CHOLIC ACID AND DERIVATIVES #55

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MONOGRAPH
ON
CHOLIC ACID
AND
DERIVATIVES

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CHOLIC ACID AND DERIVATIVES

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CHOLIC ACID AND DERIVATIVES

Summary

The bile acids (cholic acid, desoxycholic acid, glycocholic acid, taurocholic acid, and ox bile extract) have been researched extensively, especially in the past 20 years. Radioisotopic techniques have helped elucidate the absorption and normal metabolic paths of the various bile acids, but much remains unknown concerning specific dietary tolerances of different test animals.

Lindstedt and Samuelsson (468) observed that significant amounts of bile acids are absorbed in the cecum of the rat. They also estimated that 70% of the bile acids transported to the large intestine is absorbed and transported back to the liver. Weiner and Lack (875) reported that taurocholate, glycocholate, and cholate were absorbed in the ileum of guinea pigs.

Available evidence indicates that bile acids are absorbed or reabsorbed from the lower part of the small intestine in man (79). In 1968 Samuel et al. (704) studied the absorption of labelled bile acids from the large intestine of patients who had undergone cholecystectomy. They found that cholic acid is converted to desoxycholic acid in the human colon, and both of these acids are absorbed from the human large bowel in significant amounts. Their studies indicated that most of the bile salts excreted by the liver are reabsorbed during transit in the small bowel, are carried back to the liver by the portal vein, and are eventually reexcreted into the gall bladder and used again. At each cycle a fraction will escape reabsorption in the small intestine, will enter the large intestine to be reabsorbed there, and a small amount will escape into the feces.

In 1970, Norman (577) summarized the bile acid metabolism in man. Cholic acid and chenodesoxycholic acid, primary bile acids, are excreted in the bile conjugated with taurine or glycine. Lindstedt and Norman (467) had previously reported that cholic acid was completely conjugated, 1-2% with glycine, 98-99% with taurine, when excreted in rat bile. In 1971, Hanson and Williams (284) observed that when labeled sodium desoxycholate was administered intravenously to patients with external bile fistulas, it was excreted as bile conjugated with glycine (85%) and taurine (13%). In Bergstrom's (56) review of the literature, it is reported that rabbit bile contains almost pure desoxycholic acid conjugated with glycine.

In the duodenum and upper part of the jejunum, the bile acids are still present in the conjugated form. Since fecal bile acids are mainly unconjugated, the bacteria in the intestinal tract must deconjugate them (577). Earlier Lindstedt and Norman (467), reported that when labeled cholic acid was injected i.p. into rats, only minor amounts of the fecal cholic acid were found to be conjugated. Aries et al. reported that the bacteria Bacterioides spp. and Clostridia spp. were responsible for deconjugation of bile acids in man, as were Bifidobacterium spp., Veillonella spp., and enterococci (20).

Such bacteria are also important in the conversion of the primary bile acids to secondary bile acids, which then enter the enterohepatic circulation. In 1969 Aries et al. (20) noted that many enterobacteria present in the human intestine converted cholic acid to 7-ketodesoxycholic acid only. The bacteria Bacterioides spp. and Clostridia spp. produced desoxycholic acid, lithocholic acid, cholanic acid, and 7-ketodesoxycholic acid. From examining human feces, Norman (576) determined the major metabolites of labeled cholic acid to be 3,12-dihydroxycholanic acid and 3-hydroxy-12-ketocholanoic acid. About 25% of the metabolites were saponifiable derivatives of 3,12-dihydroxycholanic acid in the beta form. Norman (577) indicated through labeling experiments that the unconjugated cholic acid was the main substrate for 7-alpha-dehydroxylation in the human intestinal tract. Some cholic acid was also converted to 3-alpha, 12-alpha-dihydroxy-7-keto-5-beta-cholanoic acid and 3-alpha, 7-beta, 12-alpha-trihydroxy-5-beta-cholanoic acid.

Bergstrom (56) reported in his review of the literature that cholic acid is converted to desoxycholic acid in rabbit's gut.

Gustafsson and Norman (275) observed that the intestinal bacteria of rats converted cholic acid to desoxycholic acid (3-alpha, 12-alpha-dihydroxy-5-beta-cholanoic acid) and lithocholic acid (3-alpha-monohydroxy-5-beta-cholanoic acid). Gustaffson et al. (279) noted that when the bacteria (Lacto-bacilleae) was established in germ-free rats, cholic acid was 7-alpha-dehydroxylated. Germ-free rats were found to excrete bile acids to a lesser degree than normal rats, presumably due to a longer transit time of intestinal contents (274).

Part of these unconjugated bile acids are reabsorbed, but must be conjugated again prior to excretion in the bile (577). Species vary as to their ability to rehydroxylate desoxycholic acid back to cholic acid in the liver. Rats are able to complete the rehydroxylation, but rabbits aren't (56). Hanson and Williams (284) determined that the human liver cannot rehydroxylate either desoxycholic acid or lithocholic acid.

Bergstrom (56) stated that the half life of any carbon labeled bile acid to be 2-3 days for a normal rat with a pool size of 20-25 mg for a 200 g rat. Based on data from the oral administration of labeled cholic acid to man, Lindstedt (466) calculated the half life of cholic acid to be 2.8 days. The pool size was 1.39 g. Gordon et al. (259) determined that cholic acid half life in man depended on the fat content of the diet. It was 8.8 days on a butterfat diet, 3.7 days on a corn oil diet, but 4.2 days on a fat-free diet. In healthy individuals the bile acid pool is in a steady state. The excretion which occurs exclusively in the feces is replaced by the synthesis of a similar amount in the liver (466).

Pope et al. (645) noted that when sodium desoxycholate was perfused through segments of rat jejunum, the transport of urea, lysine, leucine, acetate, sodium and glucose was inhibited. Since the mucosa was badly damaged, they suggested that free bile acids are toxic to the upper gut; the bile acids would be tolerated by the ileum and colon.

Burke et al. found cholic and taurocholic acids to be strongly bound to plasma albumin (100).

A 20 mg/kg oral dose of sodium glycocholate or taurocholate increased the gastric movements of rabbits; a 60 mg/kg dose decreased the movement (709).

Eyssen et al. (182) and Edwards (163) found increased fat absorption in chicks consuming cholic acid.

Dietary cholic acid was found by Back et al. (32) to inhibit cholesterol synthesis in rats. Peric-Golia (632) states that dietary cholic acid delayed the normal metabolic conversion of cholesterol to cholic acid in rats. Bergman and Van der Linden (53) found similar results. Beher et al. (48) proposed that a double-feedback mechanism was involved in the rate of hepatic cholesterol biosynthesis in mice. Cholic acid caused an equal decrease in the rates of cholesterol synthesis and bile and biosynthesis.

Dietary bile acids (cholic, desoxycholic, taurocholic, glycocholic acids) have been found by several authors (1181, 41, 42, 343, 648, 861, 413) to induce hypercholesteremia and increase liver cholesterol in chicks mice and rats. Lithocholic acid induced a hypocholesteremic effect (343).

However, Howe and Hutchison (342) reported that bile acids in the absence of dietary cholesterol have no effect on plasma cholesterol levels in the rat. Friedman et al. (208) suggested that cholic acid promotes the intestinal absorption of cholesterol, producing hypercholesteremia. Member et al. (512) reached the same conclusion. Desoxycholic acid was found to increase the blepharoptic activity of reserpine in mice (495).

Several studies using chicks, mice, rats, and rabbits have been designed to study the effect of various bile acids in the diet on growth and other toxicological parameters. Most of these studies were one month or less in duration, and have produced a variety of results. A table of acute toxicity is presented on page 17.

Neither dietary cholic acid nor desoxycholic acid appear to affect growth in chicks at levels of 0.2% or lower. Edwards (163) reported growth and feed efficiency to be essentially normal in day-old White Rock chicks following one month of dietary cholic acid (0.2%). In an earlier study, Edwards (162) found similar results in 8-day-old chicks fed 0.2% cholic acid. Leveille et al. (457) found growth to be average in chicks fed 0.2% cholic acid for 3 weeks. Martin and Patrick (499) reported 0.1% cholic or 0.1% desoxycholic acid in the diet of 6 chicks increased growth over controls in a 14-day period. Eyssen et al. (182) found growth and feed efficiency to be normal in cockerels fed 0.2% cholic acid or 0.2% desoxycholic acid for 2 weeks. No ill effects were found in the liver, gall bladder, aorta, lung, spleen, small intestine, or kidneys of 9 chickens fed 0.2% cholic acid for 8 weeks. The same tissues were normal for 16 chickens following 3 weeks of dietary desoxycholic acid (348). The feeding of 0.2% cholic acid to laying hens temporarily reduced egg production at 15 days; at 25 days, production was normal (163).

Several authors have found some toxicity associated with dietary lithocholic acid. Edwards (162) reported a decreased growth in

cockerel chicks fed 0.2% lithocholic acid for 3 weeks.

Lyman et al. found a proliferation of cholangiocytes and marked fibrosis in livers of 8 cockerels that ate 0.2% lithocholic acid for 2 weeks; weight gain was reduced 20-40%. In a subsequent study, 40 chicks ate a basal diet plus 0.2% lithocholic acid for 3 months and were found to have extensive liver damage (182).

Leveille et al. (458) observed enlarged livers and biliary hyperplasia in chicks fed 0.2% lithocholic acid. In a later study, Leveille et al. (457) found dietary lithocholic acid (0.2%) to induce a decreased weight gain, and enlargement of the liver, and higher plasma cholesterol and lipid phosphorous levels.

The feeding of 0.2% lithocholic acid to 8 chicks for 2 weeks resulted in extensive bile duct proliferation, distorted and disrupted hepatic lobules, and a marked fibrosis in the liver; weight gain was reduced by 50% (181).

Dietary lithocholic acid (0.2%) induced a marked biliary hyperplasia with an increased liver size in chicks (459). Martin and Patrick reported unusually large livers in chicks fed 0.1% lithocholic acid for 14 days (499). When 10 laying hens consumed 0.2% lithocholic acid for 30 days, egg production was significantly decreased. A following study showed reduced egg production at the 0.1% level (164).

There have been a variety of short-term studies performed on rats. Besancon et al. reported dietary cholic acid in levels of 0.5% and 1% to cause gallstones and gall bladder lesions in mice, without addition of cholesterol to the diet, in an 8 month study (63).

Visek et al. observed that 6 rats fed 2.0% sodium cholate died within 7-21 days; rats fed 0.0, 0.1, and 0.5% survived the 28 day study. A 10% reduction in weight gain for every 0.1% level of sodium desoxycholate added to the diet of rats for a 28 day period was reported. At the highest level, 1%, the rats died within 5 days (856).

In 1969 Bray and Gallagher (88) found reduced liver and body weight in 7 rats fed 0.15% cholic acid; heart weight increased during the 26 day study. Desoxycholic acid (0.15%) reduced the mean weight of 7 rats tested; extensive histological examination revealed no untoward effects. Six male rats that consumed 0.15% desoxycholic acid for 120 days suffered a slow weight gain for the first 30 days. In 1968 Bray and Gallagher (86) noted that consumption of 0.15% desoxycholic acid for 28 days produced no evidence of damage to the livers of intestinal mucosa of rats.

Shiner (755) administered 63 mg/kg desoxycholic acid by stomach tube twice daily to 3 rats for 4 days; lipid deposits were found in the jejunum. No evidence of atherosclerosis was found in 8 adult male rats fed 1% cholic acid for 8 weeks (680). Kim and Ivy (413) observed an increased mortality in male rats over female rats when given 110 mg/kg desoxycholic acid daily.

Growth depression was observed in rats consuming ox bile extract at levels of 0.5% and 1.0%; hyperemia of the distal small intestine was also reported. Two percent sodium taurocholate produced a significant growth depression in rats after a 28 day period; 1.0% taurocholate produced hyperemia of the distal small intestine. Rats receiving 0.1% taurocholate showed an apparent growth stimulation (856).

Gambal and Quackenbush found dietary cholic acid (1%) to retard growth and body fat deposition in rats on a basal diet deficient in the essential fatty acids. Comparison was made to rats on a deficient diet only and to positive controls on a complete diet. When rats were fed 1% sodium glycocholate in addition to a diet deficient in essential fatty acids, deficiency symptoms appeared 3-5 weeks sooner than in control animals; growth was also more depressed (241).

No histological evidence of damage to the gallbladder was found in 3 rabbits fed 0.25% cholic acid for 4 weeks (434). Rosenman et al. reported a few scattered atherosclerotic plaques present in 5 male rabbits which consumed 1% cholic acid for 8 weeks (680). Mosbach et al. failed to find any gallstones or other histological evidence of damage in 4 male rabbits which ate 0.25% desoxycholic acid for 4 weeks (543). Bray and Gallagher (87) reported desoxycholic acid to be an appetite suppressant in man. Dietary taurocholic acid appeared helpful in removing implanted clots in man (434).

Two long-term studies have been performed on rats. No significant differences were found in rats fed 0, 0.005%, 0.025%, or 0.125% desoxycholic acid for 20 months; the stomach, respiratory tract, ears, feet, spleen and bone marrow were examined (38).

Rats fed a semi-synthetic diet, poor in proteins and riboflavin, plus 0.5 g/kg cholic acid added suffered an increase in liver weight with some steatosis; myelinization and limphoid atrophy were pronounced in the spleen (444). Although the rats were studied for up to a 1 year period, no control rats were described for comparison. Similar results were found in rats fed 0.5 g/kg lithocholic acid plus the semi-synthetic diet (444).

Several special studies have been performed. Little et al. injected chicks with Rous sarcoma virus; chicks subsequently fed 0.25% cholic acid developed 3 times as many tumors as those fed a basal diet (473). Cook (129) reported malignant tumors in mice when desoxycholic acid was applied to the skin in an alcohol-benzene mixture; i.m. injections induced spindle-celled tumors.

Dietary cholesterol and cholic acids have been shown to interact, inducing effects not brought about by the feeding of cholic acid alone. Multiple thrombi were present in the small heart vessels of rats fed both cholesterol (5%) and cholic acid (2%) for 35 weeks; a 40% mortality occurred (666). Hellerstein et al. (307) reported sudanophilia in the hearts of rats fed cholesterol (1%) and cholic acid (0.3%).

Atherosclerosis was found in Cebus monkeys fed cholesterol and cholic acid (1%) using two different protein sources, and in one protein source without cholic acid (891).

Tepperman et al. (819) found gallstones in 9 out of 10 obese mice after 9 months consumption of cholesterol and cholic acid (0.5%); grossly fatty livers were also reported. It was stated that both cholesterol and cholic acid must be fed for this effect to occur. Similar results were found in a 4 month study by Bergman and Van der Linden (50) using a 1% level of cholic acid or a 0.5% desoxycholic acid. In a later study, Bergman and van der Linden (51) observed that the omission of cholic acid from such a diet caused gallstones to dissolve in mice. Atrophy of the gallbladder but no gallstones was reported by Bergman (53) in the same type of study. Histological changes were not reversible in hamsters, however. Rozynkowa et al. (690) reported some liver damage in rats fed cholesterol (1%) and cholic acid (0.4%) for 11 weeks as did Walker and Emerson (861) with cholic acid at the 1.0% level. Gerbils fed 1% cholesterol and 0.5% cholic acid for 4 months suffered a high mortality rate, and damage to their livers, gallbladders, spleens, and kidneys. When removed from such a diet, the gerbils recovered (52).

CHOLIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Cholic acid
2. Cholalic acid

B. Chemical Names

1. 3-alpha, 7-alpha, 12-alpha-trihydroxy-5-beta-cholanic acid
2. 17-beta-(1-methyl-3-carboxypropyl) etiocholane-3-alpha, 7-alpha, 12-alpha-triol

C. Trade Name

Colalin

D. Chemical Abstracts Registry No.

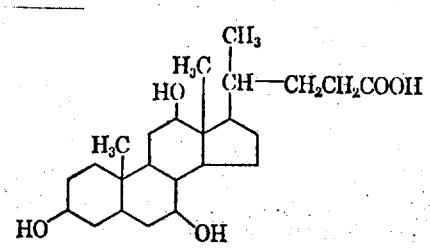
000 081 254

II. Empirical Formula

$C_{24}H_{40}O_5$

III. Structural Formula

C 70.55%
H 9.87%
O 19.58%



IV. Molecular Weight

408.56

V. Specifications

Food Chemicals Codex	
Assay	Not less than 98.0% of $C_{24}H_{40}O_5$
Melting range	Between 197° and 202°
Specific rotation	$(alpha)_D$ 25°, Not less than + 37°
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 40 ppm
Lead	Not more than 10 ppm
Loss of drying	Not more than 0.5%
Residue on ignition	Not more than 0.1%

VI. Description

A. General Characteristics

Cholic acid occurs as colorless plates or a white, crystalline powder having a bitter taste with a sweetish aftertaste.

B. Physical Properties

The melting point of cholic acid is 198 degrees. Its solubilities at 15 degrees are: in water, 0.28 g/l; in alcohol, 30.56 g/l; in ether, 1.22 g/l; in chloroform, 5.08 g/l; in benzene, 0.36 g/l; in acetone, 28.24 g/l; in glacial acetic acid, 152.12 g/l; soluble in solutions of alkali hydroxides and carbonate.

C. Stability

Store in tight containers.

VII. Analytical Methods

No methods of separating cholic acid and its derivatives from foods were found. Detection methods are available using thin layer chromatography to separate mixtures of bile acids from various body fluids. Matkovics and Tegyey (502) discussed several TLC solvent systems consisting of a high percentage of a nonpolar solvent (benzene) a polar solvent (methanol, acetone etc.) used in separating bile acids from duodenal bile. Sundaram et al. (793) discussed using an iso-octane:ethyl acetate:acetic acid: N butanol (10:5:1.5:1.5) TLC solvent system for separating free bile acids and their methyl esters from fecal and bile sources. Nystrom and Sjovall (584) developed a TLC method on lipophilic Sephadex for rapid screening of solvent systems for free bile acids. The TLC system of Gregg (267) iso-octane:isopropyl ether:glacial acetic acid:isopropyl alcohol (2:1:1:1) can be used to separate free and conjugated bile acids.

VIII. Occurrence

Cholic acid occurs in conjugation with glycine and taurine in the bile of most vertebrates.

DESOXYCHOLIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Deoxycholic acid
2. Desoxycholic acid

B. Chemical Names

1. 3-alpha, 12-alpha-dehydroxy-5-beta-cholanic acid
2. 17-beta-(1-methyl-3-carboxypropyl)etiocholane-3-alpha, 12-alpha-diol

C. Trade Names

None

D. Chemical Abstracts Registry No.

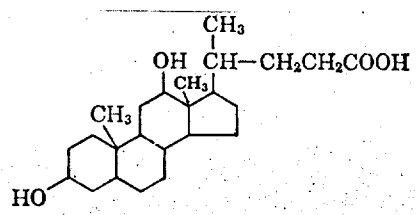
000 083 443

II. Empirical Formula

$C_{24}H_{40}O_4$

III. Structural Formula

C 73.43%
H 10.27%
O 16.30%



IV. Molecular Weight

392.56

V. Specifications

Food Chemicals Codex	
Assay	Not less than 98.0% and not more than the equivalent of 102.0% of $C_{24}H_{40}O_4$ (anhydrous)
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 40 ppm
Lead	Not more than 10 ppm
Loss on drying	Not more than 1%
Residue on ignition	Not more than 0.2%

VI. Description

A. General Characteristics

Desoxycholic acid is a white, crystalline powder.

B. Physical Properties

The melting point of the alcohol crystals of desoxycholic acid ranges from 176 to 178 degrees. Its solubilities at 15 degrees are in water, 0.24 g/l; in alcohol, 220.7 g/l; in ether, 1.16 g/l; in chloroform, 2.94 g/l; in benzene, 0.12 g/l; in acetone, 10.46 g/l; in glacial acetic acid, 9.06 g/l; also soluble in alkali hydroxides and carbonates. The solubility in water of sodium desoxycholate is greater than 333 g/l at 15 degrees.

C. Stability

Store in tight containers.

VII. Analytical Methods

See Cholic acid

VIII. Occurrence

Desoxycholic acid occurs in the bile of man, ox, sheep, dog, goat, and rabbit.

GLYCOCHOLIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Glycocholic acid
2. N-Cholyglycine

B. Chemical Name

Glycocholic acid is the chief conjugation product of cholic acid and glycine.

C. Trade Name

None listed

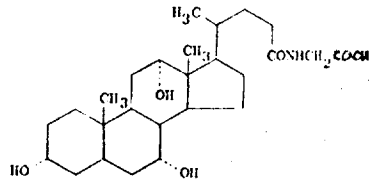
D. Chemical Abstracts Registry No.

000 475 310

II. Empirical Formula

$C_{26}H_{43}NO_6$

III. Structural Formula



C 67.06%
H 9.31%
N 3.01%
O 20.62%

IV. Molecular Weight

465.61

V. Specifications

None listed

VI. Description

A. General Characteristics

Glycocholic acid is hydrolyzed and forms crystals. Hydrolysis products are cholic acid and glycine (produced by acids and alkalies).

B. Physical Properties

The melting point of the anhydrous form of glycocholic acid is 165 to 168 degrees. Its solubility at 15 degrees is, in water, 0.33 g/l; in boiling water, the solubility is 8.3 g/l. The sodium salt, sodium glycocholate, has the following solubilities: at 15 degrees in water, more than 274 g/l; in alcohol, more than 340 g/l.

C. Stability

Keep in closed containers.

VII. Analytical Methods

See cholic acid

VIII. Occurrence

Glycocholic acid is the chief ingredient of the bile of herbivorous animals. It exists as a sodium salt in the bile, which is a weak, alkaline solution.

OX BILE EXTRACT

Chemical Information

I. Nomenclature

A. Common Names

1. Purified oxgall (1 g = 8 g ox bile)
2. Sodium choleate

B. Chemical Names

Ox bile extract consists of the sodium salts of glycocholic and taurocholic acids, cholesterol, lecithin, glycocol, and choline compounds.

