cholic acid (CA) up to about 2×10^{-3} M markedly suppressed cholesterol synthesis from [2-^{14}C]-sodium acetate (Figure 9A).

When [2-^{14}C]-DL-mevalonic acid DBED salt was used as a precursor (Figure 9B), cholesterol synthesis was greatly reduced by increasing concentrations of CDCA up to about 0.5×10^{-3} M or CA up to 2.8×10^{-3} M. A less pronounced decrease in cholesterol synthesis occurred with CDCA concentration between 0.5 to 2.8×10^{-3} M.

Experiments In Vivo

Incorporation of isotope into cholesterol was increased by both CDCA and CA (Figure 10). The stimulatory effect of CDCA (Figure 10A) on incorporation of ^{14}C-acetate into cholesterol was more pronounced than the effect of CA (Figure 10B). Rats treated with CA showed an augmented incorporation of ^{14}C-acetate (Figure 10B) of approximately the same extent as the incorporation of ^{14}C-mevalonate in rats injected with CDCA (Figure 10C). Incorporation of ^{14}C-mevalonate into cholesterol was not significantly affected by CA (Figure 10D).

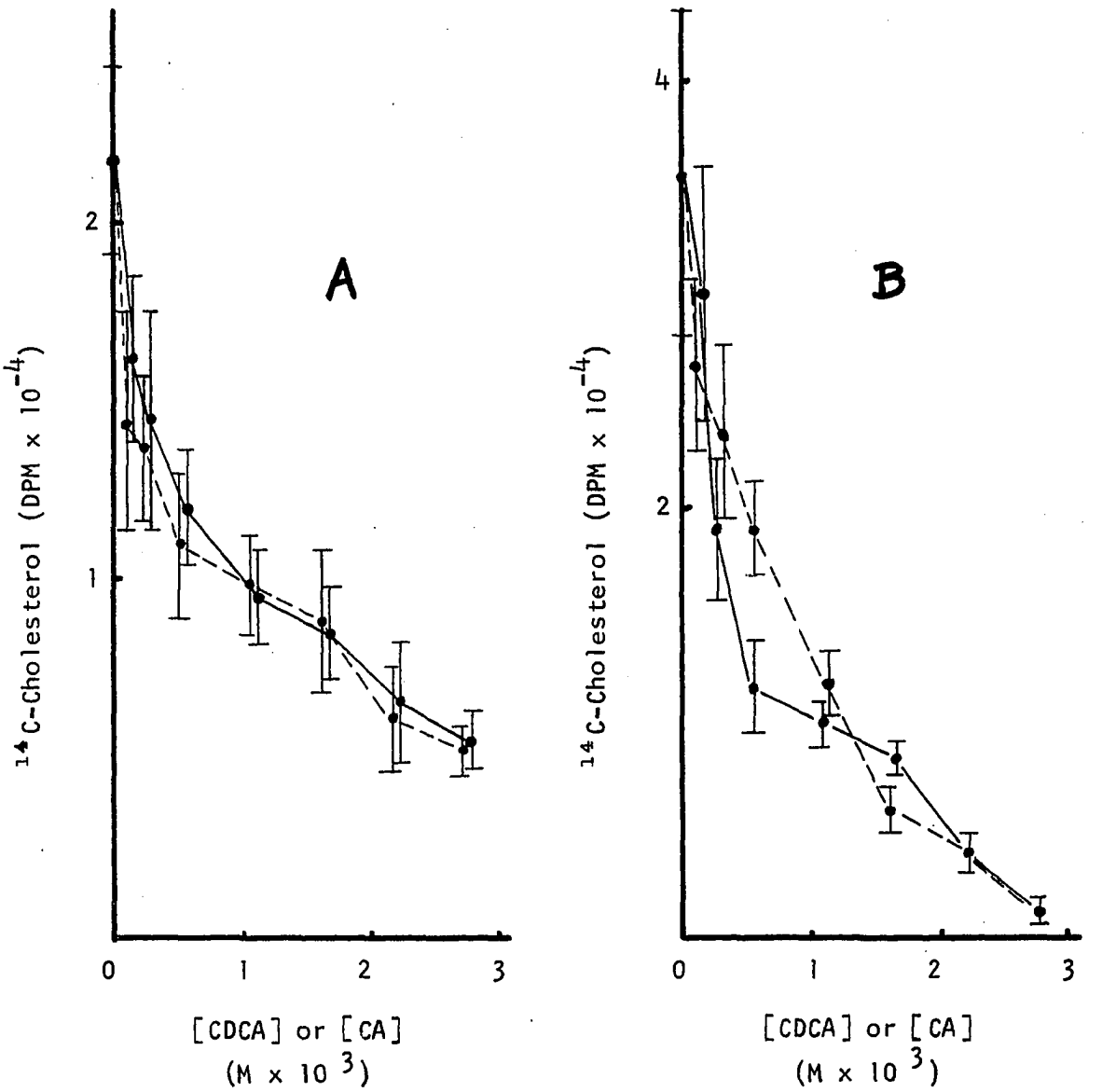


Figure 9. Effects of CDCA and CA on incorporation of [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-cholesterol in rat liver, in vitro.

●——● and ●– – –● represent incubation mixtures containing CDCA and CA respectively. Each point represents an average of 6 rats. Each incubation contained 7,000 x g liver supernatant fraction equivalent to 0.1 g fresh liver. Cofactors listed in the methods section.

I represents standard deviation calculated from 6 rats.

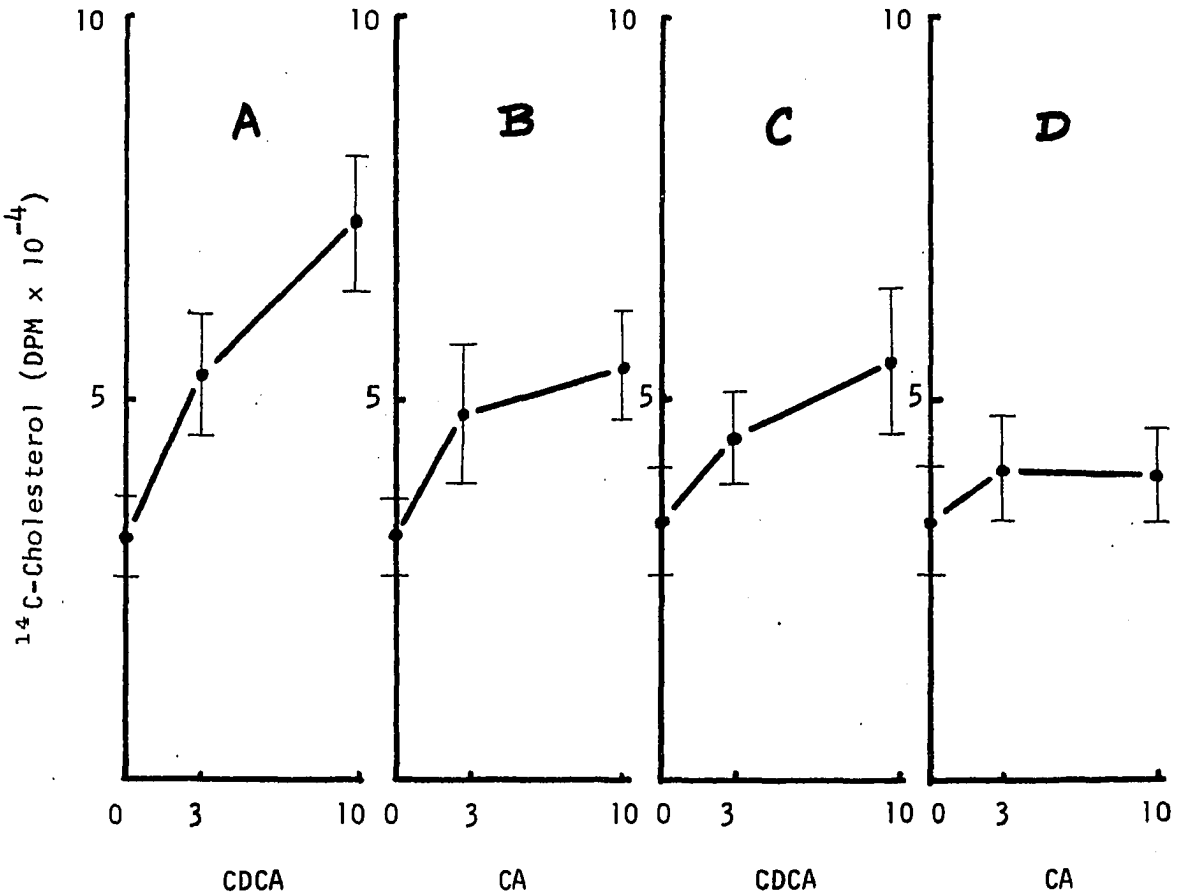


Figure 10. Effects of CDCA and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figures A & B) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figures C & D) into ^{14}C-cholesterol in one hour.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