C. Trade Names

1. Bicol
2. Bi-Ketolan
3. Bilein
4. Bilicholan
5. Cholatol
6. Colalin
7. Crescefel
8. Desicol
9. Doxychol
10. Glycotauro
11. Ketochol
12. Panoxolin
13. Plebilin
14. Valachol

D. Chemical Abstracts Registry No.

MX8008
MX8 008 637

II. Empirical Formula

Not applicable

III. Structural Formula

Not applicable

IV. Molecular Weight

Not Applicable

V. Specifications

Not listed

VI. Description

A. General Characteristics

Ox bile extract is a yellowish-green, soft, solid, with a partly sweet, partly bitter, disagreeable taste.

B. Physical Properties

Ox bile extract is very soluble in water or alcohol.

C. Stability

Keep in closed containers

VII. Analytical Methods

See cholic acid

VIII. Occurrence

Ox bile extract is the purified portion of the bile of an ox.

TAUROCHOLIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Taurocholic acid
2. Cholaic acid
3. Cholytaurine

B. Chemical Names

Taurocholic acid is the product formed from the conjugation of cholic acid with taurine.

C. Trade Name

None listed

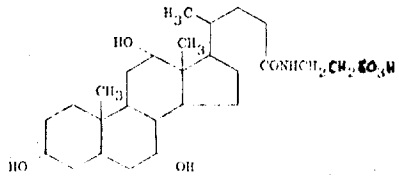
D. Chemical Abstracts Registry No.

000 081 243

II. Empirical Formula

$C_{26}H_{45}NO_7S$

III. Structural Formula



C 60.55%
H 8.78%
N 2.72%
O 21.72%
S 6.22%

IV. Molecular Weight

515.69

V. Specifications

None listed

VI. Description

A. General Characteristics

1. Amorphous, yellow, hygroscopic crystals
2. Clusters of slender, 4-sided prisms from alcohol

and ether

3. Stable in air
4. Hydrolyzed to cholic acid and taurine by acids and alkalies
5. The sodium salt has a sweet taste and a bitter after-taste.

B. Physical Properties

Taurocholic acid decomposes at about 125 degrees. It is freely soluble in water and alcohol and is almost insoluble in ether and ethyl acetate. Its sodium salt, sodium taurocholate, decomposes at about 230 degrees, is soluble in water or alcohol, and has a solvent action on cholesterol.

C. Stability

Keep in closed containers.

VII. Analytical Methods

See cholic acid

VIII. Occurrence

Taurocholic acid, in the form of its sodium salt, occurs as the chief ingredient of the bile of carnivorous animals.

CHOLIC ACID AND DERIVATIVES

Biological Data

I. Acute toxicity

Compound	Animal	No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Cholic Acid	Mice	10	s.c.	500	LD_0	747
Cholic Acid	Mice	74	i.v.	350	LD_{50}	687
Cholic Acid	Mice	Unk.	i.v.	200	LD_0	747
Cholic Acid	Mice	Unk.	i.v.	500	LD_{100}	747
Cholic Acid	Mice	39	p.o.	1520	LD_{50}	899
Desoxycholic Acid	Mice	10	s.c.	500	LD_{100}	747
Desoxycholic Acid	Mice	60	i.v.	150	LD_{50}	687
Desoxycholic Acid	Mice	Unk.	i.v.	100	LD_0	687
Desoxycholic Acid	Mice	Unk.	i.v.	200	LD_{100}	687
Desoxycholic Acid	Mice	39	p.o.	1060	LD_{50}	899
Glycocholic Acid	Mice	54	i.v.	370	LD_{50}	687
Glycocholic Acid	Mice	Unk.	i.v.	340	LD_0	687
Glycocholic Acid	Mice	Unk.	i.v.	400	LD_{100}	687
Sodium Glycocholate	Dogs	10	i.v.	562	LD_{100}	171
Taurocholic Acid	Mice	56	i.v.	330	LD_{50}	687
Taurocholic Acid	Mice	Unk.	i.v.	300	LD_0	687
Taurocholic Acid	Mice	Unk.	i.v.	360	LD_{100}	687
Sodium Taurocholate	Dogs	10	i.v.	668	LD_{100}	171

A. Cholic Acid

Mice

Subcutaneous injection of 500 mg/kg of crystalline cholic acid into 10 strain A mice (unspecified sex) resulted in no fatalities (747).

Rothlin and Schalch reported an i.v. LD_{50} equal to 350 mg/kg cholic acid for 74 white mice. An LD_0 equalled 200 mg/kg; an LD_{100} was 500 mg/kg. Immediately following the injection, strong motor agitation appeared. With increased dosage, clonic-tonic convulsions with ataxia and confusion occurred. After a moment of extreme convulsions, complete exhaustion was noted. Generally, the animals succumbed within 5-10 minutes after injection (687).

Yamasaki et al. reported an LD_{50} equal to 1520 mg/kg cholic acid administered orally to white mice, male and female, weighing 18-28 g. Doses of 408, 612, 816, 1224, 1632, and 2448 mg/kg were given to groups of 5-8 mice. Hyperactivity was followed by a reduction of "automatic"

movement. Symptoms appeared generally 1-2 hr after injection; recovery occurred within 6 hr if the animal survived. In fatal cases the animal fell on its side and remained without moving until death. No spasms were noted. Death occurred from 15 minutes to 48 hours (899).

Dogs

Gillert injected intravenously 30, 40, 50, and 60 mg/kg sodium cholate in a 1% solution to an unspecified number of rabbits. The animals showed some discomfort at the intermediate doses, but died after the 60 mg/kg dose (249).

B. Desoxycholic Acid

Mice

Shear et al. injected s.c. 500 mg/kg of crystalline desoxycholic acid mixed with glycerol into the left flank of 10 strain A mice (unspecified sex). The 3-4 month old mice were dead within 4 days. It was reported that a smaller dose (unspecified) was tolerated. Another 6 mice received s.c. 500 mg/kg desoxycholic acid plus glycerol; 5 were dead the next day. Desoxycholic acid mixed with a little water, 500 mg/kg, was again injected into 6 mice; within 3 days 5 were dead (747).

Rothlin and Schalch reported an i.v. LD_{50} equal to 150 mg/kg desoxycholic acid for 60 white mice. An LD_0 equalled 100 mg/kg; an LD_{100} was 200 mg/kg. Immediately following the injection, strong motor agitation appeared. With increased dosage, clonic-tonic convulsions with ataxia and confusion occurred. After a moment of extreme convulsions, complete exhaustion was noted. Generally, the animals succumbed within 5-10 minutes after injection (687).

Yamasaki et al. reported an LD_{50} equal to 1060 mg/kg desoxycholic acid administered orally to white mice, male and female, weighing 18-28 g. Doses of 392, 588, 784, 1176, 1568, and 2352 mg/kg were given to groups of 5-8 mice. Hyperactivity was followed by a reduction of "automatic" movement. Symptoms appeared generally about 1-2 hr after injection; recovery occurred within 6 hr if the animal survived. In fatal cases the animal fell on its side and remained without moving until death. No spasms were noted. Death occurred from 15 minutes to 48 hours (899).

Rats

Bray and Gallagher administered 100 micro moles (39.3 mg) desoxycholic acid per kg body weight to rats trained to eat in 6 hours. After one dose, feeding was depressed for 3 days. The same effect was observed when the taurine or glycine conjugate was administered. The potency of desoxycholic acid was changed when the position or total number of hydroxyl groups was shifted (86).

Rabbits

Sodium deoxycholate was given as a 1% solution by i.v. injection

to an unspecified number of rabbits. A dose of 10 mg/kg elicited violent screaming while 15 mg/kg resulted in immediate death. Dissection revealed systolic stasis of the left ventricle and diastolic stasis of the right ventricle. Also, there was venous hyperemia of all (unspecified) organs (249).

Cats

In cats, Emmelin showed that the i.v. injection of 50 mg/kg sodium desoxycholate (a toxic dose) caused a temporary branchoconstriction. Desoxycholate was not as strong as glycocholate or taurocholate in producing this effect (173).

C. Glycocholic Acid

Mice

Horrall and Carlson found that sodium glycocholate would kill mice in 30 minutes when injected i.p. in the following dosages:

6,250 mg/kg in 0.5 ml distilled H_2O
1,550 mg/kg in 0.1 ml distilled H_2O
750 mg/kg in 0.05 ml distilled H_2O

One mouse was used for each level. Pure glycocholic acid produced no toxic effects when 2 ml of a saturated solution in distilled water was injected i.p. into mice. Glycocholic acid (40 mg) dissolved in 2 ml 10 N NaOH killed a mouse in 45 min. (332).

Rothlin and Schalch reported an i.v. LD_{50} equal to 370 mg/kg glycocholic acid for 54 white mice. An LD_0 equalled 340 mg/kg; and LD_{100} was 400 mg/kg. Immediately following the injection, strong motor agitation appeared. With increased dosage, clonic-tonic convulsions with ataxia and confusion occurred. After a moment of extreme convulsions, complete exhaustion was noted. Generally, the animals succombed within 5-10 minutes after injection (687).

Guinea pigs

Emmelin reported that the intravenous injection of 15-25 mg/kg sodium glycocholate into 7 guinea pigs induced bronchoconstriction temporarily (173).

Rabbits

Gillert injected i.v. 80 mg/kg sodium glycocholate into an unspecified number of rabbits; distress and agitation were observed. A 90 mg/kg dose resulted in exitus. Dissection revealed parenchymatous hemorrhage in the liver (249).

Cats

Emmelin found that the i.v. injection of 5 to 10 mg/kg sodium glycocholate in cats (unspecified number) caused a sharp, temporary, bronchoconstrictory effect. Glycocholate was more readily able to

elicit the effect than the same dosage of taurocholate or desoxycholate (173).

Dogs

Horrall and Carlson found that pure glycocholic acid was not toxic, when injected into dogs, due to its insolubility in water, salt solutions, and blood serum. The impure salt of sodium glycocholate was toxic to dogs within 3 days after 80 to 160 mg (in 4 and 2 ml water) were injected i.p. Toxic effects included bloody diarrhea, albuminuria, pulmonary edema, decreased respiration, bradycardia, and death. When glycocoll alone was injected into dogs i.v., no toxic effects were observed. Sodium cholate was as toxic to dogs as sodium glycocholate. It was concluded that the toxic part of bile salts is cholate, which may dissolve the lipids in cell membranes and change cell metabolism and surface tension. These changes may be reversible, when a small amount of bile salt is used (332).

The average lethal dose (LD_{100}) for sodium glycocholate (3% solution) i.v. in 10 dogs was found by Emerson to be 8.5 ml/lb or 562 mg/kg. Toxic effects were, decreased blood pressure, respiratory difficulties, and myocardial failure. Glycocholic acid, injected as a sodium salt, was found to be more toxic than a similar solution of taurocholic acid (171).

D. Lithocholic Acid.

No information available

E. Ox Bile Extract

Dogs

Emerson found the average lethal dose (LD_{100}) of ox bile (i.v.) diluted 1:1 with saline, for 10 dogs was 3.1 ml/kg (concentration not given). The lethal dose for the ox bile minus 95% of the bile pigment was 3.3 ml/kg in 10 dogs. Toxic effects included decreased blood pressure, arrhythmia, and myocardial failure. Bile pigment, prepared from human gallstones, injected in alcohol into 10 dogs was found to have no toxic effects in a 11.4 ml/kg dose (171).

F. Taurocholic Acid

Mice

Horrall and Carlson reported that one mouse died within 30 min. after 125 mg sodium taurocholate (6250 mg/kg) in 0.5 ml distilled H_{20} were injected i.p. (332).

Rothlin and Schalch reported an i.v. LD_{50} equal to 330 mg/kg taurocholic acid for 56 white mice. An LD_0 equalled 300 mg/kg; and LD_{100} was 360 mg/kg. Immediately following the injection, strong motor agitation appeared. With increased dosage, clonic-tonic convulsions with ataxia and confusion occurred. After a moment of extreme convulsions, complete exhaustion was noted. Generally, the

animals succumbed within 5-10 minutes after injection (687).

Rabbits

Schiaparelli injected intrapleurally (right hemithorax) 50, 100, 120, and 150 mg/kg sodium-taurocholate in 5 ml to 1, 6, 1, and 3 fasted rabbits. Rabbits receiving 50-100 mg/kg underwent convulsions followed by dyspnea; only one (100 mg/kg) died. Animals receiving 120-150 mg/kg died in 15 minutes. A necroscopic examination revealed dark yellow liquid (hemorrhagic) on the right side of the thoracic cavity. The right parietal pleural layer showed slight superficial hemorrhage; the visceral layer and the surface of the right lung was congested. The pleura and lungs were intact. Rabbits died when 120 mg/kg in 10 ml, 150 mg/kg in 10 and 20 ml, or 140 mg/kg in 45 ml were injected. Liver, kidney, adrenals, heart, and spleen were examined histologically. Schiaparelli also injected i.v. 100 mg/kg, 200 mg/kg, and 300 mg/kg into 1 rabbit each; all survived (723).

Gillert injected intravenously 80 mg/kg sodium taurocholate into an unspecified number of rabbits. The respiratory rate decreased, bowel movement occurred, and mydriasis occurred; the rabbit recovered in 6 minutes. A dose of 90 mg/kg caused a slower recovery. At 100 mg/kg violent opisthotonus was observed. Extinction of the corneal reflex with exophthalmus was reported. Cessation of respiration was interrupted only by deep breathing separated by pauses of several seconds. Respiration was resumed abruptly, beginning with tachypnea. A dose of 110 mg/kg produced immediate exitus with tonic-clonic cramps, exophtalmus and cessation of respiration (249).

Cats

Emmelin found that sodium taurocholate injected i.v. in a 15 to 20 mg/kg dose caused a marked, temporary bronchoconstriction in cats (unspecified number). Its bronchoconstrictory properties were midway between glycocholate and desoxycholate (173).

Dogs

Emerson found that the average lethal dose (LD_{100}) of taurocholic acid administered i.v. as a 3% solution of its sodium salt, to 10 dogs was 10.1 ml/lb or 668 mg/kg. The toxic effects were decreased blood pressure, arrhythmia, and myocardial failure. Sodium taurocholate was found to be less toxic than sodium glycocholate (171).

Sodium taurocholate was toxic to dogs, when Horrall and Carlson injected upward of 700 mg/kg i.v., i.p., and s.c. Its toxic effects were bloody diarrhea, albuminuria, pulmonary edema, decreased respiration, bradycardia, and death. The cholate part of taurocholic acid salt was found to be the toxic part. Taurin had previously been found to be non-toxic, when injected in a 10,000 mg dose i.v. into humans (332).

II. Short Term Studies

A. Cholic Acid

Mice

Besancon et al. reported that cholic acid induced cholesterol stones and gall-bladder lesions in young female Swiss mice (nulliparas), without the addition of cholesterol to the diet. Levels of 0, 0.5, and 1.0% cholic acid were given to 42, 33, and 19 mice respectively. Detailed histological examination of the gall-bladders and livers were made after 2, 4, and 8 months. At the 0.5% level, the mortality rate was negligible. The lithogenic effect was demonstrated by the appearance of large birefractive crystals of cholesterol in the bile before 2 months, and of stones before 4 months. Lithiasis affected 11 out of 19 mice tested at 4 months, and 5 out of 6 at 8 months. Vesicular lesions were precocious and intense. Liver weight increased by 2 months, with an abundance of birefractive crystals in the parenchyma. Cholic acid at the 1% level produced lithiasis earlier than the 0.5% level. Crystals were present in the bile at 4 months in 8 out of 10 animals examined. The vesicular and hepatic lesions were intense, but the gall-bladder lesions appeared to be regressive (63).

Chicks

Hunt et al. fed 9 chickens a basal diet with 0.2% cholic acid added; 11 chickens served as controls. After 8 weeks, gross and histochemical examinations of the liver, gallbladder, heart, aorta, lung, spleen, small intestine and kidneys were made. No effects were noted (348).

In a second experiment, 2 groups of 16 chickens each ate a basal diet plus 0 and 0.2% cholic acid. After 3 weeks, the same examinations were made. No effects were noted (348).

Edwards conducted two 4-week feeding studies on day old White Rock cockerel chicks to see if 0.2% cholic acid affected growth when it supplemented the basal diet. No effect on growth was observed. A slight decrease in amount of feed needed for 1 unit of grain and a slight increase in fat absorption were found (163).

Edwards found that 0.2% cholic acid added to a basal diet did not significantly affect either the serum cholesterol or the growth rate in 8 day-old cockerel chicks during a 3 week feeding study. When cholic acid was added to a diet containing the same level of lithocholic acid, the effects of lithocholic acid were reduced - lithocholic acid increased serum cholesterol and retarded the growth rate (162).

Cholic acid (0.2%) did not significantly alter growth or liver weight when fed to 8 male Hyline chicks (7 day old) for 3 weeks (457).

In one experiment, a basal diet was fed to 2 groups of 10 laying hens for 30 days. Cholic acid was supplemented at levels of 0, and 0.2%. Egg production in the group fed cholic acid dropped at 15 days, but returned to normal within 25 days. Body weight was normal for all groups (164).

Chicks (6) fed cholic acid (0.1%) grew slightly better than controls over a 14 day period (499).

Cholic acid was fed at a 0.2% level to 8 three week old cockerels for 2 weeks. Eight others served as controls. Growth and feed efficiency were normal. No effects were noted upon gross or histological examination of the liver (181).

Rats

Gambal and Quackenbush studied the effects of cholic acid on rats fed a diet free from the essential fatty acids (EFA). Seven control rats ate the basal diet (devoid of essential fatty acids), 7 ate the basal diet plus 1% cholic acid, and 5 positive control rats ate the basal diet plus 3% soybean oil. Male weanling Wistar rats were used. After 5 weeks, growth impairment was obvious in all but the positive control group. On a scale in which the growth of the positive controls equalled 100%, the deficient controls ranked 75%, and the cholic acid group ranked 26%. Symptoms of EFA deficiency were reported in deficient controls and the cholic acid group at 8-10 weeks (dermatitis, loss of hair). Animals receiving cholic acid had severe gastrointestinal disturbance (diarrhea); two died prior to the 5 week period. The total lipids present in the carcass and liver were less in the cholic acid group than in the deficient controls. In general cholic acid did not accelerate EFA-deficiency symptoms, but retarded growth and body fat deposition (241).

Two groups of 7 male Gofmoor rats weighing 55-70 g were fed a semi-synthetic diet; one group had 0.15% cholic acid in addition. Body weight was recorded two to three times weekly. After 26 days the animals were sacrificed. Gross examinations were made of the intestine, liver, heart, gonads, and histological examinations were made of the heart, liver, stomach with duodenum, and ileum with colon. After the first week, the cholic acid rats weighed significantly less than control rats. The weight of the livers per 100 g body weight was reduced as well. The weight of the heart was increased (88).

No evidence of atherosclerosis was found in 8 adult male Long-Evans rats fed 1% cholic acid for 8 weeks. Rats (8) fed 2% cholesterol and 1% cholic acid were also free from atherosclerosis (687).

Visek et al. fed male, weanling, 48 to 60 g rats of Holtzman strain (6 per group) a basal diet with 0%, 0.1%, 0.5%, and 2.0% sodium cholate added for 28 days. No growth depression occurred with 0.1% cholate, but all of the 2.0% group died between 7 and 21 days. The 0.5% groups had hyperemia of the distal small intestine (856).

Rabbits

After 4 weeks of consuming 0.25% cholic acid in Purina rabbit chow, 3 male chinchilla rabbits still had normal gallbladders. Histological

examination revealed no trace of gallstones or other damage. Weight gain was comparable to the 7 control rabbits (543).

Five 6 week old male rabbits consumed 1% cholic acid for 8 weeks; 9 rabbits served as controls. The test animals displayed a few scattered atherosclerotic plaques (687).

B. Desoxycholic Acid

Chicks

Desoxycholic acid (0.2%) was fed to 16 chickens for 3 weeks; 16 chickens served as a control group. The chickens were examined grossly and microscopically (liver, gallbladder, heart, aorta, lung, spleen, small intestine and kidneys). No effects were noted (348).

Desoxycholic acid was fed at a 0.2% level in a basal diet to 8 three week old cockerels for 2 weeks. Eight others served as controls. Growth and feed efficiency were normal. No effects were noted upon gross or histological examination (181).

Chicks (6) fed desoxycholic acid (0.1%) grew slightly better than controls over a 14 day period (499).

Rats

Powdered desoxycholic acid (63 mg/kg) was mixed in the food and water and administered twice daily by stomach tube to 3 rats. One control rat received his food and water by stomach tube, as well. The treatment lasted four days, after which they were fasted for 18 hours and sacrificed. All three experimental animals developed diarrhea. One test animal died on the 2nd day of treatment; the others were in poor condition at the time of sacrifice. Jejunal segments were analyzed by electron microscopy. Dense droplets of lipid material were found within the epithelium and between the lateral membranes of surface cells in desoxycholic acid consumers (755).

Visek et al. performed a 28 day feeding study with male weanling rats of the Holtzman strain, weighing 48 to 60 g. Various levels (0, 0.1, 0.5, and 1.0%) of sodium desoxycholate were added to a basal diet and distilled water was provided. Desoxycholate proved to be more toxic than any other bile salt studied. One effect was a 10% reduction in weight gain, compared with control groups, for every additional 0.1% sodium desoxycholate used, a significant decreased food intake occurred. In the groups given as much as 1% desoxycholate, all the rats died within 5 days. At the 0.5% level, hyperemia of the distal small intestine and diarrhea were observed (856).

Kim and Ivy fed rats on different diets 1100 mg/kg desoxycholic acid daily for 1 week. Results are listed in the following table:

Diet	No. of Rats Male	No. of Rats Female	No. Dead Male	No. Dead Female
Basal	5	3	4	0
Basal and Cholesterol	4	3	3	0
Basal and Corn Oil	5	3	1	0
Basal and Cholesterol and Corn Oil	5	3	0	0

A marked sex difference is noted (413).

Two groups of 6 male rats (250 g) were fed a semisynthetic diet, with or without 0.15% desoxycholic acid for 120 days. Body weight was measured frequently. The weight gain was slow for the first 30 days but there was no detectable effect for the last 90 days (88).

Two groups of 7 male Gofmoor rats weighing 55-70 g were fed a semi-synthetic diet; one group had 0.15% desoxycholic acid in addition. Body weight was recorded two to three times weekly. After 26 days the animals were sacrificed. Gross examinations were made of the intestine, liver, heart, gonads, and histological examination of the heart, liver, stomach with duodenum, and ileum with colon. By the end of 3 weeks, the mean weight of the experimental animals was reduced (88).

Bray and Gallagher found that when rats were fed 0.15% desoxycholic acid ad lib, weight gain was reduced in 2 weeks. After 28 days, no evidence of damage to the liver or intestinal mucosa was observed (86).

Rabbits

Mosbach et al. fed 4 male chinchilla rabbits 0.25% desoxycholic acid in Purina rabbit chow for 4 weeks. Seven animals ate only chow. No gallstones were found in either group upon histological examination of the gallbladder. No obvious toxic effects were noted (543).

C. Glycocholic Acid

Rats

Gambal and Quackenbush studied the effects of sodium glycocholate on rats on a diet free from the essential fatty acids (EFA). Seven control rats ate the basal diet (devoid of essential fatty acids), 7 ate the basal diet plus 1% sodium glycocholate, and 5 positive control rats ate the same diet plus 3% soybean oil. Male weanling Wistar rats were used. After 5 weeks, growth impairment was obvious in all but the positive control group. On a scale in which the growth of the positive controls equalled 100%, the deficient controls ranked 75%, and the sodium glycocholate group ranked 66%. In the test group symptoms of EFA deficiency were reported at 5 weeks (dermatitis, loss of hair); the deficient controls showed symptoms at 8-10 weeks (241).

D. Lithocholic Acid

Chicks

Lithocholic acid was fed at a 0.2% level to 8 Hyline broiler chicks (3 week-old cockerels) for 2 weeks. Eight others served as controls. Weight gain was reduced 20-40% and feed efficiency was impaired. Proliferation of cholangiocytes and marked fibrosis were noted in the livers. Increased liver size (3 fold) was due to proliferation of bile-duct cells (181).

In a second study, 40 chicks ate the same diet plus 0.2% lithocholic acid for 3 months. Histologically the liver changes consisted of necrosis of hepatocytes, followed by proliferation of cholangiocytes and cholangiofibrosis. Within one month ascites and fibrinous exudation were present in the peritoneal cavity. Damage was noted within 12-36 hr after the start of the experiment (181).

Eyssen and DeSomer performed a study on the effects of feeding lithocholic acid to chicks. Two groups of 8 chicks (3 weeks old) were fed 0 and 0.2% lithocholic acid for 2 weeks. Food intake was reduced 25-30% and weight gain by 50% in test chicks. The livers were greatly enlarged with pale yellow-brown coloring. The bile bladders contained a viscous yellow-green fluid instead of the clear green bile of the controls. Histological changes consisted of extensive bile duct proliferation, distorted and disrupted hepatic lobules, and a marked fibrosis (182).

Edwards did a 3 week study on 8 day-old cross-bred cockerel chicks fed 0.2% lithocholic acid in a basal diet. The chicks had a significantly decreased growth rate and a 3-fold increase in serum cholesterol level. When 0.2% cholic acid was added to the same diet, the growth rate was increased and the hypercholesteremic condition was reduced to twice the normal level (162).

Leveille et al. found that male Hy-line chicks fed lithocholic acid (0.2%), with or without cholesterol, had less than normal weight gain, larger livers, higher plasma cholesterol and lipid phosphorus levels, and lower liver fat. Cholic acid feeding partially reversed these effects without cholesterol; the effects were nearly gone with cholesterol. Lithocholic acid feeding also increased liver cholesterol when cholesterol was not in the diet and decreased liver cholesterol in the presence of dietary cholesterol (457).

Leveille et al. observed that 0.2% lithocholic acid in the diet of young chicks 4 to 8 weeks old produced enlarged livers (2 fold increase) due to biliary hyperplasia, a lower amount of liver fat, no significant growth effects, and lower weight gains than control chicks (458).

Two groups of 10 laying hens were fed a basal diet plus 0 or 0.2% lithocholic acid for 30 days. Egg production was reduced significantly in the test group; serum cholesterol was increased. Body weight dropped slightly (164).

Edwards et al. fed a basal diet to 4 groups of 15 Single Comb White Leghorn hens supplemented with 0, 0.025, 0.05%, and 0.1% lithocholic acid; the feeding lasted 20 days. Egg production was reduced at the 0.1% level, weight remained normal. Moulting was increased in all of the test animals. Serum cholesterol was increased, as were total egg lipids and egg cholesterol. Egg size was unaffected (164).

Six chicks were fed 0.1% lithocholic acid for 14 days; they grew less than controls. Their livers were abnormally large (499).

Leveille investigated the effects of feeding lithocholic acid to male Hy-line White Leghorn chicks (130 g). Twenty-eight chicks were started in each of 6 groups; control, 0.2% lithocholic acid, 0.2% lithocholic acid plus 0.2% cholic acid, 2% cholesterol, 0.2% lithocholic acid plus 2% cholesterol, and finally 0.2% lithocholic acid plus 2% cholesterol plus 0.2% cholic acid. Seven chicks from each group were sacrificed at 3, 6, 9, and 12 weeks. Body weight was lower in chicks fed lithocholic acid (49). Dietary lithocholic acid increased plasma lipid levels, decreased liver fat, increased liver size and induced a marked biliary hyperplasia in chicks fed either a cholesterol-free or supplemented diet. Lithocholic acid increased liver cholesterol levels in chicks fed a cholesterol-free diet and decreased liver cholesterol in chicks fed a cholesterol-supplemented diet. Cholic acid supplementation partially prevented the changes in liver size and plasma liver lipid levels resulting from ingestion of a lithocholic acid-supplemented cholesterol-free diet. In cholesterol-fed chicks, cholic acid afforded almost complete protection against the effects of dietary lithocholic acid (459).

E. Ox Bile Extract

Rats

The addition of 0.5% or 1.0% ox bile to a basal diet of male, weanling, 48 to 60 g rats (6 per group) of the Holtzman strain produced a depression in growth. The rats were observed to have hyperemia of the distal small intestine (856).

F. Taurocholic Acid

Rats

When Visek et al. added 2.0% sodium taurocholate to a basal diet over a 28 day period, a significant growth depression, but no fatality, was observed in 6 male, weanling, 48 to 60 g rats of the Holtzman strain. Rats (6) receiving 1.0% taurocholate in a similar study, had hyperemia of the distal small intestine. Rats (6) receiving 0.1% taurocholate apparently showed a stimulation of growth (856).

III. Long-term Studies

A. Cholic Acid

Lacassagne et al. fed 10 male Wistar rats (380 g) a semi-synthetic diet, poor in proteins and riboflavin, with 0.5 g/kg cholic acid added. One animal was sacrificed at 32, 95, 153, 211, 264, 285, 319, 333, 349 and 362 days. No controls were reported. Weight loss of up to 100 g occurred, presumably due to nutritional deficiency. Liver weight increased by 20%. Some steatosis was present in the liver, as were small clusters of lympho-hystocytaric cells in the portal canals. Hypertrophy of the endothelial cells of the sinus was common. The adrenals had a slight increase of vacuolization of the cells of the internal zone of the fascicula, along with a surcharge of chromolipids in the glandula and endothelial cells. The authors reported very slight hypertrophy of the spleen, with some congestion and hemorrhaging. Myelinization and limphoid atrophy were pronounced in the spleen. Spermatogenisis was normal (444).

B. Desoxycholic Acid

Wistar rats, 6 months old, were divided into 4 groups of 14 rats each and fed a basal diet plus 0, 0.005%, 0.025%, or 0.125% desoxycholic acid. Records of weight and food consumption were maintained for 20 months. Pathological examination of the stomach, respiratory tract, ears, feet, spleen, and bone marrow revealed no significant differences between control and test groups (38).

C. Glycocholic Acid

No information available

D. Lithocholic Acid

Lacassagne et al. fed 6 male Wistar rats (330 g) a semi-synthetic diet, poor in protein and riboflavin, with 0.5 g/kg lithocholic acid added. One animal was sacrificed after 28, 49, 103, 161, 272, and 372 days. The rats lost weight initially, but stabilized later on. Liver weight increased by 20%. Some steatosis was present in the liver, as were small clusters of lympho-hystocytaric cells in the portal canals. Hypertrophy of the endothelial cells of the sinus was common. The adrenals had a slight increase of vacuolization of the cells of the internal zone of the fascicula, along with a surcharge of chromolipids in the glandular and endothelial cells. Some congestion and, occasionally, hemorrhageing of the spleen were reported. Myelinization and limphoid atrophy occurred. Spermatogenesis was normal (444).

E. Ox Bile Extract

No information available

F. Taurocholic Acid

No information available

IV. Special Studies

A. Cholic Acid

Carcinogenic

Little et al. studied the effect of various dietary constituents on tumor production in baby chicks injected with Rous sarcoma virus. The tumor-producing virus was injected into the right breasts of 1 day old chicks in doses of 1 mg in 0.25 ml of a suspension of tumor tissue in peptone. The chicks were fed a common basal diet with and without certain nutrients to see if the nutrients stimulated tumor growth, for 16 days. Three times as many tumors developed when 0.25% of the diet was cholic acid. Absence of cholic acid did not prevent tumors from developing (473).

Cholesterol - Cholic Acid Interaction

Thrombosis - Rats

Renaud and Allard fed 10 male albino rats, (108-124 g) a Purina Lab chow diet for 7 weeks. They were then given a diet containing 5% cholesterol, 2% cholic acid as the sodium salt, 53% Purina, and 40% butter. After 35 weeks, a 40% mortality had occurred. Hyperlipemia and some hypertension were present. Histological examination of most of the organs (unspecified) especially the aorta and heart revealed multiple thrombi in small vessels. Lesions of nephrosis were found in the kidneys (667).

Saudanophilia in the heart was reported in rats fed cholesterol (1%) and cholic acid (0.3%), weight gain was depressed during the 28-day study. Edematous kidneys were noted (307).

Monkeys

Wysocki et al. studied the effect of cholate feeding on atherosclerosis in monkeys. Cebus monkeys of various ages and both sexes were fed diets with either casein or alpha-protein as the protein source, with and without the addition of 1% cholic acid (CA). Cholesterol (5%) was present in all diets. Three, two, six and one monkeys were used for the casein without CA, casein with CA, alpha-protein without CA, and alpha-protein with CA diets respectively. The casein without group was studied for 25 months while the others ate the assigned diets for 1 yr. Five monkeys received the alpha-protein diet plus 30 mg cystine for 6 months. All animals were hypercholesterolemic except the casein without CA group. Gross and histochemical examination was made of tissues for atherosclerosis. The addition of cholic acid to the diets produced diarrhea initially; this was associated with weight loss, especially in the animals on the casein diet. One of the monkeys died after 16 days of the regimen (not specified). Significant aortic athersclerosis was found in all animals fed the alpha-protein with and without, and the casein with CA. Hyper-beta-lipoproteinemia was also present (891).

Gallstones - Mice

Tepperman et al. studied the induction of gallstones in mice by feeding a diet containing both cholesterol and cholic acid. Mice

(male and female) were injected i.p. with aurothioglucose (ATG); of those which survived the injection, one third became obese after 3 months on lab chow. At the end of that time ten were fed a high-fat, low-protein, cholesterol, and cholic acid (0.5%) diet for 9 months. Nine remained on lab chow. The cholesterol-cholic acid (C-CA) mice showed a significant elevation of serum cholesterol and a ten-fold increase in liver cholesterol. Nine mice on the C-CA diet had gallstones. None were found in mice on lab chow. Mice on the C-CA diet had grossly fatty livers; three had small nodules (primary hepatomata) distributed throughout the hepatic parenchyma. In a second test, AT6 was omitted; the same gallstones appeared earlier in females than in males. In a third test, various features of the C-CA diet were omitted. It was found that cholesterol and cholic acid must both be fed for the gallstones to occur; food intake had to remain sufficient and liver function unimpaired. The gallstones were proved to be practically pure cholesterol (819).

In a similar study, Bergman and van der Linden subjected 30 mice (15 males and 15 females) (10 g) to a diet containing 1% cholesterol and 0.5% cholic acid. After 4 months, the gallbladder, liver, heart, lungs, spleen, kidneys, and blood were surveyed histologically. Gallstones composed of cholesterol and fatty degeneration of the liver were found. Liver and serum cholesterol increased while cholesterol synthesis decreased (50).

In a later study Bergman and van der Linden found that the omission of cholic acid from a gallstone-producing diet (containing 1% cholesterol) caused gallstones to dissolve in mice (50).

After 1 month on a cholesterol (1%)-cholic acid (0.5%) diet, Bergman et al. found mice to have steatosis of the liver, enlargement of the liver, hypoplastic thyroid, increased stainable lipids in the adrenal cortex, and an atrophied gallbladder with a distension of the mucous membranes. No gallstones were observed. These changes proved to be reversible when cholesterol and cholic acid were removed from the diet.

Similar effects were found in hamsters, however, the histological changes persisted after termination of the experimental diet (52).

Rats

Increased liver weight was reported in male Wistar rats (9-months) fed diets supplemented with 1% cholesterol and 0.4% cholic acid for 11 weeks. Liver sections revealed clay-yellow livers with a friable parenchyma in groups with either 15% hydrogenated or 15% natural rape seed oil. A temporary enlargement of the adrenals was present at 5 weeks (690).

Walker and Emerson reported massive fatty deposits in the parenchymal cells of the liver in rats fed 1.29% cholesterol and 1.0% cholic acid. The diet contained fat corresponding to the fat level and mixture in the average American diet. After 10 weeks growth was normal (861).

Gerbils

Bergman and Van der Linden investigated the effects of cholesterol-cholic acid feeding on Mongolian gerbils. Thirty animals (male and female weighing 60 g) were fed ad libitum lab chow supplemented with 1% cholesterol and 0.5% cholic acid for 4 months. Eight others consumed lab chow only. The gerbils were sacrificed 4 months later; gross and histochemical analysis of the gallbladder, lungs, heart, spleen, lymph nodes, kidneys, adrenals, thyroid, and pituitary glands were carried out. Only 7 out of the 30 test animals survived the eight month period; 6 out of 8 controls survived. The liver weight of test animals was significantly higher than that of controls. Test livers were pale yellow, typical of extreme steatosis and the gallbladders were quite dilated. Fatty degeneration of the liver with fibrosis and modular parenchymal regeneration were observed. Gallstones were found in 5 out of the 7 test gerbils. The gallbladders had thin walls with slight fibrosis and atrophy of the mucous membrane. Spleens were enlarged with ascites present, cholesterol crystals were found in the lungs, spleen, and lymph nodes. The kidneys revealed slight fatty changes in the loop of Henle and in the proximal convoluted tubules. The test diet appeared to accentuate the hypoplasia of the thyroid gland found in normal controls. Cholesterol crystals were found in all layers of the adrenal cortex (53).

In a second study, Bergman and Van der Linden analysed the recovery capabilities of gerbils which had been fed cholic acid and cholesterol. Eighty animals were placed on lab chow supplemented with 1% cholesterol and 0.5% cholic acid. Twenty control animals ate lab chow for 1 month; they were then sacrificed. Twenty of the test animals were sacrificed after 1 month. The remaining 60 test gerbils were returned to lab chow only, and sacrificed in groups of 20 after 1, 2, and 4 months. The test gerbils sacrificed after 1 month on the test diet showed a conspicuous enlargement of the liver. An accumulation of intracellular lipid droplets and cholesterol crystals was found in the parenchymal and Kupffer cells. There were no gallstones but the gallbladder was moderately distended. Animals sacrificed 1, 2 and 4 months after the diet had been switched back to lab chow revealed similar liver changes, but to a lesser degree. The histological changes of lungs, kidney, gallbladder, spleen, and thyroid gland were of a similar nature to the ones present in the preceding study, but less marked. They disappeared when the gerbils were returned to normal diet (53).

B. Desoxycholic Acid

Appetite Suppressant

Bray and Gallagher found that bile acids may control and suppress appetite in humans. In 2 types of experiments, a total of 1400 obese patients were given one of various bile acids or a corn-starch placebo orally for one week to 3 months. The patients were asked to evaluate the effect on their appetites of the drugs. Four out of 6 patients thought desoxycholic acid suppressed their appetites, when taken daily in 1200 mg doses for 2 week intervals. Other bile acids studied were given in the same doses for 6 out of 7 days.

Lithocholic acid was moderately effective, and cholic acid was only slightly effective (no more so than the placebo) (87).

Carcinogenic - Mice

Desoxycholic acid was found to produce malignant tumors in mice when applied directly to the skin or when injected i.m. One out of 20 mice developed an epithelioma at the site of application on the skin in an alcohol-benzene mixture in 776 days. Three out of 10 mice developed spindle-celled tumors in 300 days, after receiving a total of 70 mg desoxycholic acid in 15 i.m. injections. One out of 10 mice developed the spindle-cell tumor in 155 days, after being injected i.m. with 28 mg in 6 injections (129).

Cholesterol-cholic Acid Interaction - Mice

Borgman and Van der Linden subjected 30 mice (10 g, 15 males and 15 females to a diet containing 1% cholesterol and 0.5% desoxycholic) acid. After 4 months, the gallbladder, liver, heart, lungs, spleen, kidneys, and blood were surveyed histologically. Gallstones composed of cholesterol and fatty deneration of the liver were found (50).

C. Glycocholic Acid

No information available

D. Lithocholic Acid

No information available

E. Ox Bile

No information available

F. Taurocholic Acid

Clot removal

Kroell administered 1500 mg sodium taurocholate daily (orally with meals) to 2 elderly men, in which subcutaneous, ^{131}I-labelled plasma clots had been implanted. Six control males were also studied, in which clots were implanted, but no sodium taurocholate was given. The clot-elimination rate, which indicated the fibrinolytic activity, was increased by the sodium taurocholate after a 4 day time lag. One author suggested that the effect was local, rather than systemic (332).

BIOCHEMICAL ASPECTS

I. Breakdown

No information available

II. Absorption - Distribution

Lindstedt and Samuelsson reported that significant amounts of bile acids are absorbed in the cecum of rats. They calculated that if the cholic acid pool in the small intestine, liver, and bile ducts circulates 12 times per day, about 10% of this pool is lost to the large intestine per circulation. Of the total amount transported to the large intestine per day, 70% is absorbed from the large intestine and transported back to the liver in the form of cholic acid, desoxycholic acid, and their metabolites (468).

Weiner and Lack studied the in vivo absorption of bile salts from the small intestine of the guinea pig by determining the biliary excretion of the bile salts injected into isolated regions of the small intestines. They reported that taurocholate, glycocholate and cholate were absorbed in the ileum. Absorption from the proximal mucosa was absent (875).

Borgstrom et al. reported that bile acids are absorbed or reabsorbed from the lower part of the small intestine in man (79).

Samuel et al. reported that cholic acid is converted to desoxycholic acid in the human colon, and both of these bile acids are absorbed from the human large bowel in significant amounts. Labelled cholic acid was injected into the cecal lumen, hepatic flexure of the colon, or into the transverse colon of several men (cholecystectomy patients). The cecum, ascending colon, hepatic flexure, and transverse colon may be sites of reabsorption. Fifty eight percent of the radioactivity appeared in the common bile duct within 5 days, with most of it excreted during the first 24 hours (704).

During the enterohepatic circulation of bile, most of the bile acids excreted by the liver are reabsorbed during transit in the small bowel. They are then carried back to the liver by the portal vein, and are eventually reexcreted into the gallbladder and used again. The pool of bile acids is circulated 6-10 times daily through the enterohepatic cycle. At each cycle a small fraction of transiting bile acids will escape reabsorption in the small bowel, will find its way into the large intestine, and will appear in the feces. Samuels et al. state that daily fecal excretion of cholic acid metabolites ranges from 87-2114 mg for man (704).

III. Metabolism and Excretion

In 1970, Norman summarized the bile acid metabolism in man. Cholic acid and chenodesoxycholic acid, primary bile acids, are excreted in bile conjugated with taurine or glycine. In the duodenum and upper part of the jejunum, the bile acids are present in the conjugated form. Since fecal bile acids are mainly unconjugated, the bacteria in the intestinal tract must deconjugate them. Also they convert

primary bile acids to secondary bile acids (especially the conversion of cholic acid to desoxycholic acid) which then enter the enterohepatic circulation. Part of the unconjugated bile acids are probably absorbed, but since only conjugates are in normal bile, these unconjugated acids are conjugated prior to excretion in the bile (577).

Cholic acid is excreted in the bile of rats completely conjugated, 1-2% with glycine and the rest with taurine (467).

Labeled sodium desoxycholate was excreted almost entirely as conjugated desoxycholate (85% with glycine; 13% with taurine) after intravenous administration to men with external bile fistulas. Hanson and Williams stated that these results indicated that desoxycholic acid cannot be rehydroxylated to cholic acid by the human liver. They mentioned that lithocholic acid cannot be rehydroxylated either (284).

Rabbit bile from the gallbladder contains almost pure desoxycholic acid conjugated with glycine. Cholic acid is converted to desoxycholic acid in the gut, which cannot be rehydroxylated to cholic acid in the liver (56).

Lindstedt and Norman reported that when cholic acid-24-^{14}C was injected i.p. into rats, about 85% was recovered from the feces within 10 days. Only minor amounts of the fecal cholic acid were found to be conjugated. Extensive changes on the bile acid molecule by microbial enzymes were observed (467).

From studies of the human intestine, Aries et al. have found that many enterobacteria convert cholic acid to 7 ketodesoxycholic acid only. The bacteria Bacterioides spp and Clostridia spp produce desoxycholic acid, lithocholic acid, and possibly cholanic acid together with 7 ketodesoxycholate. Bacterioides spp, Bifidobacterium spp, Clostridium spp, Veillonella Spp, and enterococci were able to deconjugate bile salts (20).

While studying human fecal extraction products of cholic acid, Norman found that over half the products in fecal homogenates were dialysable. Very few conjugated bile acids or cholic acid were left in unchanged forms. The major metabolites were 3,12-dihydroxycholanic acid and 3-hydroxy-12-ketocholanic acid. About 25% of the metabolites were saponifiable derivatives of 3,12-dihydroxycholanic acid, of which most were the 3 beta isomer. Over 90% of these were dialysable. The derivatives of cholic acid recovered were either poorly soluble or easily adsorbed to the residue in the intestinal contents (576).