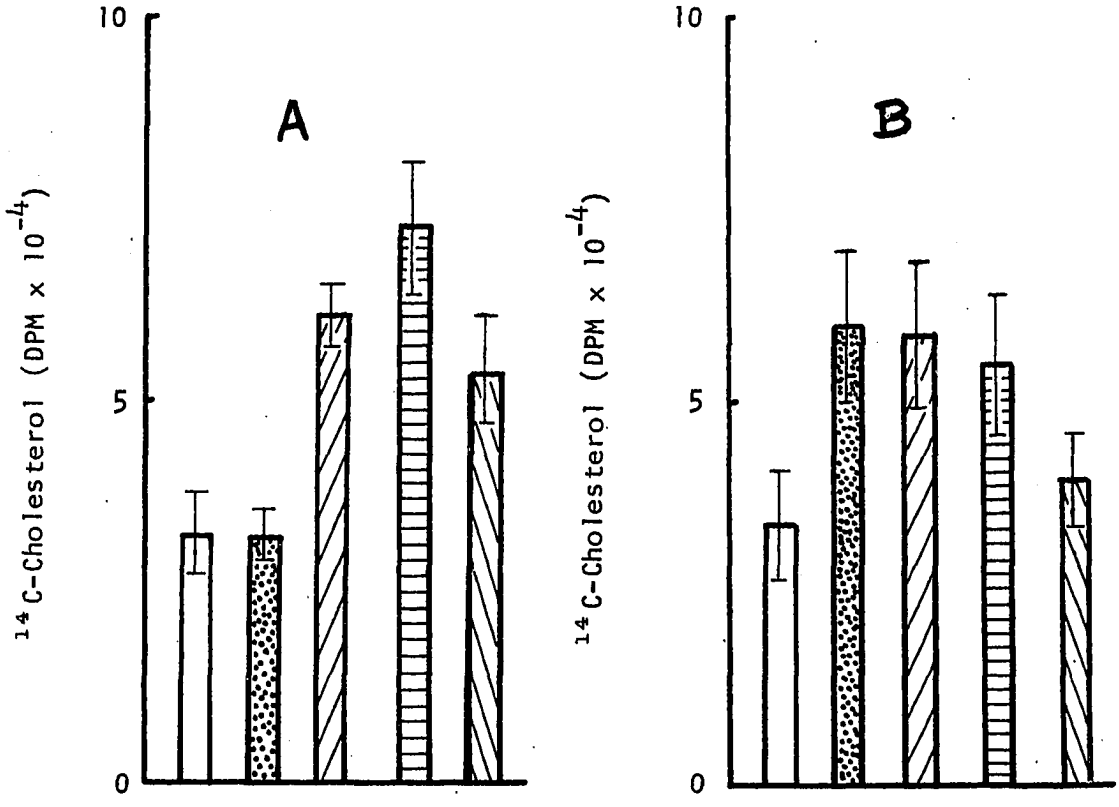


Figure 11. Effects of 0.42 M saline, Celite, CDCA, and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-cholesterol in one hour.

, , , , and represent control rats and rats treated with 0.42 M saline, Celite, CDCA, and CA, respectively. Parenteral dosage of CDCA or CA was 10 µmoles per 100 g body weight.

I represents standard deviation calculated from 6 rats.

Each bar represents an average of 6 rats.

Chenodeoxycholic acid, CA, and Celite increased the incorporation of ^{14}C-acetate into cholesterol to about the same extent, whereas 0.42 M saline failed to show any effect on incorporation (Figure 11A). Chenodeoxycholic acid, Celite, and 0.42 M saline increased the incorporation of ^{14}C-mevalonate into cholesterol to the same extent (Figure 11B), whereas the effect of CA was insignificant.

Free Fatty Acid

Experiments In Vitro

Free fatty acid synthesis from [2-^{14}C]-sodium acetate was significantly inhibited by both CDCA and CA up to 1.7×10^{-3} M (Figure 12A). Further increases in either CDCA or CA concentrations had little further effect on synthesis.

Significant quantities of free fatty acid were synthesized from [2-^{14}C]-DL-mevalonic acid DBED salt (Figure 12B). No significant effects on free fatty acid synthesis were observed at concentrations of CDCA below 0.2×10^{-3} M and CA below 0.5×10^{-3} M. Synthesis was markedly reduced by increasing concentrations of either CDCA or CA; at concentrations below 2×10^{-3} M, CDCA was significantly more inhibitory.

Experiments In Vivo

Incorporation of ^{14}C-acetate into free fatty acid was decreased in the rats treated with either CDCA or CA (Figure 13), whereas incorporation of ^{14}C-mevalonate was slightly increased by either CDCA or CA

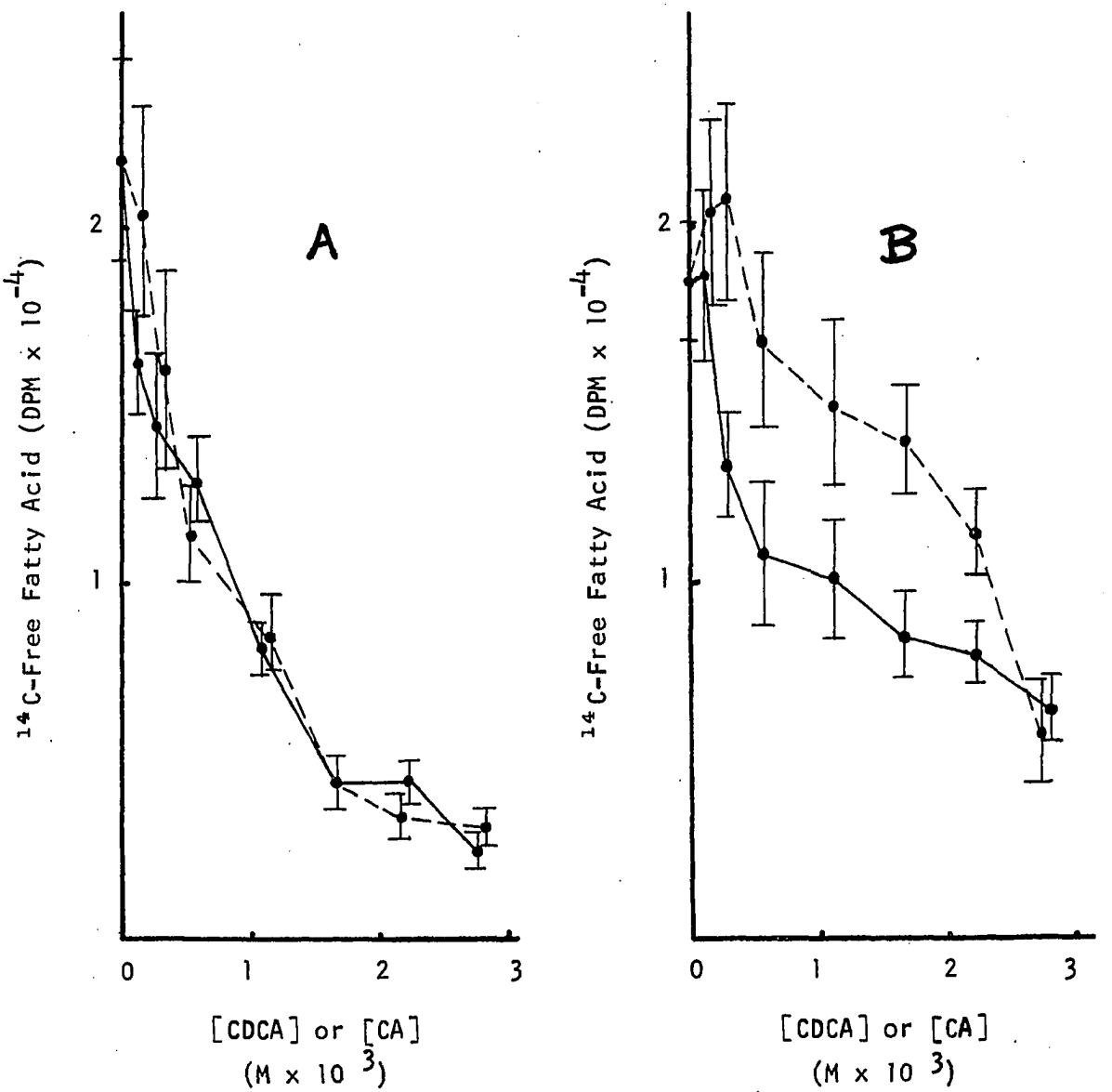


Figure 12. Effects of CDCA and CA on incorporation of [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-free fatty acid in rat liver, *in vitro*.