A rat weighing 350 g whose bile ducts were cannulated received into the upper duodenum 1 mg of cholic acid 24-14C. Within 5 hours, 19.2% of the labeled material was excreted via biliary fistulas; after 24 hours 57.5% was excreted, and after 48 hours 69.0% was excreted (648).

Norman orally administered 24-^{14}C-cholyl 1-^{14}C glycine (glycocholic acid) to man. Twenty five hours later 43-75% of the remaining 24-^{14}C labelled bile acids were present in the gallbladder as double labelled glycocholic acid; 3-6% of the labeled bile acids had been 7-alpha-dehydroxylated. Since the glycodesoxycholic acid isolated only

contained a trace of isotope in the glycine compared to the steroid moiety, Norman indicated that the unconjugated cholic acid was the main substrate for 7-alpha-dehydroxylation in the intestinal tract. While the main reactions in the intestinal tract are deconjugation and dehydroxylation, some cholic acid was converted to 3-alpha, 12-alpha-dihydroxy-7-keto-5-beta-cholanoic acid and 3-alpha, 7-beta, 12-alpha-trihydroxy-5-beta cholanoic acid (577).

Gustafsson and Norman fed 3 groups of rats 3 different diets - a semi-synthetic diet (D7), D7 + 20% cellulose, and a commercial diet (pellets). The fecal metabolites of the bile acids were labelled by givving each rat 0.1 mg sodium 24-^{14}C cholate (5 uci) orally. For the next 8 days feces were collected, the metabolites separated by partition column chromatography, and identified by TLC and autoradiography. The authors stated that cholic acid (3-alpha, 7-alpha, 12-alpha-trihydroxy-5-beta-cholanoic acid) and chenodesoxycholic acid (3-alpha, 7-alpha-dihydroxy-5-beta-cholanoic acid), which are the primary bile acids, are changed into secondary bile acids in entero-hepatic circulation through the metabolism of intestinal bacteria. The secondary bile acids are partially absorbed, modified by the liver, and then partially eliminated in the feces. The secondary bile acids, metabolites of cholic acid, which the authors investigated were desoxycholic acid (3-alpha, 12-alpha-dihydroxy-5-beta-cholanoic acid) and lithocholic acid (3-alpha-monohydroxy-5-beta-cholanoic acid) (275).

The rats fed D7 or D7 + cellulose had a low level of bile acid excretion; the pellet group had a high level. The bile acids excreted in all 3 dietary groups were mostly unconjugated, 7-alpha-dehydroxylated forms. All 3 dietary groups excreted 3-alpha, 12-alpha-dihydroxy-5-beta-cholanoic acid and 3-beta, 12-alpha-dihydroxy-5-beta-cholanoic acid in a 3 to 1 ratio. The authors did not isolate 3,12-diketo-5-beta-cholanoic acid from the feces of any of the 3 groups. The D7 + cellulose and the pellet-fed rat groups exhibited an increased formation of keto derivatives (3-alpha-hydroxy-12-keto-5-beta-cholanoic acid and 3-beta-hydroxy-12-keto-5-beta-cholanoic acid) (275).

Gustafsson et al. reported that when deconjugating bacteria had been established in the digestive tract of germ free rats, 15-25% of the excreted bile acids were unconjugated. When 7-alpha-dehydroxylating bacteria (Lactobacilleae) were also established, the 7-alpha-dehydroxylation of the unconjugated bile acids was observed (279).

Gustafsson and Norman found that male germ-free rats (Swedish strain), 107 to 235 days old, excreted bile acids to a lesser degree than normal rats. Bile acids in germ-free and normal rats were found to be mostly in the small intestine, no matter what the diet (basal diet D7, D7 + cellulose, or commercial pellets). The cholic acid excretion was higher in both normal and germ-free rats on the pellet diet than the rats on diet D7. In the rats fed diet D7, the germ-free rats were most different from the normal rats in cholic acid excretion possibly because of the longer transit time of intestinal contents in germ-free rats. The prevention of coprophagy in any rat group did not significantly influence the half-life of cholic acid (274).

Bergstrom noted in his review of bile metabolism that desoxycholic acid could be rehydroxylated to cholic acid in the rat liver (56).

Fifty percent of the cholic acid transported to the large intestine was converted to desoxycholic acid in the rat (468).

A half-life of 2-3 days has been found for any carbon labeled bile acid for a normal rat. A pool size of 20-25 mg in a 200 g rat corresponds to a daily production of 5-6 mg bile acid (56).

Lindstedt determined the half life of cholic acid to be 2.8 days on the average in man. The cholic acid pool size was 1.39. Data was based on the oral administration of cholic acid -24 ^{14}C to 8 men. The bile acids are practically confined to an anatomically restricted space consisting of liver, bile ducts, gallbladder, intestines and portal circulation. Under normal conditions the concentration of bile salts in the systemic circulation is negligible and bile acids are exclusively excreted in the feces. In healthy individuals the bile and pool are in a steady state with any excreted bile acids being replaced by synthesis in the liver (466).

Gordon et al. found that half life of cholic acid in man to depend on the amount of fat in the diet. On a butterfat diet (45% of the calories) the half life was 8.8 days. On a corn oil diet it was 3.7 days, but on a fat-free diet it was 4.2 days. The cholic acid pool size was 1.67 g on the butterfat diet, 1.49 g on corn oil, and 1.59 g on the fat-free diet (259).

IV. Effects on Enzymes and Other Biochemical Parameters

Desoxycholate (0.4 mM) inhibited the transport of urea, lysine, leucine, acetate, and sodium across perfused segments of the rat jejunum. Cholate was a weaker inhibitor (645).

Pope et al. perfused segments of the rat jejunum with solutions of the various bile acids. Eight mM sodium cholate and 0.8 mM sodium desoxycholate inhibited glucose transport irreversibly. The mucosa was damaged. Conjugated salts had no effect. The authors suggested that free bile acids are toxic to the upper gut, but tolerated by the epithelial cells of the ileum and colon. Normally, the conjugated bile salts (taurocholate and glycocholate) are hydrolyzed to the free acids in the lower intestines. Upon transport to the liver, these free acids are again hydroxylated and conjugated by the microsomes (645).

Cholic and taurocholic acids were found by Burke et al. to be strongly bound to plasma albumin, the stronger bond being with cholic acid. Cholic acid was found to be over 95% bound and taurocholic acid was 50% bound in the serum of 2 human subjects. The bile acids also bound with serum globulins to some degree. The percent of which cholic and taurocholic acid is bound to human serum or albumin is not significantly affected by the bile acid concentration, because of the strong binding capacity (100).

The oral administration of 20 mg/kg doses of sodium glycocholate or taurocholate increased the gastric movements of rabbit's

stomach; a larger dose (60 mg/kg) decreased it (709).

Eyssen et al. reported that dietary cholic acid improved intestinal fat absorption in chicks lithocholic acid decreased fat absorption. Desoxycholic acid had no effect (182). Previously, Edwards had found a similar effect for cholic acid (163).

Back et al. reported that dietary cholic acid (1%) inhibits the biosynthesis of cholesterol in rats. Levels of 0.1-0.15% cholic acid attained a maximum inhibition after 20 hours (32).

Peric-Golia found that cholic acid, chenodesoxycholic acid, and cholesterol were the major labelled components in the bile of 15 rats given ^{14}C cholesterol i.v. The unlabelled cholic acid group received 2 mg of the sodium salt of cholic acid by daily duodenal infusion; the other test group (5 rats) received 2 mg cholesterol in the same manner. The control rats were given ethanolic saline. The labelled cholic acid was no longer detected in the bile of control rats (5 rats) after the 8th day; in the group (5 rats) given unlabelled cholic acid, labelled cholic acid remained until the 15th day. Excretion of labelled compounds in the bile was at the greatest level on the 2nd day after ^{14}C cholesterol in the 3 groups. The daily cholic acid excretion of the control group was higher than the group receiving unlabelled cholic acid. The author stated that when the amount of cholic acid in the enterohepatic circulation was increased, 1) the conversion of exogenous cholesterol to cholic acid was delayed; 2) the excretion of cholic acid in the bile was prolonged; 3) the cholesterol level in the plasma was increased. These changes were not observed in the rats receiving unlabelled cholesterol (632).

The feeding of cholic acid and cholesterol to gerbils induced a rise in cholesterol, cholic acid, and desoxycholic acid concentrations and a decrease in chenodesoxycholic acid. It was proposed that dietary cholic acid reduces biosynthesis of bile acids (52).

Beher et al. studied the effects of feeding 24 female, albino, Webster strain mice a synthetic cholesterol-free diet supplemented with 0.5% cholic acid. An additional 24 mice were fed the cholesterol-free diet alone. Both groups were given labelled compounds (mevalonic acid 2-^{14}C or cholesterol 4-^{14}C) i.p. The controls had 60% of the ^{14}C activity in the sterol fraction of the feces and 40% in the bile acid fraction. Cholic acid caused equal decreases in the rates of hepatic cholesterol synthesis and bile acid biosynthesis. The authors concluded that a double feedback mechanism governed by cholic acid and its conjugates controls the rate of hepatic cholesterol biosynthesis in the mouse (48).

Feeding 0.2% lithocholic acid to chicks resulted in increased serum cholesterol and total liver cholesterol (181).

Beher and Anthony used Webster strain male albino mice, 18 to 20 g, for a 3-week feeding study, with 7 dietary groups, containing 7 to 9 mice each. Every diet was basically cholesterol-free with cholesterol, cholic acid, and/or dihydrocholesterol added as supplements. It was found that when the cholic acid content was

increased in the diets supplemented with cholesterol alone, the liver cholesterol and beta steroids were increased to a certain limit. Increasing the cholic acid in diets containing constant levels of dihydrocholesterol and cholesterol decreased the liver cholesterol and beta steroids. If dihydrocholesterol was added to diets containing cholic acid and cholesterol, the liver cholesterol and beta steroids was also decreased (41).

Portman and Bruno found that feeding cholic acid (0.4%) to male rats (adult) for 21 days increased serum cholesterol levels 25% above the controls. When cholesterol is included in the diet, the liver cholesterol increased almost 100%. Cholic acid without cholesterol resulted in a smaller and slower accumulation of liver cholesterol. Taurocholic and glycocholic acids were found to be as active in hypercholesterolemia as cholic acid. Desoxycholate was less active; lithocholic had no significant activity. It was suggested that all bile acids with a 12-alpha-hydroxy group had some hypercholesterolemia activity (648).

Howe et al. reported that dietary taurocholic acid is less hypercholesterolemic than cholic acid in the mouse. Lithocholic acid exerts a hypocholesterolemic effect and nuetralizes the hypercholesterolemic action of other bile acids (343).

Beher and Anthony found that when the cholic acid percentage was increased in a cholesterol-rich diet, the total liver cholesterol in mice was significantly increased (42).

Rats fed a mixture of cholic acid and cholesterol had high levels of plasma cholesterol after 10 weeks. Total liver lipids and cholesterol were higher than normal as well. The diet contained a fat mixture comparable to that of an average American (861).

Portman and Murphy reported that dietary cholesterol (5%) and cholic acid (1.5%) induced an increase in the number of beta-lipoproteins in sera with increased cholesterol composition in rats (650).

Cholic acid added to the diet of 8 rats increased serum cholesterol levels; the diet contained cholesterol and oleic acid. When corn oil was substituted for oleic acid, the serum cholesterol did not rise. Desoxycholic acid did not increase cholesterol absorption (413).

Howe and Hutchison reported that bile acids in the absence of dietary cholesterol have no effect on plasma cholesterol levels in the rat. Levels of 0.05% and 0.1% desoxycholic acid and 0.1% cholic acid were fed for 12 days. In the presence of dietary cholesterol, hypercholesterolemia was found for both acids (342).

Friedman et al. reported an increased lymphatic absorption of cholesterol following the oral administration of 50 mg cholate to rats. The authors state that cholate promotes the intestinal absorption of cholesterol; when in excess in the plama, cholate may produce hypercholesteremia (208).

Member et al. studied the effects of feeding bile acids on the levels of cholesterol in the blood. Cholesterol and one of the bile acids were fed to 75 female rabbits, 3 to 5 months old. The various diets were continued for 12 to 14 weeks each; the entire study lasted 66 weeks. Each group of cholesterol plus bile acid was compared with a control group fed cholesterol alone (39 rabbits). The cholesterol was mixed with the basic Ralston Purina rabbit chow in 1000 mg doses 3 times per week. The bile acid was mixed with the chow in 500 mg doses 3 times per week.

Diet Supplement	Effects
Cholesterol and Cholic Acid	Increased Cholesterol level in whole blood, aorta, and liver; increased combined cholesterol (ester) of whole blood; all levels were greater than with cholesterol alone.
Cholesterol and Glycocholic Acid	Same as above
Cholesterol and Desoxycholic Acid	Did not increase cholesterol levels

The authors suggested that cholic acid may stimulate esterification of cholesterol in the intestinal wall, and that cholic and glycocholic acids may increase the absorption of cholesterol from the intestine, which causes high cholesterol levels in the blood (512).

V. Drug Interaction

When desoxycholic acid and reserpine were concurrently administered orally to mice, the blepharoptotic activity of reserpine was increased (495).

VI. Consumer Exposure

Cholic acid and its derivatives are used in food as an emulsifying agent. The only information available from the NAS/NRC survey is on the use of ox bile extract mainly in cheese.

The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on frequency of eating and from the USDA data on mean portion size of foods in each food category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of each substance.

Table 2 reports the usage of ox bile extract in foods and table 11 reports the annual poundage data. Table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample.

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/01/72

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
OX BILE EXTRACT NAS 0140	06 CHEESE(R)	*	.00090	.00170

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/03/72

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

OX BILE EXTRACT NAS 0140	*/ *	400	470	470	470

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE -- -- SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
OX BILE EXTRACT NAS 0140	06 CHEESE(R)	*	0-5 MO.	**********	.000900	**********
			6-11 MO.	.024300	.087300	.043900
			12-23 MO.	.070200	.159800	[illegible]
			2-65+ YR.	.084600	.212400	.159800
OX BILE EXTRACT NAS 0140	ALL CATEGORIES	*	0-5 MO.	**********	.000900	**********
	**********		6-11 MO.	.024300	.087300	.043900
	**********		12-23 MO.	.070200	.159800	.132600
	**********		2-65+ YR.	.084600	.212400	.159800

CHOLIC ACID

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Degradation of bile salts by human intestinal bacteria

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We have previously reported that many strains of enterococci and the strictly anaerobic bacteria of the intestine (Bacteroides, Bifidobacteria, Clostridia, and Veillonella) hydrolyse bile acid conjugates (Drasar, Hill, and Shiner, 1966; Hill and Drasar, 1968). More detailed studies on the deconjugating enzyme, cholanylglycine hydrolase, obtained from Bacteroides spp, Bifidobacterium spp, Clostridium spp, and *Strep. faecalis* (two strains of each) are reported elsewhere (Aries and Hill, 1969). Here we report our studies on the metabolism of cholate and deoxycholate, and further studies on deconjugation.

METHODS

Samples of faeces from normal persons living on a mixed diet in England, and from normal persons living on a vegetarian diet in Uganda were examined bacteriologically using the methods described previously (Drasar, 1967), modified to facilitate the isolation and identification of Bacillus spp, Pseudomonas spp and anaerobic Sarcina. The differences between the faecal flora of Ugandans and English people are discussed elsewhere (Aries, Crowther, Drasar, Hill and Williams, 1969).

The bacteria were grown at 37 C under the conditions listed in Table I. Bacteria from 50 ml of broth culture were harvested by centrifugation and resuspended in 0.5 ml of supernatant. This suspension was mixed with 0.5 ml of a 0·1% solution of substrate (taurocholate, cholic acid, or deoxycholic acid) in 0·02 M phosphate buffer solution pH 7 and incubated at 37 C for 48 hours. Degradation products were separated by thin layer chromatography on silica gel G (Merck) using the solvents described by Eneroth (1963). Standard solutions were included on all plates; these contained sodium taurocholate (Maybridge Research Chemicals Ltd, Tintagel, Cornwall), cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid (all from Koch-Light Laboratories), cholanic acid (Steraloid Ltd), 7 keto deoxycholic acid, 12 keto lithocholic acid, and 7,12 dihydroxycholic acid, all prepared by oxidation methods from the parent hydroxyl compound (Fieser and Rajagopalan, 1949; Bergstrom and Haslewood, 1939).

TABLE I

GROWTH MEDIA AND CONDITIONS OF THE VARIOUS ORGANISMS STUDIED

Bacteria	Medium	Period of Growth (hr)	Atmosphere	Other Conditions
Enterobacteria	Glucose broth	24	Air	
Enterococci	Glucose broth	24	Air	
Str. salivarius	Reinforced clostridial medium	48	Air	
Lactobacilli	Reinforced clostridial medium	48	90% CO_2 10% air	
Clostridia	Reinforced clostridial medium	48	10% CO_2 in H_2	
Veillonella	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bacteroides	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bifidobacteria	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bacillus spp	Glucose broth	24	Air	Shaking
Anaerobic Sarcina	Reinforced clostridial medium	24	10% CO_2 in H_2	

RESULTS

Groups of organisms unable to deconjugate sodium taurocholate in the more concentrated solutions used in our previous studies were unable to do so in the dilute solutions used here.

Many strains of Bacteroides spp, Bifidobacterium spp, Clostridium spp, Veillonella spp and enterococci were able to degrade cholate and deoxycholate yielding one or more products (Table II). Strains of these genera also deconjugated bile salts. In addition, many enterobacteria, none of which were able to deconjugate bile salts, were able to metabolize the bile acids. The reaction was subject to substrate inhibition and very little reaction with 0·1% cholate was detected (Hill and Drasar, 1968).

TABLE II

ORGANISMS ABLE TO DEGRADE TAUROCHOLATE, CHOLATE, AND DEOXYCHOLATE

Organism	Source of Faeces	No. Tested	Percentage Able to Degrade Bile		
			Taurocholate	Cholate	Deoxycholate
Enterobacteria	Uganda	180	0	53	32
	England	87	0	78	39
Enterococci	Uganda	162	90	68	18
	England	90	93	81	29
Str. salivarius	Uganda	37	0	0	0
	England	45	0	0	0
Lactobacilli	Uganda and England	48	0	0	0
Clostridia	Uganda	69	96	91	43
	England	70	94	87	47
Bifidobacteria	Uganda	137	86	47	18
	England	57	74	56	21
Bacteroides	Uganda	17	71	48	18
	England	37	82	79	30
Bacilli	Uganda	36	11	25	0
	England	45	49	9	0
Veillonella	Uganda	72	36	19	1
	England	20	95	90	30
Anaerobic Sarcina	Uganda	12	67	58	0

(It is possible that a higher proportion of strains would prove to be active at still lower substrate concentrations.)

A number of strains of each of the active genera are under more extensive investigation. In general the enterobacteria produce only a single degradation product from cholic acid, usually 7 ketodeoxycholate. The Bacteroides and Clostridia are much more active, producing a number of products, some of which have been identified as deoxycholic acid, lithocholic acid, and possibly cholanic acid together with 7 ketodeoxycholate. In addition, a number of products in which the α hydroxyl groups have been inverted to the β form (presumably by way of keto intermediates) have been tentatively identified.

DISCUSSION

The conversion of cholate to deoxycholate (Midtvedt, 1967; Coccucci, and Ferrari, 1963) and other products by bacteria is now well documented. Midtvedt and Norman (1967) have screened a number of anaerobic bacteria and identified the products of cholate metabolism as 3α 12α dihydroxy 7 keto cholanate, 3α hydroxy 7,12 diketo cholanate and 3, 7, 12 triketo cholanate. We have not detected the two latter metabolites but this may be a result of the reaction conditions; Midtvedt used a much lower substrate concentration and incorporated it in the growth medium. The concentration used in our work (0·05%) is the lowest that can conveniently be used with our detection methods.

In conclusion, our results demonstrate that, in the metabolism of bile salts and acids by intestinal bacteria, the strictly anaerobic bacteria are of major importance. Our investigations on human faecal steroids indicate that this is probably true *in vivo* as well as in the situation *in vitro* described here.

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Regulation of Cholesterol Biosynthesis in Rat Liver: Diurnal Changes of Activity and Influence of Bile Acids.[1]

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Rats maintained on semisynthetic diets show a low rate of acetate incorporation into cholesterol, when compared with rats fed a commercial diet. Addition of a bile acid sequestering anion exchange resin to the semisynthetic diet causes a several-fold increase of hepatic cholesterol synthetic activity.

A diurnal rhythm is demonstrated for the incorporation of acetate-1-^{14}C (but not of mevalonate) into cholesterol by rat liver slices and liver extracts. Synthesis at midnight is increased by a factor of 3.8 over the synthesis which is measurable during the day. The rhythmic activity changes are due to activity changes of the enzyme system synthesizing mevalonate. These rhythmic changes of activity are assumed to be due to alterations of the level of enzyme protein. Cholic acid, when given with the diet, also acts at this enzymic step as inhibitor of cholesterol biosynthesis and is shown to be much more effective than dietary cholesterol, but the concentration levels of cholic acid in liver do not show alterations which can be correlated with the observed rhythmic activity changes under conditions of normal feeding.

The studies of Bucher *et al.* (1, 2) have established that the synthesis of cholesterol in rat liver is regulated by changes of activity of the rate-limiting microsomal enzyme HMG-CoA reductase.[3] This enzyme acts at the first step of the ultimate enzymic chain synthesizing cholesterol (3). It irreversibly converts HMG-CoA to mevalonate (4) and evidence has been obtained for it to be sensitive to a wide variety of nutritional and experimental conditions (1, 2, 5–8). Regen *et al.* (9) demonstrated the rapid decrease of HMG-CoA reductase activity in starved animals and showed recovery of activity of HMG-CoA reductase in refed animals. Refeeding a semisynthetic diet to starved rats failed to give the maximum values of cholesterol synthesis from acetate, which could be obtained after refeeding ordinary laboratory chow (10). This observation could be taken as an indication for the involvement of a nutrition-linked extrinsic or intrinsic factor in the regulation of the rate of cholesterol biosynthesis in rat liver. Previous investigations had already shown that commercial rat diet causes a more rapid excretion of bile acids (12) and cholesterol (13), respectively, than does semisynthetic diet. Therefore the reinvestigation of the role of the bile acids, the catabolic end products of cholesterol in liver, which undergo enterohepatic circulation and were reported to act as feedback inhibitors (13), seemed to be necessary. This was done especially with the aim to get some insight whether cholesterol synthesis is regulated by an inhibitor of HMG-CoA reductase or by synthesis and decay of this enzyme.