●——● and ●- - -● represent incubation mixtures containing CDCA and CA respectively. Each point represents an average of 6 rats. Each incubation contained 7,000 x g liver supernatant fraction equivalent to 0.1 g fresh liver. Cofactors listed in the methods section.

I represents standard deviation calculated from 6 rats.

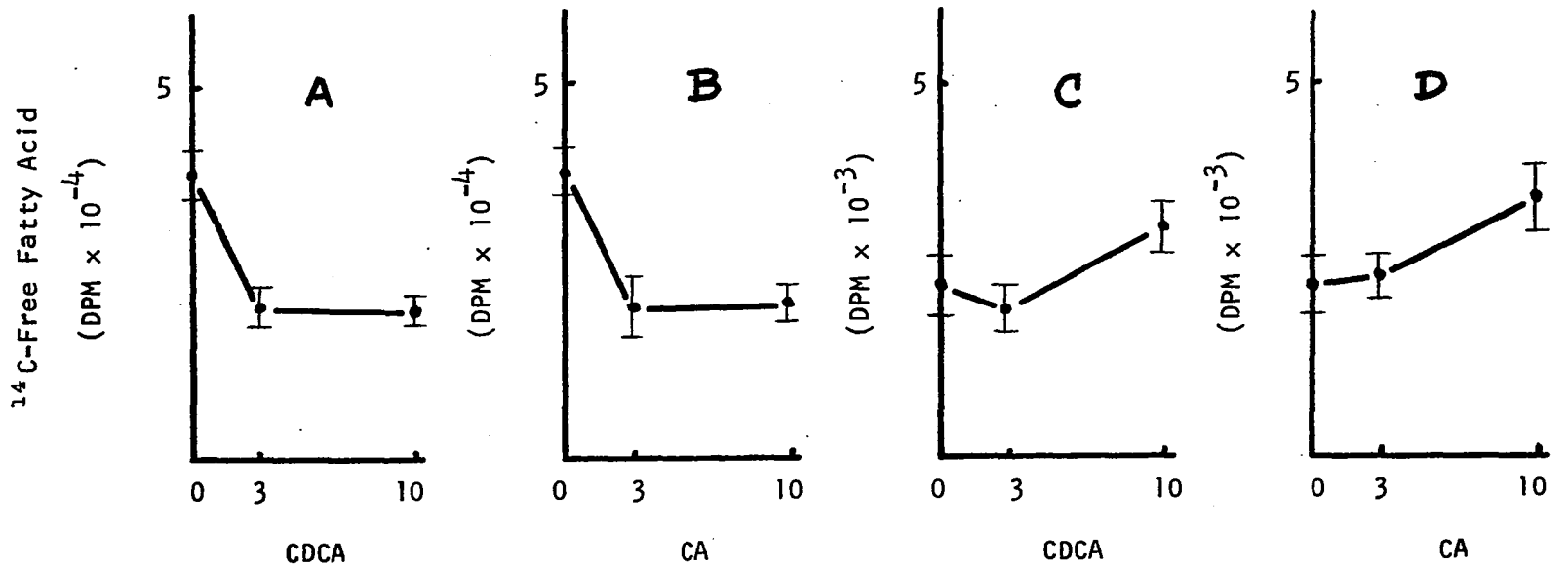


Figure 13. Effects of CDCA and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figure A & B) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure C & D) into ^{14}C-free fatty acid in one hour.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

at 10 μmoles per 100 g body weight.

Incorporation of ^{14}C-acetate into free fatty acid was significantly increased by Celite, but suppressed by CDCA, CA, and 0.42 M saline (Figure 14A); whereas the incorporation of ^{14}C-mevalonate into free fatty acid was reduced by 0.42 M saline, enhanced by CDCA and CA, and unaffected by Celite (Figure 14B).

Triglycerides

Experiments In Vitro

Both CDCA and CA at concentrations below 0.5×10^{-3} M markedly reduced triglyceride synthesis from [2-^{14}C]-sodium acetate (Figure 15A). Further increases in either CDCA or CA concentrations had only a slight effect on synthesis.

Triglyceride synthesis from [2-^{14}C]-DL-mevalonic acid DBED salt (Figure 15B) was not significantly affected by either bile acid at concentrations below 1×10^{-3} M, whereas at higher concentrations, both CDCA and CA inhibited synthesis to about the same extent.

Experiments In Vivo

Both CDCA and CA enhanced slightly the incorporation of ^{14}C-acetate into triglyceride, and were associated with a more pronounced effect on incorporation of ^{14}C-mevalonate (Figure 16).

The effects of 0.42 M saline, Celite, CDCA, and CA on the incorporation of ^{14}C-acetate into triglyceride were negligible (Figure 17A);

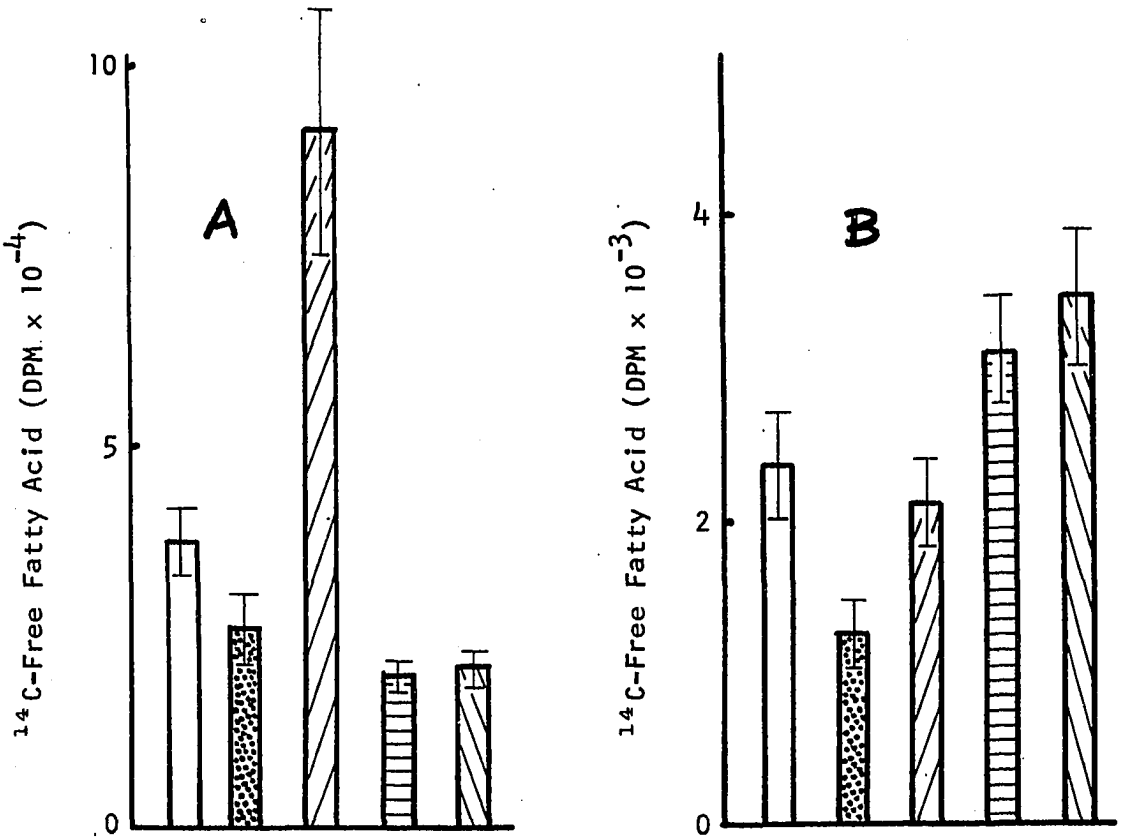


Figure 14. Effects of 0.42 M saline, Celite, CDCA, and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-free fatty acid in one hour.

, , , , and represent control rats and rats treated with 0.42 M saline, Celite, CDCA, and CA, respectively. Parenteral dosage of CDCA or CA was 10 µmoles per 100 g body weight.

I represents standard deviation calculated from 6 rats.

Each bar represents an average of 6 rats.