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[3] Abbreviations used are as follows: CoA, Coenzyme A; HMG-CoA, β-hydroxy-β-methylglutaryl coenzyme A; HMG-CoA reductase, mevalonate: NADP oxidoreductase (acylating CoA), EC 1.1.1.34; NAD, nicotinamide adenine dinucleotide (oxidized form); NADP, nicotinamide adenine dinucleotide phosphate (oxidized form).

The results to be presented in this paper demonstrate a diurnal rhythm of cholesterol biosynthesis in rat liver. This rhythm is shown for the activity of mevalonate formation from acetate and presumably is due to a diurnal rhythm of enzyme synthesis. Evidence is also provided for the action of bile acids at the level of the synthesis of the microsomal enzyme HMG-CoA_2 reductase.

MATERIAL AND METHODS

Animals. Male Sprague-Dawley rats of 100-200 g body weight were used in all experiments. For each experiment rats of almost equal weight were selected. The animals were kept in metal cages and had free access to food and water. The room temperature was held at 26°. The illumination followed the day-night cycle.

Diets. Altromin R from Altrogge (Lage/Westf.) was used in pelleted form as normal diet. For preparation of cholic acid-supplemented diet the Altromin R pellets were ground to powder and mixed with two times recrystallized cholic acid (E. Merck AG, Darmstadt) in powder form. The mixture was stirred with distilled water to give a sticky mass, from which new pellets could be formed easily. After air-drying the supplemented diet was fed *ad libitum*. Cholesterol (E. Merck AG) two times recrystallized from ethanol was dissolved in acetone:ether 1:1 (v/v) and the solution was mixed with Altromin to give a homogeneous mass. The solvent mixture was evaporated in vacuo and pellets were formed according to the procedure described above. Semisynthetic diets contained casein (E. Merck AG), fat in form of Mazola-oil (Maizena-Werke, Hamburg), D-(+)-glucose, a vitamin mixture, and a salt mixture according to Grab (14) to provide all requirements for sufficient growth. Cholestyramine anion exchange resin was obtained from Merck, Sharp and Dohme, Munich. Pellets of semisynthetic diet were prepared after addition of distilled water and dried. For the composition of semisynthetic diets see Table I.

Measurement of acetate-1-^{14}C incorporation into the nonsaponifiable lipid fraction by liver slices. Liver slices were prepared freehand from fresh ice-cold liver by means of razor blades and transferred to ice-cold bicarbonate-buffer (15), which was continuously bubbled through by an O_2/CO_2 (95%/5%) gas mixture. Tests were performed with 500 mg of blotted slices. The slices were equilibrated 10 min at 37° in 3.5 ml of bicarbonate buffer, pH 7.4, under the same gas atmosphere; the incubation was started by addition of 10 μmoles of sodium acetate-1-^{14}C (Radiochemical Centre Amersham; specific activity 18 nCi/μmole) dissolved in 0.5 ml of water. After 2 hr of shaking under O_2/CO_2 atmosphere the reaction was stopped with 1 ml of a saturated solution of potassium hydroxide. One milligram of carrier cholesterol was added in ethanolic solution and the reaction mixture was hydrolyzed for 1 hr in an autoclave at 120°. The nonsaponifiable lipids were extracted three times with petroleum ether under careful elimination of traces of the watery phase. After evaporation of the solvent the residue was dissolved in 2.5 ml of dioxane and an aliquot of 2.0 ml was counted in a liquid scintillation counter (Tri-Carb Packard) after addition of 0.5 ml of distilled water and 10 ml of a premixed scintillator containing 0.07 g of 2,5-diphenyloxazole, 0.003 g of 1,4-bis-2-(5-phenyloxazolyl) benzene, and 1 g of naphthalene in dioxane. The radioactivity of the substrate was counted under the same conditions. The calculations of the incorporation rates of radioactive acetate into the nonsaponifiable lipids were expressed in percentages of radioactivity introduced by the substrate.

Measurement of incorporation of acetate-1-^{14}C and mevalonate-2-^{14}C into nonsaponifiable lipids by liver extract. The liver extract (5000*g* supernate) was prepared according to Bucher *et al.* (16). The final volume of the incubation mixture was 2.45 ml containing 2 ml of liver extract, 20 μmoles of glucose-6-phosphate, 2 μmoles of NADP, 2 μmoles of NAD, and 10 μmoles of sodium acetate-1-^{14}C (sp radioact 18 nCi/μmole) or 15 μmoles of sodium RS(±)-mevalonate-2-^{14}C (Tracerlab, Waltham, Mass.) with a specific radioactivity adjusted to 1.55 nCi/μmole.

The incubation and extraction were performed in the same manner as described for the incubation of liver slices. Protein determinations were done by the biuret method. The incorporation proceeded linearly for acetate-1-^{14}C and mevalonate-2-^{14}C over the incubation period of 2 hr under all experimental conditions.

Thin-layer radiochromatography of the nonsaponifiable fraction of liver lipids. After incubation and extraction a representative fraction of the nonsaponifiable lipids was chromatographed at room temperature with the solvent system chloroform/diethylether 9:1 (v/v) on heat-activated Silica-Gel G (E. Merck AG). Plates were prepared by the dip-in method (17). 78% of the total radioactivity was found in the cholesterol fraction (R_f = 0.35-0.52). The rest of the radioactivity was found in three minor peaks, which were not identified.

Experiments with cycloheximide. Cycloheximide (Nutritional Biochemicals Corp.) was injected intraperitoneally once in doses of 1 and 10 mg/200 g body weight into animals of two experimental groups. Controls were injected with saline. Ani-

mals appeared to have eaten during the experiment since their stomachs at autopsy were filled with food. The animals were killed after 4 and 6 hr, and liver extracts were prepared as described above. The incubation and determination of incorporation of the radioactive substrates were performed in the manner already described.

Quantitative gas-chromatographic determination of the bile acid content of liver extracts. Fifteen-milliliter aliquots of 5000*g* supernatant fluids were hydrolyzed in metal beakers after addition of 5 ml of 5 N potassium-hydroxide and 200 μg of 3 α-, 6 α-dihydroxycholanic acid (hyodeoxycholic acid) as an internal standard. Hydrolysis took place at 120° under a pressure of 1 atm for 3 hr. The extraction of the free bile acids was performed by the method of Sjövall (18). The free bile acids were methylated according to the procedure of Schlenk and Gellerman (19). Before evaporation of excess diazomethane and solvent, 50 μg of 5 α-cholestane (Aldrich Chemical Comp.) was added as a second internal standard and the methyl esters were dried overnight in vacuo. For gas chromatography trimethylsilyl-ethers of the methylesters were prepared according to the procedure of Makita and Wells (20). After 30 min standing at room temperature the reaction mixtures were injected into the gas chromatograph (Varian-Aerograph 1520 equipped with flame-ionization-detection system). The gas-chromatographic system was almost the same as described by Makita and Wells (20). The working temperatures were 280° for the flash-heater, 238–248° for the column, and 300° for the detector. Helium at a flow of 30 ml/min served as carrier gas. The 3.6 m × 0.32 cm steel column used for the analyses contained as stationary phase 0.75% HiEff-8 Bp (Applied Science Lab.) coated on acid-washed, silanized Chromosorb W (80–100 mesh). The number of theoretical plates (21) calculated for the trimethylsilylethermethylester derivative of cholic acid was 5850. Linearity of the detector signal was given within the range of 5×10^{-2}–10 μg of injected methylester.

The calculations of the amounts of bile acids were performed by means of calibration curves for each bile acid. Pure methylesters of cholic acid (E. Merck and Co., Darmstadt) (mp 154–155°) (22), deoxycholic acid (Schuchardt, München) (mp 93°) (23), hyodeoxycholic acid (Schuchardt) (mp 111–112°) (24) and lithocholic acid (Schuchardt) (mp 126–128°) (25) were prepared and used as standard substances. Chenodeoxycholic acid (Calbiochem) was methylated by the diazomethane procedure (19) and trimethylsilylated as described above. Hyocholic acid, a generous gift of Dr. H. Danielsson, was used in the same way as standard substance without previous purification by synthesis of the methylester. The amounts corresponding to two unknown peaks with retention times equal to the reported values for α- and β-muricholic acid were calculated using the calibration curve of cholic acid. Each analysis was repeated three times and the mean value was determined. By using the internal extraction standard hyodeoxycholic acid corrections for the recovery and for differences of the volume injected were made based upon the two added internal standards. This procedure gave an error of no more than ±5%, when known mixtures of bile acids were tested.

Statistics. The significance of differences between mean values was determined by means of the Student *t*-test.

RESULTS

The experiments presented here were performed with the aim to compare rates of cholesterol formation *in vitro* with those *in vivo*. Therefore time-consuming isolation procedures which might diminish enzyme activity have been avoided and the preparatory steps for tissue slices and Bucher-homogenates have been performed as quickly as possible. From alterations of acetate or mevalonate incorporation into cholesterol in the same liver preparation conclusions could be drawn concerning the activity of the rate-limiting enzyme system.

The findings (10) that refeeding of starved rats with semipurified diet resulted in low activity of cholesterol formation from acetate could be correlated with observations (11) of low bile acid excretion during administration of a semisynthetic diet in contrast to the high excretion rates after feeding normal laboratory chow. Therefore we tried to increase the excretion by addition of a bile acid-sequestering agent to the semisynthetic diet in analogy to experiments of Huff *et al.* (26). The experimental results after addition of the anion exchange resin Cholestyramine, which is able to remove bile acids from the enterohepatic circulation (27), are shown in Table I. In spite of an even lower dietary protein content, the activity values of cholesterol synthesis from acetate in liver slices of rats refed the semisynthetic diet supplemented with Cholestyramine were higher than in liver slices of rats fed the semisynthetic diet without the resin. This result prompted investiga-

TABLE I

INCORPORATION OF ACETATE-1-^{14}C INTO STEROL FRACTION BY LIVER SLICES AFTER REFEEDING OF DIFFERENT DIETS OVER 4 DAYS TO RATS PREVIOUSLY STARVED FOR 24 HR. VALUES TAKEN AT 1:00-3:00 PM.[a]

Diet	No. of animals	Radio activity incorporated (mean values) in %
Normal laboratory chow	4	1.03
Semisynthetic I 21% protein 5% fat 58% glucose	5	0.39
Semisynthetic II (Cholestyramine: semisynthetic I 1:49 (w/w))	2	1.54
Semisynthetic III (Cholestyramine: semisynthetic I 1:24 (w/w))	2	1.76
Semisynthetic IV (Cholestyramine: semisynthetic I 1:9 (w/w))	2	1.24
Semisynthetic V (Cholestyramine: semisynthetic I 1:4 (w/w))	2	2.28

[a] 500 mg liver slices were incubated for 2 hr at 37° under an atmosphere of 95% O_2 and 5% CO_2 in 3.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4. The incubation was started by addition of 10 μmoles of acetate-1-C^{14} (sp act 18 nCi/μmole) dissolved in 0.5 ml of water. For further details see Materials and Methods.

tions of the particular role of bile acids in the regulation of cholesterol biosynthesis.

Other authors had already shown a direct influence of bile acids on the mevalonate-forming enzyme system of rat liver *in vitro* (28), but there was no convincing explanation for the mechanism of the observed inhibition which occurred at concentrations of 0.25–2.5 × 10^{-3} M. The inhibitory action of feeding bile acid on cholesterol biosynthesis from acetate was also already well documented by Pihl (29) and by Beher *et al.* (13, 30), who postulated a double feedback mechanism for regulation of cholesterol synthesis, mediated by an increased level of liver cholesterol. They fed bile acids over a rather long time of 8 days to 3 weeks. Reinvestigating some of these findings, the effect of cholic acid supplementation in the food on cholesterol formation from acetate in liver slices was tested after short-time feeding. The result is shown in Fig. 1. Cholic acid was given in a concentration of 1% in the laboratory chow. Triple incubations of liver slices were performed. The inhibitory effect of cholic acid was already apparent after 4–6 hr. Cholesterol biosynthesis in liver of animals fed the ordinary unsubstituted chow follows rhythmic activity changes with a maximum at midnight (Fig. 1). In liver slices an increase of activity up to 372% of the noon value can be observed.

Knowledge of this fact allowed a more standardized test of the influence of different amounts of cholic acid ingested with the food. Figure 2 shows that maximum inhibition after 20 hr is already apparent after application of 13–18 mg of cholic acid (0.10–0.15% cholic acid in the food) to rats of 110 g body weight. This value is about twice as high as the value of the cholic acid pool-size of 7 mg for the total body of a 100-g rat measured by Eriksson (31) and the value of 9.9 mg found by Portman and Murphy (11).

It was not possible to obtain total inhibition of cholesterol synthesis from acetate in liver slices by means of cholic acid feeding, not even with high concentrations of bile acid. However, in the 5000-*g* supernatant fluid of liver homogenates from cholic acid-fed rats almost total suppression of synthetic activity was observed (see Table IV).

Localization of the enzymic step controlling diurnal activity changes. Incubations of 5000-*g* supernatant fluids of liver homogenates with acetate and mevalonate revealed that the day-night rhythm is restricted to the formation of mevalonate. Table II shows the results from different animals killed at definite times during the day or night. The synthesis of cholesterol from acetate increased by a factor of 3.8 from noon to midnight, while mevalonate incorporation did not change significantly. This is in keeping with the generally accepted functional role of HMG-CoA-reductase as the regulatory enzyme for cholesterol synthesis. These experiments with Bucher homogenates revealed the same ratio of midnight and noon values as has been found in experiments with liver slices (see Fig. 1, controls).

Influence of cycloheximide on cholesterol

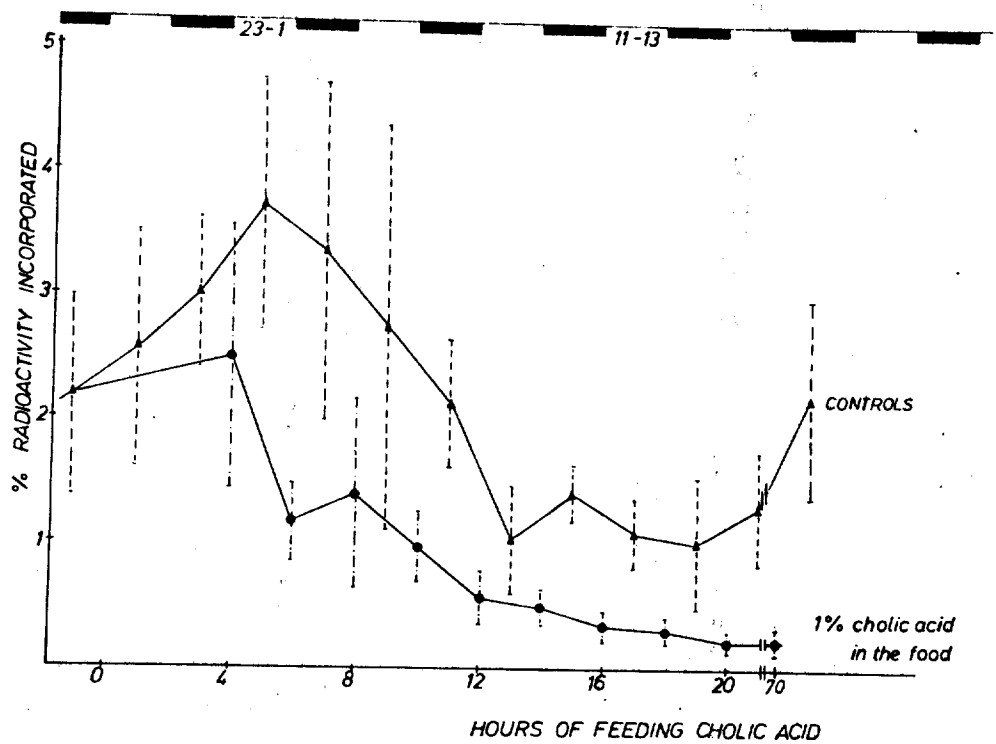

FIG. 1. Effect of cholic acid feeding on the rate of acetate-1-^{14}C incorporation into the sterol fraction by rat liver slices. Time of day dissected into 2-hr intervals (▬▬▬); Control values in normally fed animals; each point represents the mean of determinations in eight animals (—▲—▲—); Activity values at different times after begin of cholic acid-supplemented food intake. Each value is the mean of determinations in three animals (—●—●—). With both curves the standard deviations from the mean are given by the dotted vertical lines. The incubation procedure was the same as indicated in Table I. The light and dark periods were the natural periods in the months February and March; i.e., light was from 7:00 ± 1:00 AM to 6:00 ± 1:00 PM.

formation from acetate and mevalonate. Four hours after injection of low doses of cycloheximide (1 mg/200 g body weight) the conversion of acetate into cholesterol by rat liver extracts seems to be less depressed than that of mevalonate. Using the tenfold amount of the drug we obtained nearly complete inhibition of cholesterol formation from acetate. Under the same conditions utilization of mevalonate was also strongly depressed, but not markedly lower than after injection of the low doses (see Table III). The respiratory activity of liver slices of the cycloheximide-treated animals did not show significant differences from the activity of the controls, when testing the oxygen uptake during 60 min of time of incubation. These results might be taken as an indication that enzyme activities involved in cholesterol synthesis depend on protein *de novo* synthesis.

Localization of cholic acid action. With the same set of experiments, evidence was obtained that cholic acid develops its inhibitory action at some step before mevalonate formation (Table IV). Almost complete suppression of cholesterol synthesis from acetate in 5000*g* supernatant fluids of livers is demonstrable after *in vivo* administration of 0.5% cholic acid with the food, while mevalonate incorporation into cholesterol only decreased to 58% of the control value. It is noteworthy that cholic acid-dependent inhibition takes place much sooner than the inhibition exerted by cholesterol feeding (Table IV); 0.75% of cholesterol within the food was given to the animals in order to provide a sufficiently high absorption from

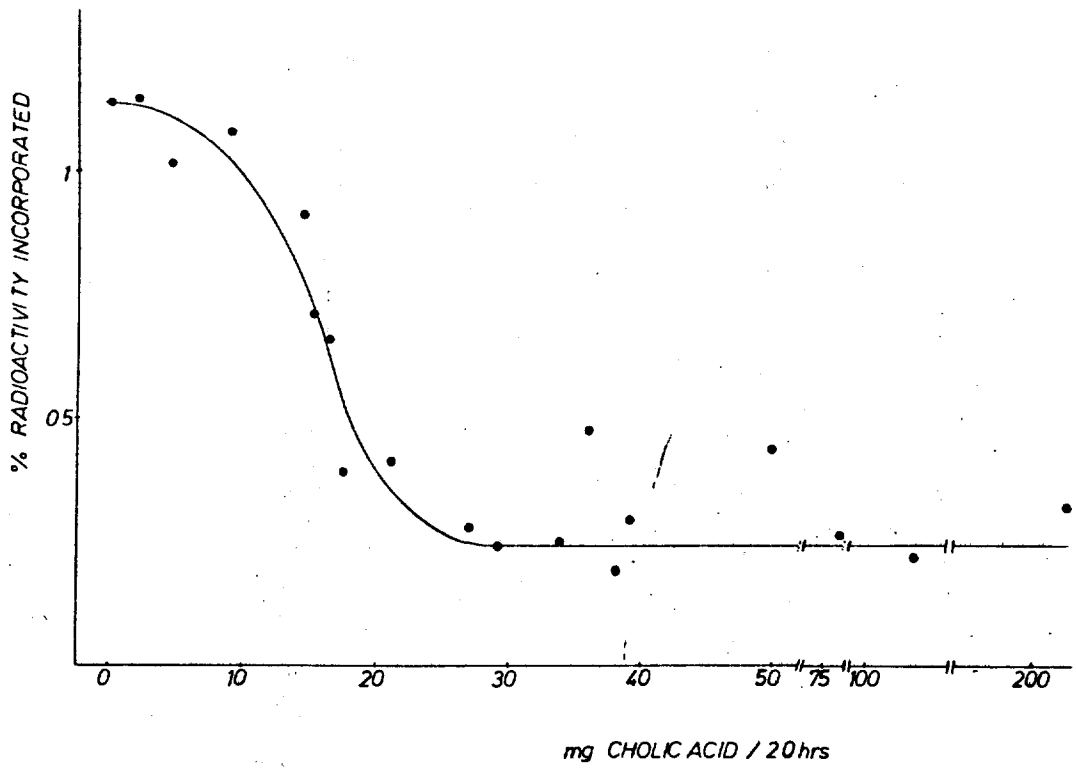

FIG. 2. Activity of incorporation of acetate-1-^{14}C into the sterol fraction by liver slices after the continuous uptake of different amounts of cholic acid over 20 hr. Each value represents the mean of determinations from three animals. All determinations were made at 0:00–2:00 PM. The experimental details are the same as in Fig. 1.

the intestinal tract assuming an uptake of at least 30% of the cholesterol administered (32–34).

Quantitative analysis of bile acid concentration in rat liver under different experimental conditions. From Fig. 2 it can be seen that a critical amount of cholic acid ingested per 20 hr is able to inhibit cholesterol synthesis. This observation prompted us to look for an inverse relationship between actual concentration of bile acids in the liver and cholesterol synthetic activity. The determinations of bile acids were performed in the same homogenates from which aliquots had been taken for the enzyme assays at day and night (cf. Table II) and after cholic acid feeding and starvation (cf. Table IV).