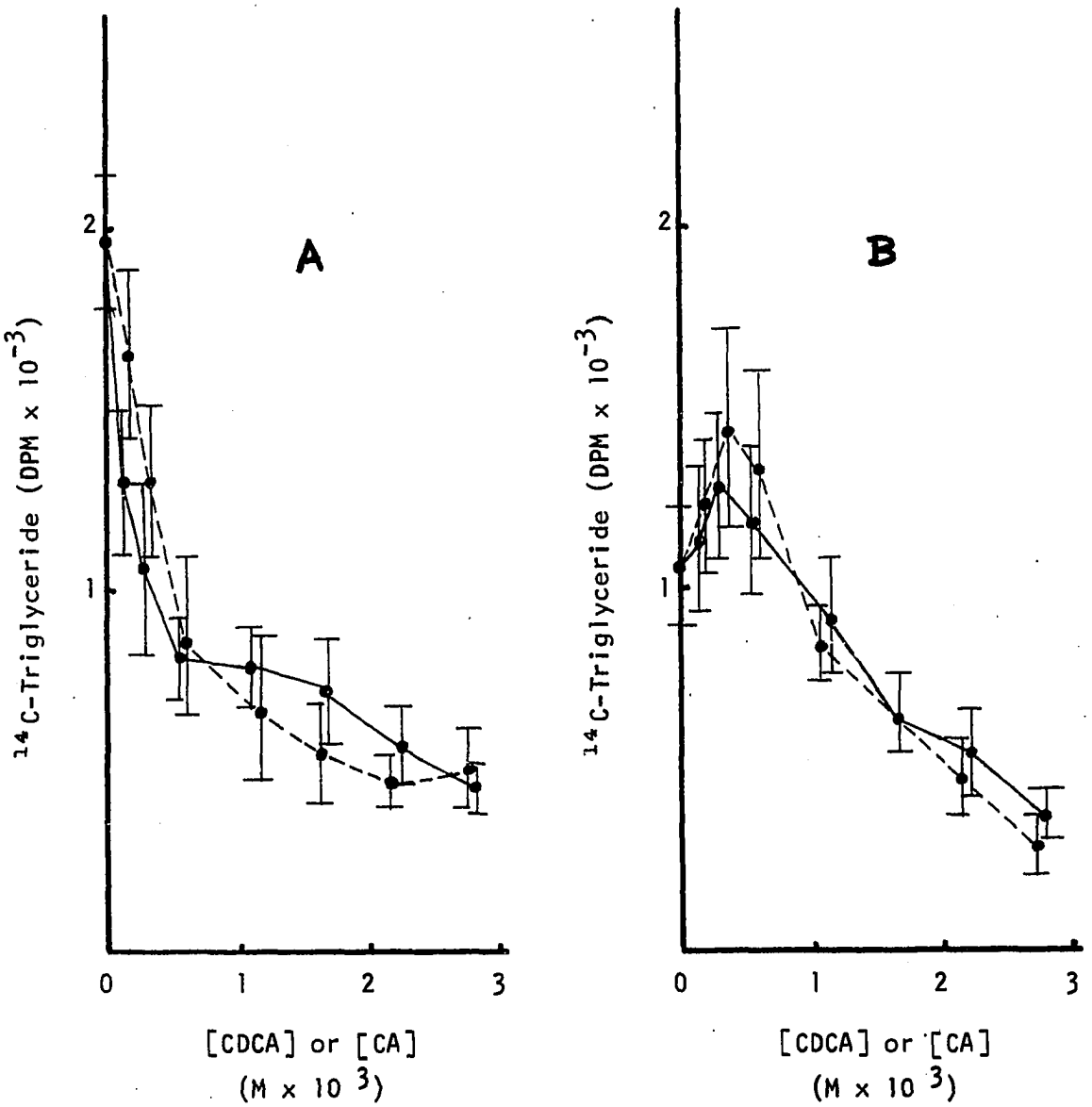


Figure 15. Effects of CDCA and CA on incorporation of [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-triglyceride in rat liver, <u>in vitro</u>.

●——● and ●- - -● represent incubation mixtures containing CDCA and CA respectively. Each point represents an average of 6 rats. Each incubation contained 7,000 x g liver supernatant fraction equivalent to 0.1 g fresh liver. Cofactors listed in the methods section.

I represents standard deviation calculated from 6 rats.

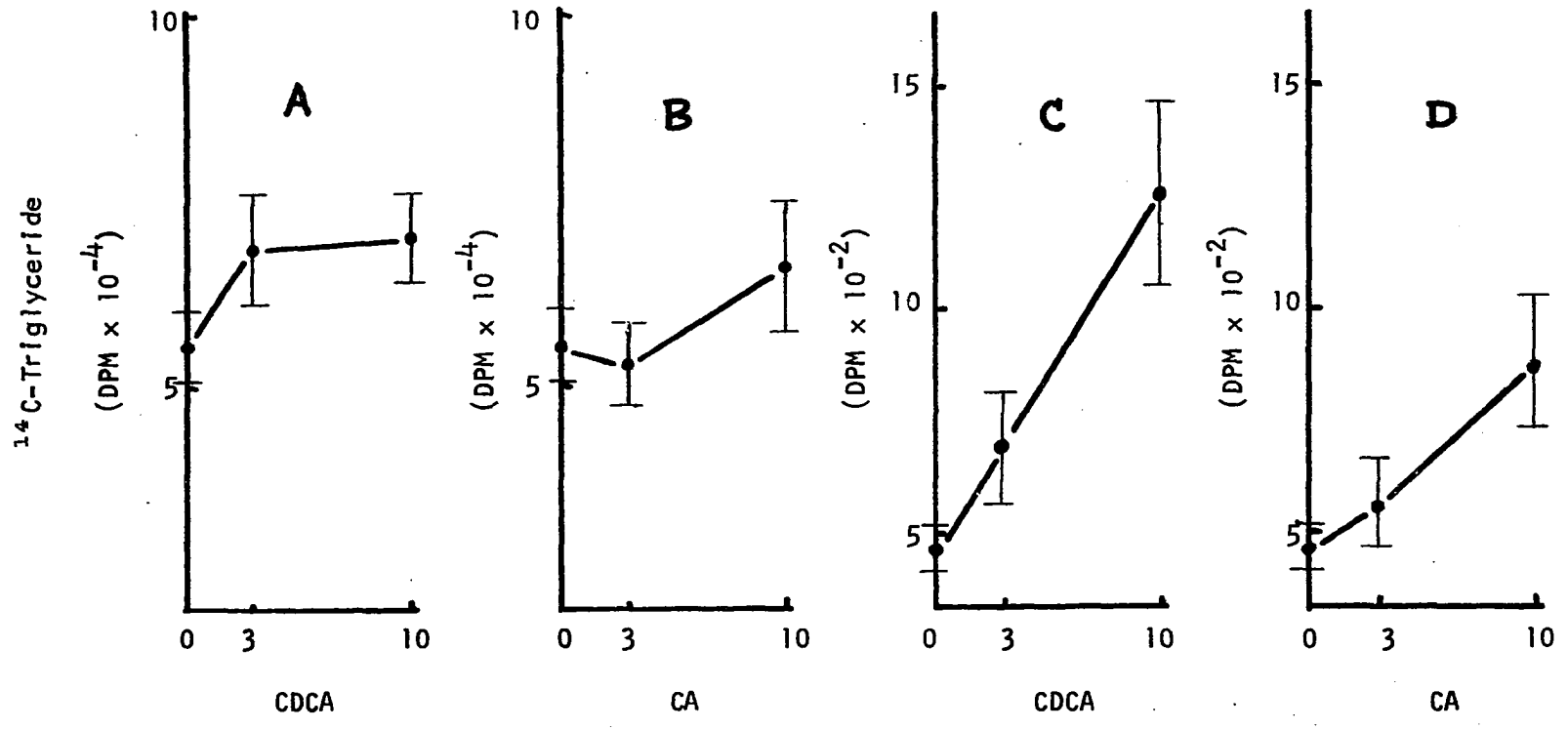


Figure 16. Effects of CDCA and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figures A & B) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figures C & D) into ^{14}C-triglyceride in one hour.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