The bile acids were extracted as free acids after hydrolysis of the conjugated acids. 3 α-,6 α-dihydroxycholanic acid was added to all samples as internal standard of extraction recovery. This acid showed no overlap in our gas chromatographic system with the naturally occurring bile acids in rat liver. It also had not been detected in 22 foregoing qualitative analyses of bile acid mixtures extracted from rat liver. With the exception of two peaks, the identification was complete in the usual bile acid chromatogram by means of reference substances and agreed well with the reported relative retention times (20). The tentative identification of the unknown two peaks was based only on reported retention data and agreed with the values given for α- and β-muricholic acids. The absolute amounts of these two bile acids in liver homogenates could not be determined owing to lack of purified reference substances. Each bile acid was determined using specific calibration curves as described under Methods. The analytical data given in Table V are means of seven triple

TABLE II

CONVERSION OF ACETATE-1-^{14}C AND MEVALONATE-2-^{14}C TO STEROLS BY LIVER EXTRACT AT NOON AND AT MIDNIGHT[a]

Time of day	Per cent radioactivity incorporated into the sterol fraction after incubation of liver extract (80 mg protein) for 2 hr with[b]:	
	Acetate-1-^{14}C	Mevalonate-2-^{14}C
0:00 PM	0.45	18.3
0:00 PM	0.86	28.8
0:40 PM	0.59	26.9
1:30 PM	0.79	29.3
2:15 PM	0.64	25.5
2:45 PM	0.50	18.3
0:00–2:45 PM Mean ± SD	0.64 ± 0.11	24.5 ± 5.0
11:40 PM	2.20	22.3
12:00 PM	1.63	25.3
12:00 PM	1.80	22.7
0:15 AM	2.77	28.0
0:45 AM	3.31	12.4
1:00 AM	2.86	19.7
11:40 PM–1:00 AM Mean ± SD	2.3 ± 0.66	21.7 ± 5.4

[a] Animals were fed *ad libitum* with normal diet as described under Materials and Methods.

[b] The incubation mixture (2.45 ml final volume) contained: 2 ml of liver extract (5000 *g* supernatant fluid), 20 μmoles of glucose-6-phosphate, 2 μmoles of NADP, 2 μmoles of NAD, and 10 μmoles of sodium acetate-1-^{14}C (sp act 18 nCi/μmole) or 15 μmoles of sodium RS-(±)-mevalonate-2-^{14}C (sp act 1.55 nCi/μmole). Incubation was for 2 hr.

TABLE III

CONVERSION OF ACETATE AND MEVALONATE TO STEROLS BY LIVER EXTRACT AFTER *in Vivo* INJECTION OF CYCLOHEXIMIDE AT 2:00 PM[a]

Cyclo-heximide (mg/200 g of body weight)	No. of animals	Time after injection (hr)	% Radioactivity found in the sterol fraction after incubation of liver extract for 2 hr with acetate-1-^{14}C, mevalonate-2-^{14}C (mean values)	
1	3	4	0.25	4.7
0	2	4	0.55	16.8
10	2	6	0.02	4.2
0	2	6	3.50	30.0

[a] Incubations were performed as described in Table II.

TABLE IV

INCORPORATION OF ACETATE-1-^{14}C AND MEVALONATE-2-^{14}C INTO THE STEROL FRACTION BY LIVER EXTRACT (5000 *g* supernatant fluid). COMPARISON OF EFFECTS EXERTED BY CHOLIC ACID FEEDING AND BY CHOLESTEROL FEEDING OVER 20 HR AS WELL AS INFLUENCE OF STARVATION FOR 20 HR[a]

Experimental conditions	% Radioactivity incorporated into the sterol fraction after incubation with:	
	Acetate-1-^{14}C	Mevalonate-2-^{14}C
Controls without food supplement[b]	0.64	24.5
Cholic acid 0.5% as food supplement	0.004	16.9
	0.002	13.1
	0.004	15.3
	0.003	10.7
	0.003	13.0
	0.000	15.6
Cholesterol 0.75% as food supplement	0.380	19.4
	0.310	17.6
Starvation	0.001	1.3
	0.000	0.4
	0.002	2.3

[a] Feeding was started at 6:00 PM and animals were killed at 2:00 PM. For preparation of diets see Materials and Methods; incubation procedure was the same as in Table II.

[b] Controls are the mean values for 0:00–2:45 PM from Table II.

determinations. It was somewhat surprising to find no keto-bile acids in liver, possibly an indication for the selective uptake of hydroxy-bile acids by the intestinal wall. The alternative, however, that enzymic reduction had taken place in the homogenates before hydrolysis could not be excluded. The absolute amounts of values determined at noon corresponded very closely to those taken during the night (Fig. 3, Table V). There was only an inversion of the ratio cholic acid/chenodeoxycholic acid from 3.8:3.5 to 3.4:3.9. In accordance with the conclusions of Bergström and Danielsson (35) the sum remained constant. The level of deoxycholate did not change significantly from day to night. Lithocholic, α- and β-muricholic acids showed slightly higher levels over day than

during night. This was significant for β-muricholic acid ($p < .01$). Under the conditions of cholic acid feeding (0.5% in the laboratory chow) a significant increase of cholic acid occurred up to 254% of the normal amount ($p < .001$). Likewise the concentration of deoxycholic acid rose up to 249% of the normal value. For all other bile acids a partial compensatory decrease of their levels was the consequence. After 20 hr of starvation the concentration in liver of all bile acids decreased (Table V).

TABLE V

CONCENTRATIONS OF DIFFERENT BILE ACIDS IN LIVER EXTRACT UNDER DIFFERENT EXPERIMENTAL CONDITIONS[a]

Bile acid	μg bile acid/100 mg protein			
	Noon	Midnight	20 hr after onset of feeding cholic acid	after starvation for 20 hr
Cholic acid	38.2 ± 11.2 (7)	33.6 ± 9.3 (7)	96.8 ± 22.1 (7)	15.0 ± 6.2 (6)
Chenodeoxycholic acid	35.1 ± 11.3 (7)	39.4 ± 15.0 (7)	13.6 ± 4.6 (7)	9.3 ± 4.4 (6)
Deoxycholic acid	7.9 ± 6.1 (7)	7.8 ± 3.3 (7)	19.7 ± 6.2 (7)	8.0 ± 4.4 (6)
Lithocholic acid	13.4 ± 4.6 (7)	8.7 ± 0.9 (7)	5.0 ± 0.9 (7)	5.0 ± 4.1 (6)

[a] The data are given as mean ± standard deviation; number of analyses in parentheses. For experimental details see Materials and Methods.

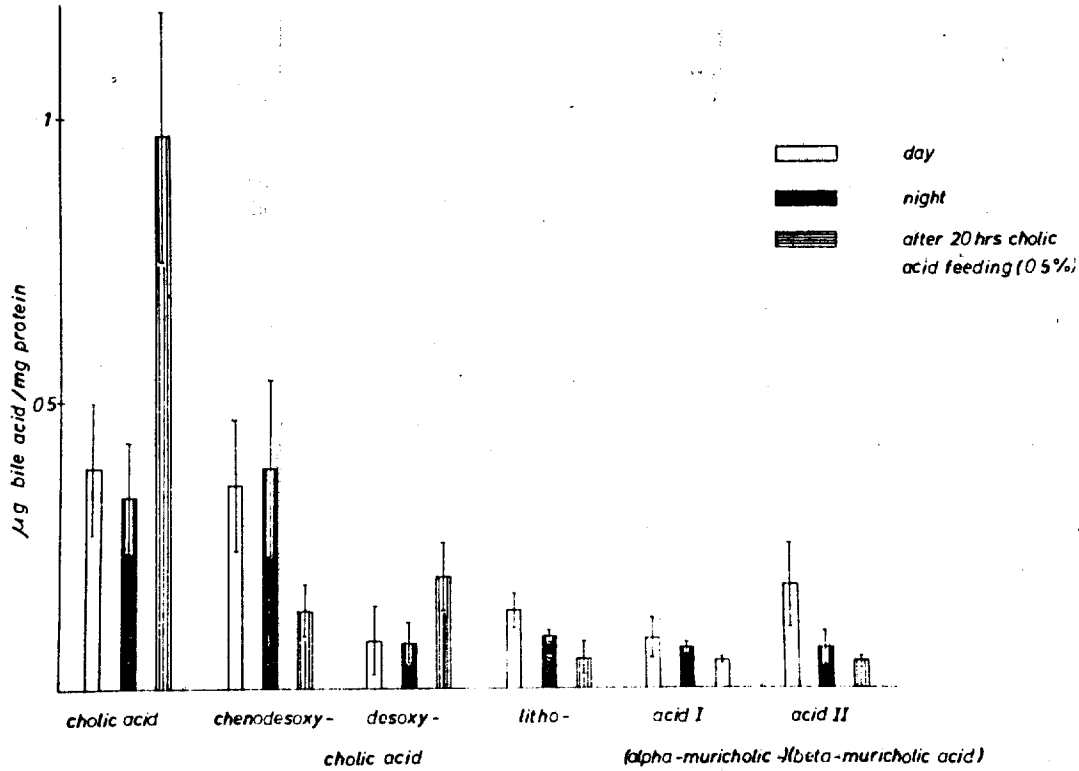

FIG. 3. Concentrations of different bile acids in liver extract (5000 g supernatant fluid). For experimental details of the gaschromatographic determination of bile acids see Materials and Methods.

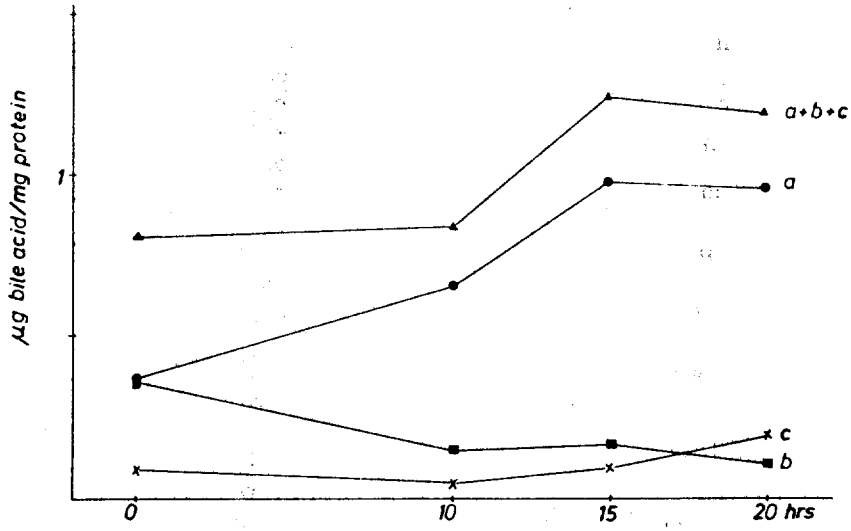

FIG. 4. Bile acid content of liver extract at different times of cholic acid feeding (0.5%). a = cholic acid; b = chenodeoxycholic acid; c = deoxycholic acid. For experimental details of the gaschromatographic determination of bile acids see Materials and Methods.

Following the time course of the bile acid concentrations in liver under conditions of feeding 0.5% cholic acid, one could see a steady rise of the partial concentration of cholic acid up to 15 hr, after which time no further increase was observed (Fig. 4). Presumably the capacity of the liver to accumulate bile acids is restricted to a certain threshold that cannot be surpassed.

From the quantitative data of the four main bile acids, cholic, chenodeoxycholic, deoxycholic, and lithocholic acid, given in Fig. 3, the molar concentration of bile acids in the liver under normal conditions can be calculated to be 3.4×10^{-4} M considering (1) the dilution of the liver contents by the homogenization medium; (2) the protein concentration of the 5000 *g* supernatant fluid (40 mg/ml); (3) the assumption that the bile acid concentration is the same in supernatant and sediment; and (4) the assumption that bile acids are equally distributed within the liver. Studies on compartmentalization of bile acids in the liver cell have not been performed in this work.

After feeding cholic acid, the bile acid content of liver rises and the maximum concentration reaches a value of 4.6×10^{-4} M. From this and the figure mentioned above the concentrations within the test system for liver extracts can be calculated to be 0.8×10^{-4} M (normal chow) and 1.1×10^{-4} M (cholic acid supplemented chow) for the sum of the four main bile acids.

DISCUSSION

The findings that the inhibition of cholesterol formation in liver by cholesterol-feeding is effected slower than that caused by feeding bile acids cannot be reconciled with the hypothesis of a double feedback (13) as the regulatory mechanism. From this hypothesis should also follow that removal of bile acids increases the formation of bile acids from cholesterol before any stimulatory effect on the synthesis of cholesterol from acetate could be observed. The bile fistula experiment of Myant and Eder (36), however, revealed that an inverse time course of events occurs.

Instead of applying a bile fistula we tried to remove bile acids by feeding the anion-exchange resin cholestyramine. We observed high stimulation of cholesterol synthesis from acetate. This was demonstrated for a semisynthetic diet, which itself caused only low cholesterol synthetic activity in liver. The results obtained confirm previous findings of Huff *et al.* (26) and Gallo *et al.* (37).

The analyses of liver concentrations of cholic acid—a metabolic endproduct, which could be demonstrated to be a potent in-

hibitor at the rate-limiting step of cholesterol biosynthesis, when given with the diet—did not reveal an inverse correlation to the activity changes of cholesterol biosynthesis during the day–night cycle in the livers of normal animals.

An increase of the liver concentration of cholic acid, however, which is obtained shortly after feeding the acid, is followed by an immediate decrease of cholesterol synthesis from acetate, but not from mevalonate. But the increase of the bile acid concentration within the test system with liver extracts did not amount to the levels which are reported to inhibit synthetic activity after addition *in vitro* (28), even after feeding extremely high amounts of cholic acid. Interference also occurs with the physiological increase of cholesterol biosynthesis during the diurnal rhythm, which remains suppressed after prolonged ingestion of cholic acid.

The concentration of total bile acids in liver of normally fed rats has been found to be 3.4×10^{-4} M and is in good agreement with the value of 2.8×10^{-4} M, which can be calculated from the data reported by Okishio and Nair (38). A possible explanation for the effects of some bile acids after feeding or removal from the enterohepatic circulation could be the involvement of these substances in repression of enzyme synthesis. The mechanism by which a bile acid might act and the exact target of its operation still remain obscure. The results presented suggest that the bile acids are not responsible for the diurnal changes of activity of cholesterol formation.

From the determinations of bile acid concentrations in livers of fasting animals (Table V), which showed diminished values for the main bile acids, it might be excluded that bile acids could be activators or inducers of HMG-CoA reductase-degrading enzymes. The half-life of HMG-CoA reductase has not yet been determined. However, the enzyme seems to have a high turnover rate (cited from 7). Another suggestion for the regulatory mechanism might be implicated in more recent findings: The experiments of Regen *et al.* (9) had demonstrated a rapid decrease of activity of the rate-limiting HMG-CoA reductase with fasting and the recovery of approximately 40% of the activity 10 hr after refeeding. The suggestion of Regen *et al.* (9), that enzyme synthesis might be involved in regulation of cholesterol biosynthesis is supported by the finding that cycloheximide is able to suppress activity increase in the dark period. This antibiotic is assumed to act as an inhibitor of protein synthesis at the translational level (39, 40). Actinomycin D, another antibiotic interfering with protein synthesis and postulated to block the transcriptional step (41, 42), has been reported to stimulate cholesterol synthesis in rat liver at some reaction before mevalonate formation, probably at the enzyme HMG-CoA reductase (8). Protein synthesis has also been suggested to be the target of thyroid-hormone action on activity of HMG-CoA reductase (7). Similar regulatory phenomena; i.e., diurnal rhythm, influence of hormones and of antibiotics which inhibit protein synthesis have been shown for the adaptive enzymes tryptophane pyrrolase and tyrosine transaminase (43–46).

A plausible explanation for the bile acid action and for the rhythmic changes of the activity of cholesterol biosynthesis from acetate might be given by assuming independent mechanisms, which vary the ratio of velocities of enzyme formation and degradation.

Therefore, together with the results of Regen *et al.* (9) our findings are likely to be reconciled with the suggestion of a regulatory mechanism which acts on synthesis of HMG-CoA reductase.

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TEST OF DESOXYCHOLIC ACID FOR CARCINOGENICITY IN RATS AND MICE

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INTRODUCTION

During the last decade, many contributors to the cancer literature have suggested that there may be some relationship between the genesis of cancer and the bile acids, hormones, or sterols. The most potent of known carcinogenic hydrocarbons, 20-methylcholanthrene, has been prepared from two of the more predominant bile acids by admittedly drastic laboratory methods. The reactions involved in the laboratory conversion of desoxycholic (3,12-dihydroxycholanic) acid and cholic (3,7,12-trihydroxycholanic) acid to methylcholanthrene consist of four types: oxidation, hydrogenation, cyclization, and dehydrogenation. Similar types of reaction are known to take place in the animal body. Absence of proof of like or similar reactions involved in the metabolism of bile acids or other steroids does not necessarily rule out the possible elaboration of as yet undetectable traces of carcinogenic substances during the normal or perhaps abnormal metabolism of steroids.

Methylcholanthrene was first prepared not by synthesis but by degradation of desoxycholic acid by Wieland and Dane (*1*). The relationship of this hydrocarbon to benzanthracene and its derivatives was recognized, and Cook and Haslewood (*2*) first reported the strong carcinogenicity of methylcholanthrene. Subsequently, Fieser and Newman (*3*) prepared methylcholanthrene by degradation of cholic acid. The discovery that a very potent carcinogenic hydrocarbon could be prepared from bile acids raised the question in several laboratories of the possible carcinogenicity of the bile acids, of which desoxycholic acid received the most attention. Ghiron (*4*) reported that desoxycholic acid, when injected subcutaneously into mice and rats, induced transplantable fibrosarcomas in a high proportion of the animals, but no details are available. Law (*5*) reported a significant increase in the incidence of pulmonary tumors in strain A mice following subcutaneous injection of sodium desoxycholate in olive oil. Mice similarly treated with cholic acid did not differ from the controls. Cook, Kennaway, and Kennaway (*6*) observed sarcomas in three of five surviving mice following repeated subcutaneous injections of desoxycholic acid in sesame oil. These sarcomas failed to grow when transplanted into mice of a mixed stock. On the other hand, Shear (*7*) was unable to induce tumors in mice with this agent or with cholic acid. This lack of agreement may have been the result of differences in technique, strain of test animal, vehicle, etc.

The presence of bile in the small intestine has long been recognized as necessary for optimum absorption of a variety of dietary components. The effects of disruption of normal bile secretion, as a result of disease or of surgical procedures, have been alleviated by the oral administration of bile preparations. The biochemical literature contains many references (*8-15*) demonstrating the efficacy of bile preparations, particularly desoxycholic acid, in facilitating the absorption of lipids, cholesterol, vitamins, hormones, and other substances in cases of disturbed bile secretion, and in the enhancement of the absorption of these substances in the normal animal. These reports describe experiments in which bile preparations or desoxycholic acid have been fed to mice, rats, rabbits, and guinea pigs for relatively short periods, usually less than 100 days. In no instance was there any description of the histologic appearance of the stomachs of the experimental animals.

It became necessary, during the course of relevant experiments in this

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laboratory (*16, 17*), to investigate the effects of prolonged feeding of desoxycholic acid upon the histologic appearance of the gastric mucosa of rats. Concurrently, the effects of subcutaneously implanted desoxycholic acid pellets in rats and mice were observed. This paper presents the results of these investigations.

EXPERIMENTAL PROCEDURE

Desoxycholic acid, "purified", obtained from a biologic supply house, was subjected to intensive purification as recommended by Sobotka (*18, pp. 77–78*). The final product, free of all solvent, melted at 174° C. This pure acid was ground in a mortar, passed through a 40-mesh screen, and preserved in a moisture-free atmosphere. One cannot place too much stress upon the importance of subjecting commercially available "purified" desoxycholic acid to further purification. In several instances we found that samples sold by the trade as pure acid contained as much as 30 percent of impurities.

ORAL ADMINISTRATION

Fifty-six male rats, 6 months of age, were procured from a dealer, who certified them as being of the Wistar strain. These animals varied in weight between 245 and 363 gm. They were divided into 4 groups of 14 rats each, in a manner that resulted in equal total weights for each group. Group 1, which served as a control, received the basic diet which consisted of the following:

	Percent
Skim-milk powder	22.75
Whole-wheat flour	61.52
Brewers' yeast (Harris' medicinal)	4.00
Sodium chloride	1.40
Ferric citrate	.13
Cod-liver oil	2.00
Corn oil	8.20

Groups 2, 3, and 4 were also fed the foregoing diet to which was added 5 mg., 25 mg., or 125 mg. desoxycholic acid per 100 gm. of diet, respectively. The rats were housed individually in screen-bottom cages and were provided with food cups that were practically spill-proof. Individual animal weights and food consumption were recorded at weekly intervals. Average animal weights and food consumption, at monthly intervals, are recorded in figure 1.

SUBCUTANEOUS IMPLANTATION

Desoxycholic acid pellets were cut from the hard lumps that result from the final step of the purification of the acid. These rough pellets were surgically implanted, under ether anesthesia, in the right inguinal region of 25 male and 25 female rats, 10 to 12 weeks of age. The incision was made in the groin, and the pellet was placed anteriorly so that it lay about 3 cm. from the incision. The skin was closed with 1 silk stitch. The implanted pellets ranged from 14 to 19 mg. in weight, with an average of 16.5 mg. Almost invariably a marked induration about the site of the pellet was noted during the first few days. The overlying skin remained intact but appeared reddened in many cases. After 2 weeks the pellet had been completely absorbed, the reaction appeared to have subsided, and healing seemed to be complete in most cases. The animals were examined at regular intervals for signs of any delayed reaction. Nine rats died or were killed because of illness during the 12 months following pellet implantation. Gross examination of the pellet site gave uniformly negative results. The remaining 41 rats were killed and examined 13 to 16 months after insertion of the pellet. Grossly and microscopically, the site of injection was consistently without neoplastic change.

Rough pellets of desoxycholic acid, 11 to 13 mg. in weight, were similarly placed under the skin of 46 strain C3H male mice, 8 weeks of age. Within 48 hours an inflamed area of induration developed about the site of the pellet. This reaction was followed by necrosis, sequestration, and sloughing. Five days after implantation the pellet had been sloughed in 38 cases. Sequestration and sloughing failed to develop in

the other 8 mice; however, the pellet appeared to be completely absorbed, in every case, within 7 days. After 4 months, autopsy of 26 mice that died of a pneumonia epidemic revealed a uniformly normal implantation site. The experiment was terminated at the end of 6 months. The remaining 20 mice showed no evidence of neoplasia.