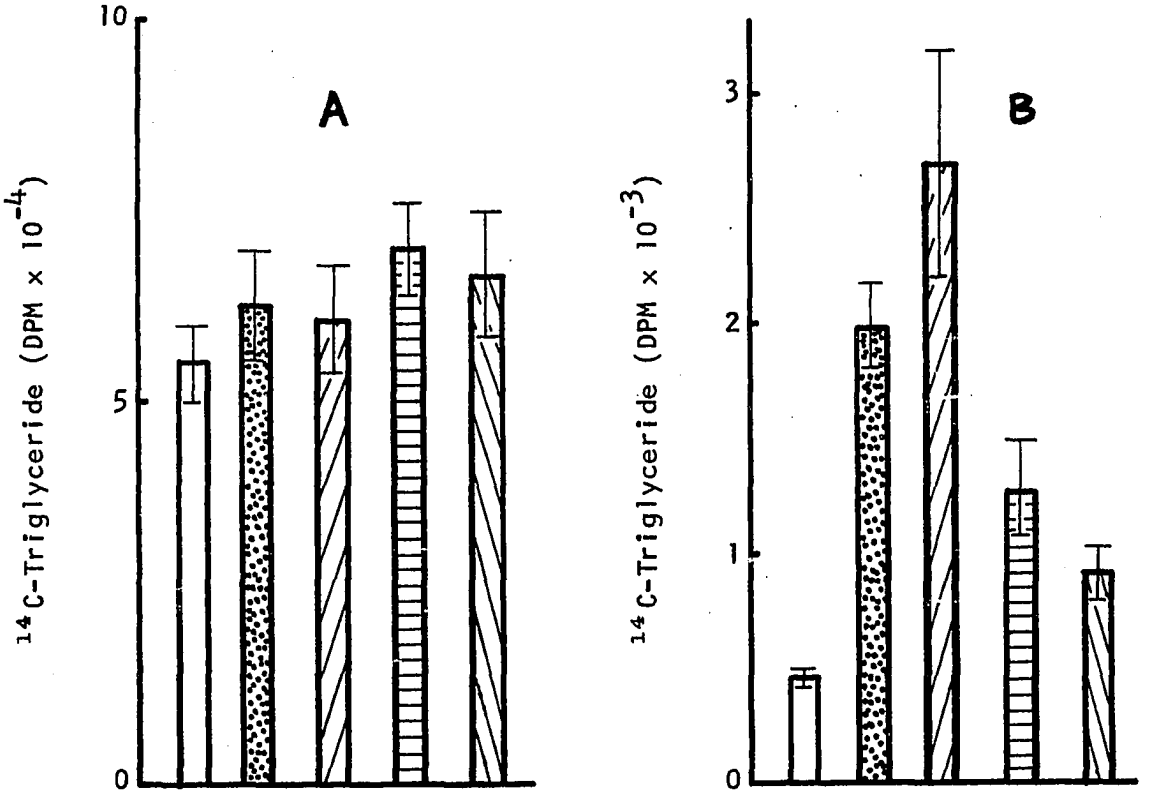


Figure 17. Effects of 0.42 M saline, Celite, CDCA, and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-triglyceride in one hour.

, , , , and represent control rats and rats treated with 0.42 M saline, Celite, CDCA, and CA, respectively. Parenteral dosage of CDCA or CA was 10 μmoles per 100 g body weight.

I represents standard deviation calculated from 6 rats.

Each bar represents an average of 6 rats.

but the incorporation of ^{14}C-mevalonate into triglyceride was significantly increased by all of them, with the most pronounced effect for 0.42 M saline and Celite (Figure 17B).

Cholesteryl Esters

Experiments In Vitro

A significant decrease in cholesteryl ester synthesis from [2-^{14}C]-sodium acetate occurred at CDCA or CA concentrations below 1 x 10^{-3} M (Figure 18A). A further increase in the concentrations of either primary bile acid showed little further effect on synthesis.

When [2-^{14}C]-DL-mevalonic acid DBED salt was used as a precursor (Figure 18B), a marked drop in cholesteryl ester synthesis occurred at CDCA concentrations lower than 0.5 x 10^{-3} M, then the synthesis decreased slowly at higher concentrations. At concentrations below 0.5 x 10^{-3} M, CA had no marked effect on synthesis; whereas at higher concentrations, the effects of the two bile acids became more nearly equal.

Experiments In Vivo

Both primary bile acids inhibited incorporation of ^{14}C-acetate into cholesteryl ester, but they stimulated incorporation of ^{14}C-mevalonate (Figure 19).

The incorporation of ^{14}C-acetate into cholesteryl ester (Figure 20A) was not affected by 0.42 M saline and Celite, but suppressed by CDCA and CA. Incorporation of ^{14}C-mevalonate into cholesteryl ester

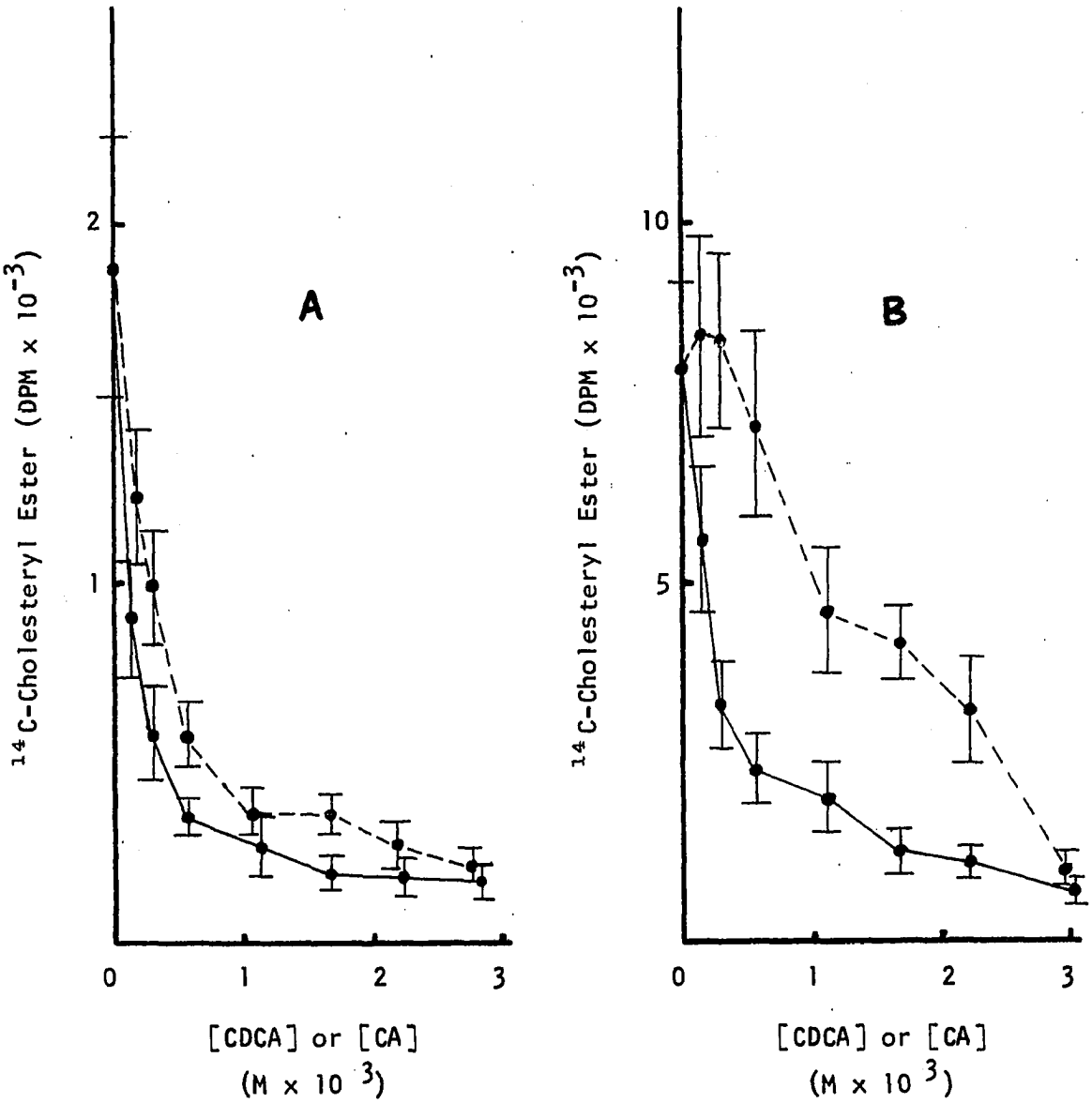


Figure 18. Effects of CDCA and CA on incorporation of [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-cholesteryl ester in rat liver, in vitro.

●——● and ●– – –● represent incubation mixtures containing CDCA and CA respectively. Each point represents an average of 6 rats. Each incubation contained 7,000 x g liver supernatant fraction equivalent to 0.1 g fresh liver. Cofactors listed in the methods section.

I represents standard deviation calculated from 6 rats.

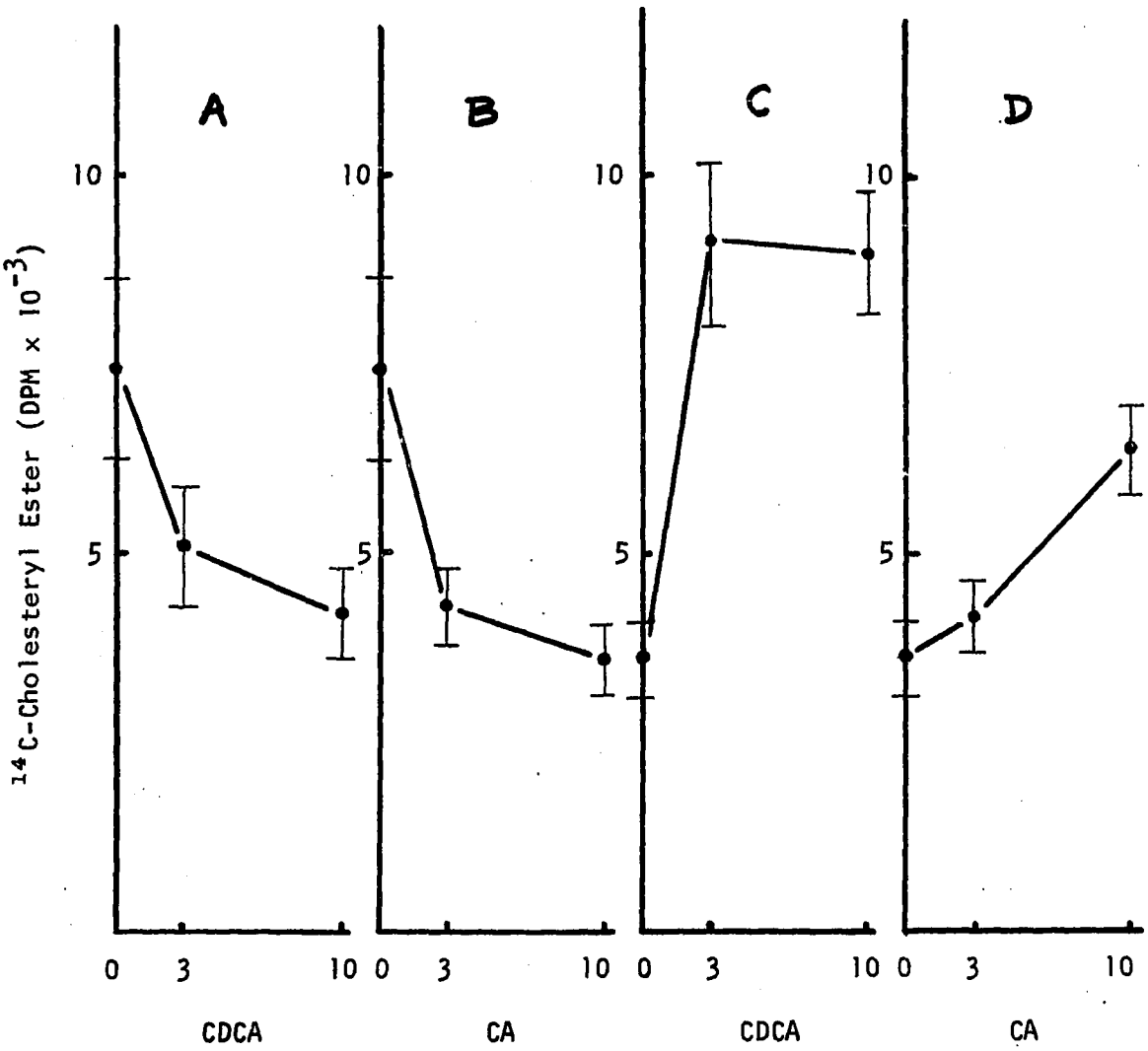


Figure 19. Effects of CDCA and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figures A & B) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figures C & D) into ^{14}C-cholesteryl ester in one hour.

I represents standard deviation calculated from 6 rats.

Each point represents an average of 6 rats.

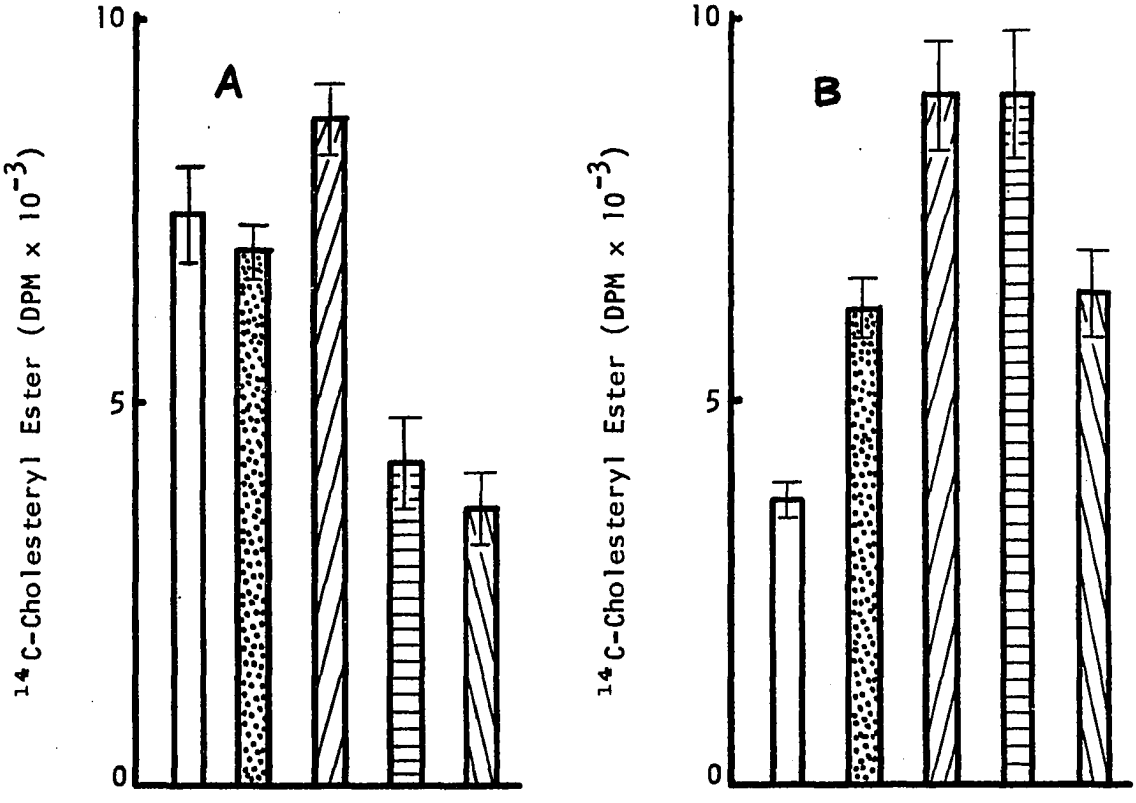


Figure 20. Effects of 0.42 M saline, Celite, CDCA, and CA on hepatic incorporation of intraperitoneally administered [2-^{14}C]-sodium acetate (Figure A) and [2-^{14}C]-DL-mevalonic acid DBED salt (Figure B) into ^{14}C-cholesteryl ester in one hour.

, , , , and represent control rats and rats treated with 0.42 M saline, Celite, CDCA, and CA, respectively. Parenteral dosage of CDCA or CA was 10 µmoles per 100 g body weight.

I represents standard deviation calculated from 6 rats.

Each bar represents an average of 6 rats.

(Figure 20B) was significantly stimulated by 0.42 M saline, Celite, CDCA, and CA, with the most pronounced effect for Celite and CDCA.

CHAPTER IV

DISCUSSION

Knowledge concerning the hepatic uptake and retention of intraperitoneally administered bile acid is necessary to evaluate the effect of administered bile acid on hepatic metabolic processes *in vivo*. Because parenteral ^{14}C-bile acid is largely transported to the liver and excreted into the intestine, the total recovery of labeled bile acid from liver and from the small and large intestinal contents may represent the total quantity of parenteral bile acids taken up by the liver. This is not true in the case of orally administered labeled bile acids, because the entire dose will pass into the intestine and mix with the bile acids excreted by the liver. The knowledge derived from parenteral administration could provide a more accurate comparison between the results obtained in the experiments *in vitro* and *in vivo*.

Uptake of Injected Bile Acid into Liver and Excretion into Intestine

A significant increase in the hepatic uptake of injected bile acid occurred with increasing doses up to 10 μmoles per 100 g body weight (Figure 7). A further increase in parenteral dosage caused no further significant change in total recovery from liver and from the small and large intestinal contents. This indicates that the mechanism responsible

for absorption of bile acids from the intraperitoneal space becomes saturated at a dose level of about 10 μmoles per 100 g body weight.