In order to test further the effects of desoxycholic acid on subcutaneous tissue, it became apparent that the pellet required modification in a manner that would reduce its caustic action and likewise retard its rate of absorption. Accordingly, pellets were prepared which contained equal parts by weight of desoxycholic acid and cholesterol. Equal quantities of cholesterol and the acid were powdered, mixed intimately, and melted. The molten mixture was drawn into lightly oiled capillary tubes, cooled, and extruded. The pellets were rinsed with a mixture of equal parts of alcohol and ether, dried, and cut into suitable lengths for implantation.

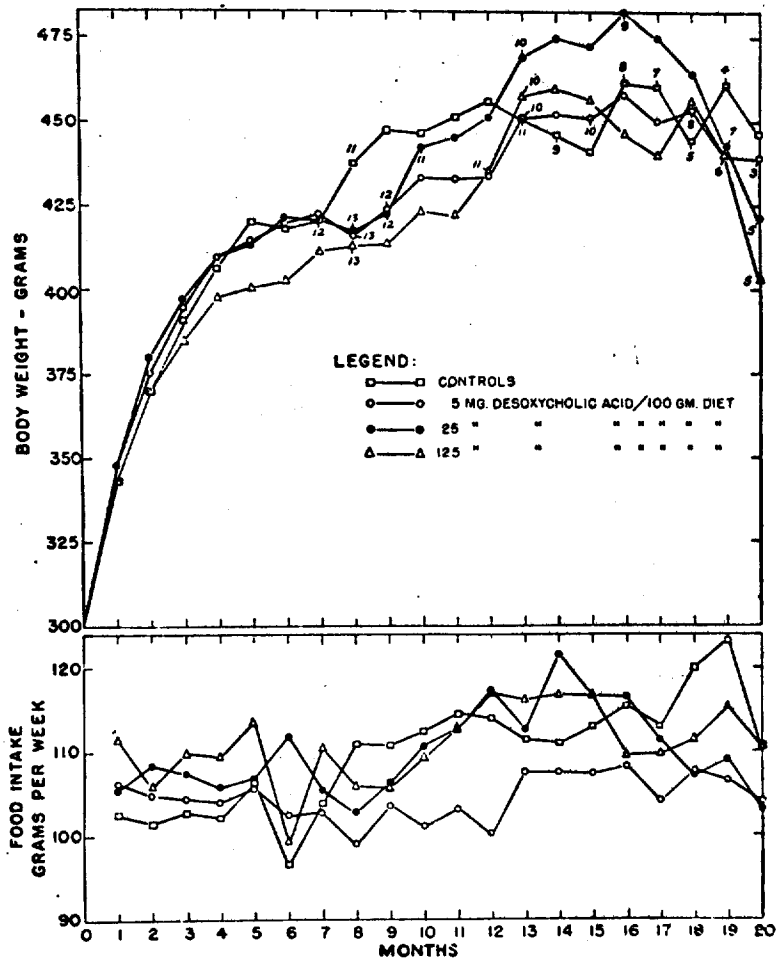

FIGURE 1.—Food intake and body weights of rats on basic diet and on basic diet plus desoxycholic acid.

Fifty-two male Wistar rats 4 months of age received implantations of the modified pellet which was placed, by means of a trocar, in the right groin just cephalad and dorsal to the inguinal fat pad. The pellets weighed 15 to 16 mg. The reaction about the site of the pellet was similar to that noted previously for pellets of desoxycholic acid alone. Seven days later the reaction had subsided, and a movable nodule, roughly 10 mm. in diameter, was noted in each animal. At the end of 2 weeks the nodule was approximately 6 mm. in diameter, on the average. Five weeks after implantation the nodules had shrunk to barely palpable size. From 9 to 12 months after pellet implantation, 12 rats were autopsied. In 6 animals the pellet had completely disappeared: a barely perceptible pellet was found in each of the other 6 rats. The pellet site appeared normal in all animals. The remaining 40 rats, autopsied from 12 to 20 months after pellet implantation, showed no residual pellet, and the areas of implantation were apparently normal.

Similarly, desoxycholic acid-cholesterol pellets were implanted subcutaneously in 50 strain C3H male mice 12 weeks of age. The pellets, which weighed 11 to 13 mg., were placed, with the aid of a trocar, in the right groin just cephalad to the inguinal fat pad and directly under the skin. The reaction about the site of implantation was similar to that encountered when pellets of the pure acid were used. Inflammation, induration, necrosis, sequestration, and sloughing were noted in 21 mice after 10 days. Of the other 29 mice, in which the reaction subsided short of sequestration and sloughing, 16 were found to have soft nodules under the skin after 20 days; absorption of the pellet material appeared to have been effected in 13 mice. Absorption of the pellet in all animals appeared to be complete within 35 days. The experiment was terminated at the end of 8 months. No evidence of hyperplasia at the pellet site was found in any case.

DISCUSSION

On examination of several reports in the literature (*8–14*), relative to oral administration of desoxycholic acid and bile salts to small experimental animals, it would appear that desoxycholic acid is not toxic when given orally. In but one case, insofar as we are aware, was there an exception. Wells, Burrill, and Greene (*15*) found that 0.6 mg. desoxycholic acid per day, when given orally along with testosterone propionate, caused 150-gm. rats to lose weight over a 10-day period. This loss in weight was apparently avoided when the acid was reduced to 0.5 mg. per day. It must be emphasized that, according to our findings, commercially available "purified" desoxycholic acid may contain as much as 30 percent of impurities. In contrast with the findings of Wells, et al., we have found that young adult Wistar rats suffer no ill effects while ingesting as much as 20 mg. of the acid per day (group 4), purified in this laboratory, during 20 months. As is shown in figure 1, the growth curves for the control group and for each of the experimental groups are similar, except that the curve for group 4, which received 125 mg. of the acid per 100 gm. of the diet, is slightly lower than the others from the fourth to the eleventh month. Similarly, the food consumption for all groups was comparable, except that group 2, which was fed the low level of the acid (5 mg./100 gm. diet), appeared to consistently eat slightly less food than did the other groups from the seventh to the eighteenth month. We are of the opinion that these small differences in average weights and food consumption are without significance owing to the relatively small number of rats in each group.

PATHOLOGY

All 56 rats of the control and desoxycholic acid-fed groups were autopsied. Six small papillomas of the forestomach and five superficial ulcers of the glandular stomach, found rather evenly distributed among the several groups,

were considered not to be a result of the experimental procedures. Fourteen animals were found to be normal on gross examination. Many animals had minor lesions, such as superficial ulcers of the foot pad, alopecia, papillomas of the ear on a parasitic basis, and an occasional parasitic cyst in the abdomen. Obesity was common in the control and the acid-fed groups. Nineteen cases of bronchiectasis, usually associated with chronic bronchitis and peribronchial inflammation, were observed. Numerous mucinous cysts of the bronchi, bronchopneumonia, with or without abscesses, and extensive metaplasia of the bronchial epithelium commonly accompanied the bronchiestasis. These lesions of the respiratory tract were rather evenly distributed among the control and acid-fed groups. Eight cases of pyelonephritis were seen, 2 in each group of 14 rats. Three cases of otitis media interna, randomly distributed among the several groups, were encountered. All of these subacute and chronic inflammatory processes were accompanied by hyperplasia of the spleen and bone marrow. A few neoplasms were observed in old rats. One animal in group 2 showed a hygroma. A carcinoma of the testis was found in a rat in group 3. A rat in group 4 had a spindle-cell sarcoma on a foot pad with retroperitoneal metastases. Two anaplastic carcinomas of the lung were noted in animals in groups 2 and 4, respectively. In our opinion, it is doubtful whether these neoplasms were attributable to the imposed experimental conditions.

SUMMARY

Three groups of rats were fed diets containing desoxycholic acid at graded levels for as long as 20 months without evidence of toxic effects. No characteristic pathology, which would distinguish one experimental group from another or from the controls, was noted in the gastric tissues.

Pellets of desoxycholic acid, implanted subcutaneously in rats, incited a temporary local tissue reaction which subsided within 2 weeks. Autopsy of 41 animals after 13 to 16 months revealed neither gross nor microscopic evidence of neoplasia or hyperplasia. Subcutaneous implantation of pellets consisting of equal parts of the acid and cholesterol likewise led to negative results as far as tumor genesis was concerned.

Subcutaneous implantation of desoxycholic acid pellets in 46 strain C3H mice led to necrosis, sequestration, and sloughing of the pellet in most cases. Twenty-six mice which survived for 6 months showed no evidence of hyperplasia at the pellet site. Similar implantation of acid-cholesterol pellets was followed by sloughing in about half the mice. Pellets that were retained in the remainder of the mice appeared to be completely absorbed within 35 days. These mice, autopsied 8 months later, showed no reaction at the pellet site.

Desoxycholic acid, as administered in these experiments, showed no evidence of carcinogenicity.

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PREVENTION OF THE CHOLESTEROL TYPE OF FATTY LIVERS IN MICE BY DIETARY DIHYDROCHOLESTEROL

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The relationship of the diet to cholesterol absorption, distribution and balance has been the subject of extensive investigation for many years. Recently a number of papers have appeared indicating the usefulness of soybean sitosterols (Sperry and Bergmann, '37; Peterson, '51; Peterson, Nichols and Shneour, '52; Pollak, '53; Peterson, Shneour, Peek and Gaffey, '53) and dihydrocholesterol (Siperstein, Nichols and Chaikoff, '53) in the control of cholesterol absorption. These efforts so far have been chiefly concerned with the prevention of cholesterol-induced atherosclerosis in chicks and rabbits. Efforts to lower organ and blood cholesterol in mice by means of soybean sitosterols have been unsuccessful (Schettler, '48). Our present experiments have been designed to determine whether the cholesterol type of fatty livers in mice can be prevented by dihydrocholesterol, and to gain some knowledge about the mechanism of this action.

EXPERIMENTAL

Webster strain male albino mice weighing 18 to 20 gm were used. After an observation period of two weeks, the mice were weighed, housed in separate cages and divided into 7 groups, each with 7 to 9 animals. Each group was fed a basal cholesterol-free diet supplemented as listed in table 1 with cholesterol, cholic acid and dihydrocholesterol.[1]

[1] Generously supplied by the Schering Corporation, Bloomfield, New Jersey.

The basal cholesterol-free diet used in all of the experiments had the following composition: vitamin-free casein 25, sucrose 55, non-nutritive diet 16 (alphacel)[2], and salt mixture 4% (Phillips and Hart, '35). This diet was supplemented with the following quantities of vitamins, expressed as milligrams per kilogram: thiamine hydrochloride 40.4, riboflavin 60.8, calcium pantothenate 151, niacin 1,008, pyridoxine hydrochloride 40.4, 2-methyl-1,4-naphthoquinone 5.12, alpha-tocoperol 30.2, folic acid 20.3, biotin 2.02, choline chloride 20,200, *p*-aminobenzoic acid 202, inositol 10,100 and ascorbic acid 100.

After an experimental period of three weeks, all mice were weighed and sacrificed by decapitation. The livers were rapidly removed, weighed and analyzed for total cholesterol and total β steroids as follows:

The excised liver was placed in a round-bottom flask containing 20 ml of 70% ethyl alcohol and 1.5 gm of potassium hydroxide. The mixture was refluxed on a steam bath for three hours. The resulting clear brown liquid was adjusted to pH 7 and transferred to a 50-ml volumetric flask using 95% alcohol. A 20-ml aliquot was placed in a separatory funnel, 40 ml of water added, and the mixture extracted with four 20-ml portions of ether. The ether extracts were evaporated to dryness and then transferred to a 25-ml volumetric flask by means of a 1 : 1 ethyl alcohol-acetone mixture. Cholesterol was determined in aliquots of this solution by the Schoenheimer-Sperry method as modified by Sperry and Webb ('50).

Total β steroids were determined by utilizing the well-known reaction of digitonin and anthrone. The technique, as developed in this laboratory, is as follows: The digitonin precipitate was prepared and washed as in the Schoenheimer-Sperry method, except that an additional washing with 2 ml of 50% ethyl alcohol was found necessary before washing with acetone ether. The washed precipitate was dissolved in glacial acetic acid and diluted with glacial acetic acid to 10 ml. A 2-ml aliquot

[2] Purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

was placed in a cuvette and 3 ml of a 2% solution of anthrone in glacial acetic acid were added. At one-minute intervals, 5 ml of concentrated sulfuric acid were added to successive tubes as rapidly as possible with vigorous agitation. The tubes were placed in the dark at room temperature for 30 minutes and then read in the spectrophotometer at 670 mμ. Blanks and standards were prepared by using 2 ml of glacial acetic acid and 2 ml of digitonin in glacial acetic acid (0.1 mg %) in place of the unknown. This method, which is about 10 times as sensitive as the Lieberman-Burchard reaction, is useful for the determination of digitonin-precipitable steroids and especially for dihydrocholesterol, which does not yield a color under the conditions of the Lieberman-Burchard reaction.

RESULTS

The results presented in table 1 show that the addition of cholesterol and cholic acid to the cholesterol-free diet (I) increases total liver cholesterol and total β steroids (diets II, III and IV). When the cholic acid of the diet is doubled (diet III) or quadrupled (diet IV), total liver cholesterol and β steroids increase, but reach a limiting value (diets III and IV). The addition of dihydrocholesterol to diets containing cholic acid and cholesterol (diets V, VI and VII) results in a significant decrease in total liver cholesterol and total β steroids. If cholesterol and dihydrocholesterol concentrations are held constant, and cholic acid concentration is increased stepwise, as in diets V, VI and VII, total liver cholesterol and β steroids decrease significantly as cholic acid concentration rises. Total liver cholesterol and total β steroids in group VII were significantly lower than in the control group (group I). Comparison of the results obtained using the anthrone method and the Lieberman-Burchard method shows that, under our experimental conditions, no dihydrocholesterol is deposited in the liver. In all cases, total β steroid concentration parallels total cholesterol concentration.

TABLE 1

Effect of dietary dihydrocholesterol and cholic acid on total liver cholesterol and β steroids

GROUP AND DIET	NO. OF MICE	ADDITIONS TO CHOLESTEROL-FREE DIET			TOTAL CHOLESTEROL IN LIVER		TOTAL β STEROIDS IN LIVER	
		Cholesterol	Cholic acid	Dihydro-cholesterol	Per organ [2]	Per gram body weight	Per organ [2]	Per gram body weight
		%	%	%	mg	mg	mg	mg
I	9	..	...	..	9.7 ± 1.6 [1]	0.39 ± 0.07 [1]	12.2 ± 3.0 [1]	0.48 ± 0.18 [1]
II	9	1.0	0.25	..	21.4 ± 6.3	0.85 ± 0.14	22.2 ± 6.2	0.96 ± 0.23
III	7	1.0	0.5	..	30.2 ± 5.5	1.50 ± 0.36	33.2 ± 5.0	1.6 ± 0.22
IV	7	1.0	1.0	..	32.1 ± 5.3	1.50 ± 0.30	32.0 ± 5.4	1.5 ± 0.40
V	8	1.0	0.25	2.5	11.4 ± 4.5	0.51 ± 0.15	13.3 ± 5.5	0.79 ± 0.32
VI	9	1.0	0.5	2.5	7.6 ± 3.1	0.35 ± 0.11	9.3 ± 4.3	0.44 ± 0.18
VII	7	1.0	1.0	2.5	5.1 ± 0.8	0.28 ± 0.04	6.8 ± 1.1	0.35 ± 0.18

[1] Standard deviations.

[2] Significance of the difference in results of the following groups: (a) II and III, $P < 0.01$; (b) II and V, $P < 0.01$; (c) V and VI, $P \cong 0.05$; (d) V and VII, $P < 0.01$; (e) VI and VII, $P \cong 0.05$.

DISCUSSION

It is clear from the data shown above that under our dietary conditions the concentrations of dihydrocholesterol employed were very effective in preventing the cholesterol type of fatty liver in mice. In an experiment reported by Hernandez et al. ('53), it has been shown that soybean sitosterols inhibit the absorption of dietary cholesterol. It is reasonable to assume that dihydrocholesterol acts in a similar manner. At least three possible mechanisms are suggested: (1) some type of nonabsorbable complex is formed; (2) dihydrocholesterol combines with all the available bile salts, forming stable complexes or emulsions, so that insufficient bile salts remain for the absorption of cholesterol; or (3) dihydrocholesterol inhibits intestinal cholesterol esterase or other enzymes necessary for cholesterol absorption. The second possibility has been eliminated by the present experiment, inasmuch as an increase in bile acid, in the presence of cholesterol and dihydrocholesterol, decreased rather than increased liver cholesterol. The other possibilities are being investigated.

SUMMARY

1. Increasing the cholic acid content of a diet containing cholesterol significantly increased mouse liver cholesterol and β steroids, up to a limiting value.

2. The addition of dihydrocholesterol to diets containing cholic acid and cholesterol significantly decreased mouse liver cholesterol and β steroids.

3. Increasing the cholic acid content of a diet containing constant amounts of dihydrocholesterol and cholesterol significantly decreased mouse liver cholesterol and β steroids.

4. Under all conditions, total liver cholesterol and β steroid concentrations were parallel.

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Effects of β-Sitosterol and Ferric Chloride On Accumulation of Cholesterol in Mouse Liver.* (21989)

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Since repeated experimental evidence has associated high cholesterol levels with atherosclerosis, various attempts have been made to lower blood and organ cholesterol content. The effects of several substances on cholesterol absorption have been studied using various experimental animals. Soybean sterols, consisting mainly of β-sitosterol, have been shown to decrease the accumulation of dietary cholesterol(1-5). Studies on dihydrocholesterol treated animals have shown that this substance is also effective in reducing cholesterol absorption(6-8). Moreover, it has been found that, in the cockerel, precipitation of bile acids by ferric chloride results in a reduction of blood cholesterol(9,10). On the other hand, in the case of the mouse and rat, evidence has been produced that soybean sterols fail to lower organ and blood cholesterol(11,12).

The object of this experiment is to study: first, the effectiveness of β-sitosterol in prevention of liver cholesterol accumulation; and second, the effectiveness of ferric chloride in a mammalian species.

Methods. Exp. I. Dietary β-sitosterol. Webster strain female albino mice weighing 15 to 20 g were used. Throughout the experiment all mice were fed *ad lib.* a basal fat-free, cholesterol-free diet, described in a previous paper(7), supplemented by adequate amounts of vit. A and D. After a 2-week preliminary period on this basal diet, the mice were divided into 7 groups. The controls, Group I, were continued on the basal diet. For the other 6 groups the basal diet was supplemented with 1% cholesterol. Cholic acid was also added to the various diets as follows: Groups II and V received 0.25%; Groups III and VI, 0.5%; and Groups IV and VII, 1%. In addition, diets for Groups V, VI, and VII contained

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β-Sitosterol used in this experiment was generously donated by Eli Lilly Co.

TABLE I. Effect of β-Sitosterol and Cholic Acid on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	β-sitosterol	Per organ†	Per g body wt‡
I	12	—	—	—	10.6 ± 2.1*	.53 ± .11*
II	10	1	.25	—	36.1 ± 6.3	1.88 ± .28
III	9	1	.5	—	47.1 ± 9.1	2.40 ± .52
IV	10	1	1.0	—	48.0 ±13.3	2.20 ± .60
V	10	1	.25	2.5	30.0 ± 6.1	1.37 ± .23
VI	11	1	.5	"	24.9 ± 6.4	1.27 ± .33
VII	9	1	1.0	"	34.2 ± 6.8	2.04 ± .41

* Stand. dev.

† Significance of the difference in results of the following groups: (a) II & V, $P \cong 0.04$; (b) III & VI, $P < 0.01$; (c) IV & VII, $P < 0.01$.

‡ Significance of the difference in results of the following groups: (a) II & V, $P < 0.01$; (b) III & VI, $P < 0.01$; (c) IV & VII, $P \cong 0.5$.

2.5% β-sitosterol. All diets were fed *ad lib.* for a 3-week experimental period, during which food consumption for the 7 groups varied from 2.9 g to 3.4 g per mouse per day. After the experimental feeding, the mice were sacrificed, and the livers removed, weighed, and quick frozen. Total liver cholesterol was assayed by a procedure similar to the one used in our previous study(7).

Exp. II. Dietary ferric chloride. National Institute of Health strain female albino mice weighing 15 to 20 g were used. As in Exp. I, all animals were fed *ad lib.* a basal fat-free, cholesterol-free diet for an initial 2-week period. The mice were then divided into 5 groups. For all groups, the basal diet was supplemented with 1% cholesterol and 0.5% cholic acid. In addition iron, as ferric chloride, was added to the diets as follows: Group I received no iron; Group II received 0.05%; Group III, 0.5%; Group IV, 1%; and Group V, 2%. After being fed *ad lib.* on these experimental diets for 3 weeks, the mice were sacrificed, and the livers quickly removed, weighed and frozen. Total cholesterol determinations were made as in Exp. I.

Results. Table I presents the data on the effect of β-sitosterol and cholic acid on the accumulation of mouse liver cholesterol. As can be seen, increasing the dietary cholic acid increased the concentration of liver cholesterol up to a maximum value attained in Group III. Comparison of Groups II and V, III and VI, and IV and VII shows the effectiveness of β-sitosterol in preventing liver cholesterol build-up. It is to be noted that β-sitosterol is most effective in the presence of 0.5% cholic acid. However, in none of these groups did total cholesterol concentration approach the control values.

Results of simultaneous feeding of cholesterol and ferric chloride are shown in Table II. The data indicate that ferric chloride failed to decrease cholesterol accumulation. In fact, increasing the concentration of ferric chloride seemed to increase the cholesterol level. Even though comparison of Groups I and II may suggest a slight decrease, the significance of this difference is doubtful in view of the large standard deviation.

Discussion. From the data in Table I, it can be seen that β-sitosterol is effective in decreasing liver cholesterol accumulation under certain conditions. However, comparison of the present results with those obtained in an identical experiment(7) with dihydrocholesterol, shows that, in the presence of equal amounts of cholic acid, dihydrocholesterol is more effective than β-sitosterol.