At a dose level of 3 μmoles per 100 g body weight (Figure 8), the low recoveries of injected cholate from the contents of large intestine might indicate that there was insufficient time for the small intestine to deliver labeled cholate into the large intestine. The nearly constant uptake of parenteral cholate into liver was reasonable, because the normal hepatic function is to synthesize and to excrete bile acids, but not to retain them. The increasing recoveries of parenteral cholate from the contents of the small intestine with time might indicate that large quantities of injected cholate were being taken up by the liver and excreted into the small intestine.

Administration of a large dose of bile acid to a subject might cause a sharp increase in the bile acid pool contained in the liver and small intestine. This could produce irritation and even cause tissue damage. In view of these disadvantages, frequent administration of small doses of bile acid to a subject might prevent many side-effects, especially those resulting from stress and irritation. The latter will be discussed in subsequent sections.

The twenty-hour incubations in the experiments performed in vitro could have favored bacterial growth in the incubation media, especially in control preparations and in preparations with low bile acid concentrations. To prevent this from happening, penicillin and streptomycin were added to the incubation mixture.

In the experiments in vivo, stress and irritation in the rats receiving intraperitoneal injections of bile acids could have had an

effect on some metabolic processes (48). This effect of stress, which would mask the true effect of bile acid, had to be evaluated. In this work, Celite (5 mg per 100 g body weight) and 0.42 M saline (84 μmoles NaCl per 100 g body weight) were used as stress-producing agents. However, the effect of stress caused by parenteral 0.42 M saline might not be exactly the same as that caused by parenteral Celite. Possibly the control animals were also affected by the physical stress of handling, and by the stress resulting from intraperitoneal injection of labeled precursor. However, these physical stress should also occur in the animals treated with 0.42 M saline, Celite, CDCA, or CA.

The aim of this research was to study the effects of parenteral administration of primary bile acids on the hepatic synthesis of cholesterol and related neutral lipids. In the experiments discussed herein, the bile acid concentrations that were employed were at levels which correspond to those that could result from oral administration of bile acid to human subjects in the treatment of cholelithiasis (1-4).

Cholesterol

In vitro, the inhibitory effects of both primary bile acids on hepatic synthesis of cholesterol from ^{14}C-acetate and ^{14}C-mevalonate were approximately the same (Figures 9A & B). The more pronounced effect of CDCA at lower concentrations on synthesis from ^{14}C-mevalonate might indicate either that CDCA is a better inhibitor of the cholesterol synthetic pathway than CA or that CDCA is more toxic to enzymes.

In vivo, the stimulatory effect of either CDCA or CA on the incorporation of labeled precursors into cholesterol (Figure 10) might be

due to increased cholesterol synthesis. This could occur in several possible ways:

1. Since augmented incorporation of both ^{14}C-acetate and ^{14}C-mevalonate into hepatic cholesterol was also observed in the rats treated with parenteral Celite (Figure 11), the result observed in the rats treated with CDCA and CA might be due to the effect of stress (48). The postulated increase in the incorporation of ^{14}C-acetate into cholesterol in the rats treated with either primary bile acid might be caused by enhanced enzymatic activity or increased enzyme levels in the steps prior to mevalonate, and is consistent with the results reported by de Matteis (48). However, it is improbable that enzyme levels increased significantly in such a short time. Parenteral 0.42 M saline also increased incorporation of ^{14}C-mevalonate into cholesterol, but failed to show any effect on incorporation of ^{14}C-acetate into cholesterol. These results, apparently, suggest that the effect of stress caused by Celite suspension and the effect of stress produced by 0.42 M sodium chloride solution are not the same. An inhibitory effect of sodium ion on acetyl-Co A synthetase *in vitro* has been reported by Webster (49). It is not clear whether this inhibition also occurs in the system *in vivo*, since we do not know to what extent, if any, parenteral 0.42 M saline or sodium salts of CDCA and CA affect the intracellular sodium ion concentration of liver cells.

2. Hepatic cholesterol synthesis *in vitro* can be increased by steroid carrier protein (SCP), which is contained in the 105,000 x g liver supernatant fraction (6-8). *In vivo*, CDCA and CA might be able to stimulate synthesis or release of SCP. An elevated SCP level

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would enable cholesterol synthetic enzymes to work more efficiently. There is no evidence, however, to support this speculation. The postulated increase in SCP level could be measured after incubating labeled cholesterol or intermediates of the cholesterol synthetic pathway with purified liver soluble fractions isolated from untreated rats and from rats treated with parenteral bile acid. The steroid-SCP complex could then be isolated, and quantitatively determined.

3. The detergent properties of bile acids might alter the transport mechanism of liver cell membranes, so that the entrance of labeled precursors and other extracellular components into liver cells would be facilitated.

4. Since cholesterol excretion and the specific activities of hepatic cholesterol were not determined, and since bile acids, especially chenodeoxycholic acid, can promote intestinal reabsorption of cholesterol, there is a possibility that cholesterol excretion was diminished by the intraperitoneally administered bile acid. Therefore, ^{14}C-cholesterol synthesized from labeled precursors would accumulate in the liver, and the increased radioactivity of liver cholesterol would reflect accumulation, not increased synthesis.

5. It is also possible that some other unknown mechanism might be involved in this increased incorporation of precursors into cholesterol.

According to some investigators, bile acids can inhibit both cholesterol and bile acid synthesis in the rat in vitro and in vivo (50-55), whereas others (56-57) have concluded that bile acids play no direct role in the control of hepatic cholesterogenesis. They assert

that the hepatic cholesterol synthesis is directly regulated by the quantity of cholesterol itself in a pool which is undergoing enterohepatic circulation.

The inhibitory effect of bile acids on hepatic cholesterol synthesis *in vitro* in this study was consistent with previous reports (52-54) although there is a difference in procedure. For instance, an inhibition of cholic acid on hepatic cholesterol synthesis has been shown by Back *et al.* (54). They fed male Sprague-Dawley rats weighing 100-200 g with a diet supplemented with 1% cholic acid for various periods of time, and incubated liver slices from these animals with ^{14}C-acetate in a medium equilibrated with O_2/CO_2 (95%/5%) at 37^{o}C for 10 minutes.

In vivo, the discrepancies between results obtained in this study and those in other investigations (55) may be due to differences in procedures and animals. For example, Schriewer *et al.* (55) treated male Wistar rats, weighing approximately 200 g, with a single intraperitoneal injection of 5.75-23 mg sodium deoxycholate to produce, within 6-8 hours, severe liver injury, ultrastructural alterations, and a general decrease in hepatic neutral fat, free fatty acid, and phospholipids. In contrast, in the present study female Holtzman rats weighing 110-150 g were used, which had been treated with intraperitoneal injections of bile acid and labeled precursor simultaneously; one hour after injection, the animals were killed, the livers were homogenized, and the levels of ^{14}C-cholesterol in these homogenates were measured. The male Wistar rats used in the experiments of Schriewer *et al.* were much older than the female Holtzman rats used in this study. Cholesterol synthesis might be affected by sex, age, and strain. Schriewer *et al.* also used a different bile acid

at different parenteral dosages and carried out their experiment for a longer time.

The general decrease in neutral fat and free fatty acid caused by the parenteral bile acid in the experiments of Schriewer et al. (55) might have been the consequence of liver injury. This injury has become increasing severe with prolonged experimental time, because the injected bile acid was being incorporated into the enterohepatic circulating pool, eventually reaching a toxic level and leading to progressive tissue damage. In contrast, the present study did not involve these problems, because time did not allow extensive recirculation of the administered bile acid.

In the course of clinical dissolution of human cholesterol gallstones with CDCA, the oral dosage is large enough to cause diarrhea (3). This means that oral administration of CDCA to a patient can also produce stress. Although the effect of stress caused by oral CDCA might differ from that produced by parenteral bile acid, caution is indicated in using CDCA for treating cholesterol gallstones, since CDCA has been shown in the present study to cause increased synthesis or retention of cholesterol, at least when administered parenterally.

In this study, one of the metabolic effects of parenteral bile acids was an increased synthesis or accumulation of hepatic cholesterol. Will this accumulated cholesterol, or the increased quantity that is synthesized, be catabolized and excreted? Or will it be incorporated in the β-lipoprotein and undergo systemic circulation, eventually to be sequestered by arterial walls and lead to atherosclerosis? These questions might be answered after solving the following problems:

1. Determination of cholesterol catabolism during and

after bile acid treatment to see whether it is affected by bile acid.

2. Determination of the excretion rates of bile acid and cholesterol during and after bile acid treatment.

3. Determination of β-lipoprotein patterns in the liver and in the circulatory system during and after bile acid treatment. Knowledge concerning the effect of bile acid on the formation or dissociation of the cholesterol-β-lipoprotein complex in blood or liver might help to evaluate the possibility that cholesterol might be deposited in the arterial walls as a result of bile acid treatment.

Since bile acids, especially chenodeoxycholic acid, are known to be capable of altering many subcellular sites (55, 58), their toxicity to other hepatic regulatory systems might be worth studying before the start of long term treatment.

Free Fatty Acids

In vitro, free fatty acid synthesis from ^{14}C-acetate and ^{14}C-mevalonate (Figure 12) was inhibited by both CDCA and CA. The insignificant effect of CA at very low concentrations on free fatty acid synthesis from ^{14}C-mevalonate might be because CA is less toxic to enzymes (58).

Mevalonic acid is supposed to be a specific precursor for cholesterol synthesis. However, the experiments *in vitro* and *in vivo* showed that substantial quantities of free fatty acid were synthesized from ^{14}C-mevalonate (in a private communication, Dr. W. H. Elliott of St. Louis University School of Medicine, reports similar yields of free fatty acids from ^{14}C-mevalonate *in vitro*). This unexpected result might be attributed to several factors:

1. Separation of microsomal and soluble fractions from mitochondria might have been imperfect, allowing the presence of the latter in the 7,000 x g liver supernatant fraction. This would have allowed cholesterol side-chain cleavage enzymes to split the cholesterol side-chain into propionyl-CoA, which could have been incorporated into free fatty acid (10).

2. One of the three methyl groups (attached to the steroid nucleus before demethylation) was labeled, having been derived from the labeled carbon of the mevalonic acid precursor. These labeled methyl groups eliminated from the 4-position as $^{14}CO_2$ could have been captured by a carboxylase system and then incorporated into free fatty acid (59).

3. Microsomes may have contained some other steroid side-chain cleavage enzymes, which might have caused the liberation of ^{14}C which subsequently became available for free fatty acid synthesis. However, there is no evidence to support this.

4. There might be some enzymes which can convert mevalonate directly into a precursor for free fatty acid synthesis (59).

Among these possibilities, number 4 may account best for the conversion of labeled mevalonate to free fatty acids. According to Popjak's suggestion (59), dimethylallyl pyrophosphate synthesized from mevalonate could be attacked by a phosphatase to yield dimethylallyl alcohol; this could then be metabolized by a route involving CO_2 fixation to 3-hydroxy-3-methylglutaryl-CoA, which is convertible to acetyl-CoA, a precursor of fatty acids.

The inhibitory effect of CDCA and CA on incorporation of ^{14}C-

acetate into free fatty acid *in vivo* (Figures 13A & B) is consistent with the inhibition by both primary bile acids of free fatty acid synthesis from ^{14}C-acetate *in vitro* (Figure 12A).

The negligible effect of either bile acid at low concentrations on free fatty acid synthesis from ^{14}C-mevalonate *in vitro* (Figure 12B) is also consistent with the insignificant effect *in vivo* (Figures 13C & D).

Triglycerides

Since triglyceride is synthesized from free fatty acid, the observed inhibitory effects of CDCA and CA on triglyceride synthesis from ^{14}C-acetate and ^{14}C-mevalonate *in vitro* (Figures 15A & B) might be the consequence of the limited availability of ^{14}C-free fatty acid.

In vivo, an increased incorporation of ^{14}C-mevalonate into triglyceride (Figure 17B) was observed in the animals treated with parenteral Celite and 0.42 M saline, i.e. as a result of stress. The stimulatory effects of both bile acids on the incorporation of ^{14}C-mevalonate into triglyceride (Figures 16C & D) might be interpreted as being due to the effect of stress. The negligible effects of CDCA and CA on the incorporation of ^{14}C-acetate into triglyceride (Figures 16A & B) are consistent with the negligible effect of stress (Figure 17A).

Cholesteryl Esters

In vitro, cholesteryl ester synthesis from ^{14}C-acetate and ^{14}C-mevalonate was decreased by increasing bile acid concentrations (Figures 18A & B). The more pronounced inhibition of cholesteryl ester synthesis from ^{14}C-mevalonate by CDCA (Figure 18B) might have been due to the fact that ^{14}C-free fatty acid synthesized from ^{14}C-mevalonate occurred in

smaller amounts, or that CDCA is more toxic to enzymes (58).

In vivo, the observed inhibitory effects of both bile acids on the incorporation of ^{14}C-acetate into cholesteryl ester (Figures 19A & B) might result from reduced availability of ^{14}C-free fatty acid (Figures 13A & B), inasmuch as the effect of stress is negligible (Figure 20A). The increased incorporation of ^{14}C-mevalonate into cholesteryl ester under the effect of either CDCA or CA (Figures 19C & D) might be caused by the increased amounts of labeled cholesterol (Figures 10C & D) and free fatty acid (Figures 13C & D) and the significant effect of stress (Figure 20B).

CHAPTER V

SUMMARY

1. *In vitro*, the hepatic synthesis of cholesterol, free fatty acid, triglyceride, and cholesteryl ester in the female rat were inhibited by both cholic acid and chenodeoxycholic acid, although the latter may have had a more pronounced effect. But the generality of the effect, and the fact that relatively high concentrations (10^{-3} M) of bile acid were necessary for inhibition, suggest that bile acids were acting as enzyme poisons, and not as specific regulatory agents.

2. The results of the study *in vivo* on uptake of intraperitoneally injected cholic acid into liver and excretion into intestine show that the mechanism responsible for processing parenteral bile acid becomes saturated at a bile acid dose of about 10 µmoles per 100 g body weight.

3. *In vivo*, the incorporation of intraperitoneally injected radioactive precursors into hepatic cholesterol, free fatty acid, triglyceride, and cholesteryl ester in one hour was usually altered by simultaneous injection of one of the following agents: Celite (5 mg per 100 g body weight), 0.42 M saline (200 µl per 100 g body weight), sodium cholate, and sodium chenodeoxycholate (bile acid doses were 3 or 10 µmoles per 100 g body weight). Celite was used as stress-producing control (irritant); 0.42 M saline was also used as a stress control.

A. Incorporation of injected ^{14}C-acetate into hepatic cholesterol was stimulated by Celite, but not affected by 0.42 M saline; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic cholesterol was enhanced by both Celite and 0.42 M saline.

B. Incorporation of injected ^{14}C-acetate into hepatic free fatty acid was stimulated by Celite, but inhibited by 0.42 M saline; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic free fatty acid was not affected by Celite, but reduced by 0.42 M saline.

C. Incorporation of injected ^{14}C-acetate into hepatic triglyceride was not affected by either Celite or 0.42 M saline; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic triglyceride was stimulated by both Celite and 0.42 M saline.

D. Incorporation of injected ^{14}C-acetate into hepatic cholesteryl ester was not affected by either Celite or 0.42 M saline; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic cholesteryl ester was augmented by Celite and 0.42 M saline.

These results (A-D) suggest that hepatic synthesis of cholesterol, free fatty acid, triglyceride, and cholesteryl ester was usually stimulated by stress.

E. Incorporation of injected ^{14}C-acetate into hepatic cholesterol was enhanced by both CDCA and CA; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic cholesterol was stimulated by CDCA, but not significantly affected by CA.

F. Incorporation of injected ^{14}C-acetate into hepatic free fatty acid was inhibited by both CDCA and CA; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic free fatty acid was increased

by both CDCA and CA.

G. Incorporation of parenteral ^{14}C-acetate and ^{14}C-mevalonate into hepatic triglyceride was stimulated by both CDCA and CA.

H. Incorporation of injected ^{14}C-acetate into hepatic cholesteryl ester was suppressed by CDCA and CA; whereas incorporation of parenteral ^{14}C-mevalonate into hepatic cholesteryl ester was enhanced by both CDCA and CA.

These results (E-H), taken together with the results from the investigations on the effects of stress (A-D), indicate that the alterations in neutral lipid metabolism under the effects of parenteral CDCA and CA may be caused in part by stress. It is not clear whether the increased incorporation of labeled precursors into cholesterol, caused by bile acids, reflects accumulation or an augmented synthetic rate.

4. No effect of bile acids on hepatic cholesterol synthesis was found, either *in vitro* or *in vivo*, that could not be explained by factors such as stress or general toxicity. No support has been found for the role of bile acids as specific regulators of hepatic cholesterol synthesis.

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ERRATA

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Page 11 line 7
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Page 18 line 20
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Page 19 line 9
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Page 21 line 3
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Page 22 line 12
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Page 24 line 7
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Page 46 line 9
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Page 47 line 24
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Page 50 line 6
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Page 50 line 23
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