Data on the ferric chloride experiment indicate that this compound does not reduce dietary cholesterol accumulation in liver. This finding does not coincide with that of Siperstein *et al.*(9), who postulated that ferric chloride binds the bile acids in the intestinal tract, thereby suppressing cholesterol absorption. However, it must be noted that two major differences exist between their experiment and the present one: First, Siperstein *et al.* experimented on cockerels, while in our experiment, mice were used; and second, they studied blood cholesterol levels, while our data

TABLE 11. Effect of Ferric Chloride on Total Mouse Liver Cholesterol.

Group and diet	No. of mice	Additions to basal fat-free diet (%)			Total cholesterol in liver (mg)	
		Cholesterol	Cholic acid	Iron as $FeCl_3$	Per organ	Per g body wt
I	7	1	.5	—	56.3 ± 11.7*	2.81 ± .65*
II	8	1	.5	.05	43.9 ± 10.4	2.22 ± .38
III	7	1	.5	.5	53.5 ± 11.5	2.54 ± .46
IV	7	1	.5	1.0	68.6 ± 17.4	3.36 ± .65
V	7	1	.5	2.0	67.6 ± 7.6	3.76 ± .50

* Stand. dev.

are measurements of liver cholesterol concentrations. The differences found in these experiments may be explained by the hypothesis that ferric ions have a toxic action on liver cells, which prevents the normal distribution of cholesterol between the blood stream and the liver. In agreement with Siperstein *et al.*, we found that ferric chloride, fed in concentrations of 2% or more, proved to be highly toxic and, in some cases, fatal after a few days. The possible physiological after-effects of even small amounts of this compound cannot be overlooked.

Summary. 1. Increasing the cholic acid content of a diet rich in cholesterol, significantly increased total liver cholesterol. 2. The addition of β-sitosterol to diets containing cholic acid and cholesterol, decreased total liver cholesterol. 3. Addition of ferric chloride, in varying amounts, to diets rich in cholesterol and cholic acid, resulted in practically no change in total cholesterol content of mouse liver.

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Feedback Control of Cholesterol Biosynthesis in the Mouse.* (27360)

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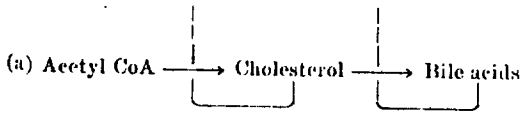
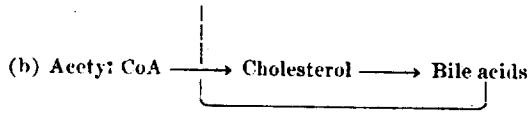
The effects of cholesterol and of the end products of cholesterol metabolism on rate of cholesterol biosynthesis have been the subject of several investigations(1). It is known that cholesterol itself lowers the rate of hepatic cholesterol synthesis, but it appears to have little effect on cholesterol synthesis in extrahepatic tissues. Bile acids also have been shown to control the rate of hepatic cholesterol metabolism through a feedback reaction(2). Two schemes have been proposed(2,3) to explain the mechanism of this feedback:



In this set of reactions bile acids, major end products of cholesterol metabolism, which are present in the enterohepatic circulation, reduce the rate of their own synthesis, causing an accumulation of small amounts of cholesterol at the intracellular level. This in turn reduces the rate of cholesterol biosynthesis.



In this scheme, the bile acids directly reduce the rate of cholesterol synthesis from acetyl CoA, thus reducing the amount of substrate available for conversion to bile acids.

Two approaches have been used in the study of the bile acid feedback reaction. Bergström and Danielsson(4) and Myant

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and Eder(3) have shown that bile acid synthesis is increased in bile fistula rats several hours after operation. The latter authors demonstrated an increased rate of hepatic cholesterol synthesis *in vitro* in these animals. We previously found(5) that bile acid synthesis in mice is inhibited by cholic acid and its conjugates. This study was made on animals with accumulated liver cholesterol, and under this condition, hepatic cholesterol synthesis is nearly at zero level. No decision between mechanisms *a* and *b* could be reached from the results of any of these experiments.

The present investigation was designed to study the bile acid feedback reaction and to locate the position or positions of feedback in normal mice maintained under normal conditions.

Methods. Forty-eight female albino mice of the Webster strain, weighing 24-30 g, were placed on a synthetic cholesterol-free diet(6) for a 2-week observation period. The diet of one-half of the mice was then supplemented with 0.5% cholic acid. Both diets were fed *ad lib.*

After 3 weeks the control and cholic acid fed mice were each divided into 3 groups of 8. For determination of hepatic cholesterol synthesis rates, a group of controls (Group I) and a group of cholic acid treated animals (Group II) received intraperitoneal injections of 5 μc sodium acetate-1-C^{14} (0.191 mg) in 0.9% saline. One hour later the animals were sacrificed and livers removed for analysis of cholesterol and cholesterol-4-C^{14}(7).

In the fecal steroid excretion studies, controls (Group III) and treated animals (Group IV) were injected intraperitoneally with 1 μc mevalonic acid-2-C^{14} (0.196 mg) in 0.9% saline. Mevalonic acid was used in this study since it has been shown that over a wide range of hepatic cholesterol biosynthesis rates, this substance (in limited quantities) is equally incorporated into liver cholesterol. An additional group of control (Group V) and treated mice (Group VI) received intraperitoneal injections of 1 μc cholesterol-4-C^{14} (17 μg) in a fine suspension. This suspension was prepared by dissolving cholesterol-4-C^{14} in absolute ethanol, homogenizing it with a suitable amount of 0.9% saline, and removing the ethanol by evaporation.

Both the mice injected with mevalonic acid (Groups III and IV) and those injected with cholesterol (Groups V and VI) were placed in metabolic cages. Feces were collected daily for 7 days. The mice were then sacrificed, and cholesterol and β-sterol-C^{14} levels determined in liver and carcass as previously described(8). Fecal steroids were fractionated (5) by extraction with ethanol, followed by hydrolysis with aqueous sodium hydroxide. $\alpha + \beta$ sterols were removed from the hydrolysate by petroleum ether extraction. The aqueous phase was acidified, and extracted with petroleum ether to remove fatty acids. Bile acids were recovered by further extraction with ethyl ether. The petroleum ether fraction containing $\alpha + \beta$ sterols was washed with dilute alkali to remove fatty acid contamination. The ethyl ether fraction containing the bile acids was washed with water to remove impurities, including mevalonic acid. Suitable aliquots of the washed extracts were plated in cup planchets and counted. All C^{14} activities were measured in an automatic windowless flow counter.

Results. Results of the study on hepatic cholesterol synthesis as affected by cholic acid are presented in the upper half of Fig. 1. Cholic acid caused a 10-fold reduction in the rate of acetate-1-C^{14} incorporation into liver cholesterol.

The time pattern of fecal steroid excretion was studied in control and cholic acid fed mice following single intraperitoneal injections of mevalonic acid-2-C^{14} or cholesterol-4-C^{14} (Table I). In control animals which received mevalonic acid (Group III), C^{14} activity was equally distributed between the $\alpha + \beta$ sterol and bile acid fractions during the first 24 hours; while in subsequent periods, activity in the former fraction was about double that in the latter one. Comparison of Groups III and IV (Table I) indicates that cholic acid did not significantly alter either the time course of C^{14} excretion, or the total activity excreted in the 2 fractions.

After a single injection of a fine suspension of cholesterol-4-C^{14}, in the control group (V) bile acid-C^{14} excretion was 40% lower than

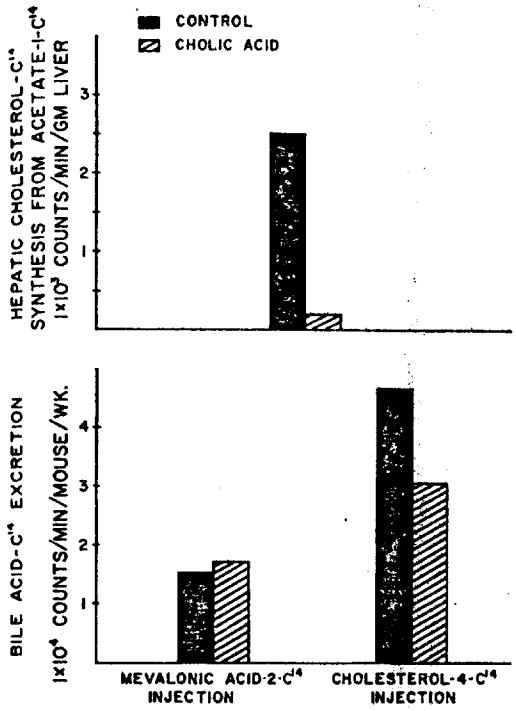

FIG. 1. Effect of cholic acid on hepatic cholesterol and bile acid synthesis.

the $\alpha + \beta$ sterol-C^{14} throughout the collection period. C^{14} excretion in both fractions was fairly constant after the first 24 hours. In the cholic acid treated group (VI), the fecal bile acid-C^{14} was uniform during the 7-day period, but after the first 24 hours, was reduced $\cong$ 40% with respect to the controls (Group V). Total excretion of bile acid-C^{14} was 36% lower in the treated animals than in the controls (Table I). The rate of $\alpha + \beta$ sterol excretion was not altered by cholic acid.

After sacrifice of the injected mice, β-sterol-C^{14} activity remaining in the livers and carcasses was determined (Table II). In animals injected with mevalonic acid-2-C^{14}, total β-sterol-C^{14} activity in the livers of cholic acid treated animals (Group IV) was higher than in controls (Group III). Carcass β-sterol-C^{14} and total C^{14} activity accounted for, was the same in these 2 groups.

When cholesterol-4-C^{14} was injected, cholic acid treated animals (Group VI) retained a significantly larger amount of hepatic β-sterol-C^{14} than controls (Group V). There was no difference in carcass sterol-C^{14} in the 2 groups (V and VI).

Liver cholesterol levels were increased significantly in the cholic acid groups (IV and VI), whereas carcass cholesterol levels remained constant.

Discussion. The experimental results reveal several interesting points. In normal mice the distribution of C^{14} activity in fecal steroids was approximately 60% in the $\alpha + \beta$ sterol fraction and 40% in the bile acid fraction, regardless of whether the steroids originated from intraperitoneal injections of mevalonic acid-2-C^{14} (Group III) or from cholesterol-4-C^{14} (Group V). Studies in normal rats(9,10), on the other hand have shown that 80-90% of the activity of cholesterol-4-C^{14} injected intravenously or intraperitoneally, is excreted in the fecal bile acid-C^{14} fraction. It seems that a species difference may exist between mice and rats in this distribution; this point is being investigated.

Excretion of fecal steroid-C^{14} in both control (Group III) and treated (Group IV) mice receiving mevalonic acid-2-C^{14} intra-

TABLE I. Effect of Cholic Acid on Time Course of Fecal Steroid-C^{14} Excretion in Mice.

	Mevalonic acid-2-C^{14}				Cholesterol-4-C^{14}			
	Group III, control		Group IV, cholic acid		Group V, control		Group VI, cholic acid	
Collection period, days	$\alpha + \beta$ sterols	Bile acids	$\alpha + \beta$ sterols	Bile acids	$\alpha + \beta$ sterols	Bile acids	$\alpha + \beta$ sterols	Bile acids
	Counts/min./mouse/24 hr							
1	7,821	7,577	8,619	9,336	7,549	4,040	4,525	4,531
2	6,010	3,071	5,486	3,220	15,663	10,130	12,893	6,456
3	3,227	1,666	4,878	1,512	18,143	11,151	20,429	6,051
4	2,830	1,234	3,741	1,027	18,447	13,110	24,751	7,471
5-7	2,783	1,490	1,713	1,987	17,044	8,475	15,305	5,734
Total excretion	22,671	15,038	24,437	17,082	76,846	46,906	77,903	30,243

TABLE II. Effects of Cholic Acid on Fecal and Tissue Steroid-C^{14} and Steroid Levels 7 Days after C^{14} Injection.

Source	Mevalonic acid-2-C^{14} Group III, control	Mevalonic acid-2-C^{14} Group IV, cholic acid	Cholesterol-4-C^{14} Group V, control	Cholesterol-4-C^{14} Group VI, cholic acid
	Counts/min./mouse			
Fecal steroids-C^{14}, total excreted	37,709	41,519	123,752	108,146
β-Sterol-C^{14} in liver	14,994	21,090	77,285	180,552
" in carcass*	45,585	44,864	330,510	323,080
Total C^{14} activity accounted for	98,288	107,473	531,547	611,778
	mg/g tissue			
Liver cholesterol	5.34 ± .67	7.53 ± 2.54	4.19 ± .97	7.52 ± 3.08
Carcass " *	2.24 ± .39	2.30 ± .39	2.06 ± .35	2.20 ± .38

* Carcass wt = body wt minus liver wt. ± stand. dev.

peritoneally was at a maximum during the first 24-hour interval and declined rapidly through the fourth day. In the case of cholesterol-4-C^{14} injection (Groups V and VI), after the first 24-hour interval, during which C^{14} excretion was low, rate of steroid-C^{14} excretion increased and then remained relatively constant. The difference between these groups was due to the slow rate of absorption of cholesterol-4-C^{14}, which was administered not as a solution but as a fine suspension, in an effort to maintain a constant hepatic cholesterol-4-C^{14} specific activity.

Certain data which need to be reconciled are shown graphically in Fig. 1. Total excretion of fecal bile acid-C^{14} in control and cholic acid treated mice which received mevalonic acid-2-C^{14} was not significantly different; thus it might appear that cholic acid has no effect on bile acid synthesis. However, Fig. 1 also shows that hepatic cholesterol synthesis was reduced 10 times in these animals. These findings might seem incompatible because:

A. Liver cholesterol level is slightly higher in treated animals than in controls; and yet B. Cholesterol synthesis rate is decreased 10 times in cholic acid treated animals; while C. Bile acid-C^{14} excretion rate proceeds at control rate. It is conceivable that in cholic acid treated mice the liver cholesterol level could be maintained *via* an extrahepatic source. This explanation, however, is inconsistent with the results because there was no significant difference in carcass β-sterol-C^{14} levels in controls and in cholic acid treated animals at the end of 7 days (Table II). Therefore cholesterol *could not* have entered the liver from the carcass. Furthermore, extrahepatic tissues have been shown to be poor sources of plasma cholesterol(11,12).

Consideration of the kinetics of hepatic cholesterol metabolism offers an explanation of the experimental results. If non-labeled cholesterol molecules are supplied to a liver containing a number of C^{14}-labeled cholesterol molecules, and all of these molecules are subject to the same chance of being metabolized to bile acids, the chances that a cholesterol-C^{14} molecule will be degraded in a given interval will be inversely related to the rate of supply of unlabeled cholesterol molecules. Keeping this fact in mind, it is possible to set up two situations relating cholesterol and bile acid synthesis rates, in order to determine which one agrees with the experimental data.

1. If non-labeled cholesterol synthesis were reduced 10 times, thus increasing the ratio of labeled/unlabeled cholesterol to be metabolized, one would expect a many-fold increase in bile acid-C^{14} excretion in 24 hours as compared with controls, even though bile acid synthesis was kept constant.

2. On the other hand, if *both* non-labeled cholesterol synthesis and bile acid synthesis were reduced 10 times, the ratio of labeled/unlabeled cholesterol would remain unchanged, and so no appreciable change in bile acid-C^{14} excretion would be expected per 24 hours, as compared with controls.

(Obviously these conditions would not necessarily apply in the first hour or two after introduction of labeled cholesterol.)

Since our data (Table I) on mice injected

with mevalonic acid show no significant difference between excretion of bile acid-C^{14} in control and cholic acid treated groups, the second of the above sets of conditions would apply, and it would follow that cholic acid must reduce both cholesterol and bile acid syntheses in normal mice. The reduction of bile acid synthesis by cholic acid is demonstrated (Fig. 1 and Table I) in the cholesterol-4-C^{14} injected mice in which the specific activity of liver cholesterol-C^{14} was maintained at a relatively constant level in control and cholic acid treated mice. It is now clear that both the rates of conversion of acetate to cholesterol and of cholesterol to bile acid are lowered in mice by cholic acid.

Since 5-8 mg of cholic acid was administered per day to each treated mouse, it seemed possible that this addition could have diluted the pool of bile acids in the enterohepatic circulation. If per unit of time a fixed fraction of the circulating pool had been removed *via* the fecal excretion, the amount of bile acid-C^{14} in the feces would have been reduced even though the rate of bile acid-C^{14} synthesis remained constant. However, Table II shows that such a dilution does *not* explain the inhibition of bile acid-C^{14} excretion in the treated animals. At the end of the experiments, the cholic acid treated animals retained more cholesterol-C^{14} in their livers than the controls. Since the excretion of sterol-C^{14} was identical in control and treated animals, liver cholesterol-C^{14} retention in cholic acid treated mice must have been due to inhibition of the conversion of cholesterol to bile acid by cholic acid. Kinetic considerations similar to those outlined above, show that inhibition of bile acid synthesis must have been very strong.

It was previously demonstrated(5) that cholic acid inhibits bile acid synthesis in mice whose hepatic cholesterol synthesis is blocked by cholesterol elevation. This showed that cholic acid can inhibit bile acid synthesis independently of its effect on rate of cholesterol synthesis. It follows that the rate of hepatic cholesterol synthesis in mice is controlled by cholic acid and its conjugates(13) through a double feedback reaction. Since taurocholic acid is the chief bile acid found in mouse bile (14), this substance must be important as a regulator in the homeostatic control of mouse hepatic cholesterol metabolism.

Summary. Effects of cholic acid on biosynthesis of hepatic cholesterol and bile acids were studied. Mice maintained on a basal diet, or this diet supplemented with 0.5% cholic acid, received single intraperitoneal injections of mevalonic acid-2-C^{14} or cholesterol-4-C^{14}. Feces were collected at 24-hour intervals, fecal steroid-C^{14} was isolated and fractionated. $\alpha + \beta$-sterol-C^{14} and bile acid-C^{14} were determined. In control mice the distribution of C^{14} activity in fecal steroids was 60% in the sterol fraction and 40% in the bile acid fraction. This ratio was constant with time after the first 24-hour interval, regardless of the substance injected. It was shown that cholic acid brings about equal decreases in the rates of both hepatic cholesterol and bile acid biosyntheses. Since a preceding study had shown that the rate of bile acid synthesis in mouse liver is inhibited by cholic acid independently of its effect on cholesterol synthesis, it was concluded that the rate of hepatic cholesterol biosynthesis in mice is controlled by cholic acid and its conjugates by means of a double feedback reaction.

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INFLUENCE OF DEOXY- AND HYODEOXYCHOLIC ACIDS ON CHOLESTEROL-CHOLIC ACID-INDUCED GALLSTONE FORMATION IN MICE

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Abstract. Hyodeoxycholic acid, which has previously been shown to protect syrian hamsters against cholesterol gallstone formation, was found to be unable to prevent gallstone formation in mice fed a cholesterol-cholic-acid diet. It did, however, prove capable of protecting these animals against the accumulation of cholesterol crystals in the liver. Deoxycholic acid was found to have an effect similar to that of cholic acid when added to a cholesterol rich diet. Treatment with this bile acid resulted in cholesterol gallstone formation and accumulation of cholesterol crystals in the liver.

In the study of gallstone formation in animals a certain parallelism has been found between experimental gallstone formation and experimental atherosclerosis. This parallelism is perhaps most pronounced in cholesterol-cholic acid-induced gallstone formation in mice. In fact, this kind of gallstone formation was first observed in the course of experiments on atherosclerosis when an "atherogenic" diet was given to a species with a gallbladder—the mouse—instead of to a species without—the rat—in which it had been tried many times before. It therefore seemed of interest to study the effect of other bile acids on this kind of gallstone formation and especially the effect of hyodeoxycholic acid. This bile acid has been found to reverse the hypercholesteremic effect of cholic acid in mice and to prevent cholesterol accumulation in the liver (Beher, Baker & Anthony, 1960).

EXPERIMENTS

60 male and 60 female mice weighing 10–12 gram were divided in a strictly random fashion into 4 groups each consisting of 15 males and 15 females. These groups were treated for 4 months with a diet consisting of ground laboratory chow to which the following ingredients were added:

Group I Cholesterol 1%, cholic acid 0.5%
Group II Cholesterol 1%, cholic acid 0.5%, hyodeoxycholic acid 0.5%
Group III Cholesterol 1%, deoxycholic acid 0.5%
Group IV Cholesterol 1%, cholic acid 0.5%, deoxycholic acid 0.5%

All animals were sacrificed after 4 months. At autopsy the gallbladder was removed and incised, its contents being permitted to run out on blotting paper so that even small concrements could be noticed. The liver was weighed and fixed together with gallbladder, lungs, heart, spleen and kidneys in 10% formalin solution. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin. Frozen sections of liver, heart and kidneys were stained with Scharlach-Rot and also analysed with the polarizing microscope. The histological examinations were performed with the assessor unaware of the group to which the animal belonged. Serum cholesterol levels were determined according to a modified Liebermann–Burchard reaction.

Survival. 7 animals in group I died, leaving 23 survivors at the end of the experimental period. In group II 4 mice died, leaving 26 survivors, while in groups III and IV there were 24 and 21 survivors respectively.

RESULTS

The results of the experiments are compiled in Table I, from which it will be seen that gallstones occurred in all groups and that no significant differences were found between the four groups in this respect. It may seem that gallstone formation was somewhat less marked in group II, treated with cholic acid and hyodeoxycholic acid, as there are a few more animals without gall-

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Table I. *Gallstones, liver histology and serum cholesterol levels in mice treated with different bile acids*

Group	N	Gallstones[a]		Liver: Fatty degeneration[b]			Liver: Birefractive crystals[c, d]		Serum chol. level	
		+	−	++	+	−	+	−	Mean	Range
I	23	13	10	13	9	1	23	0	259.3	133–477
II	26	12	14	13	8	5	1	25	176.6	92–262
III	24	14	10	20	3	1	18	6	284.1	177–385
IV	21	12	9	19	2	0	21	0	235.7	121–385

[a] +, gallstones present, −, gallstones absent. [b] ++, serious fatty degeneration of the liver; +, moderate; −, no fatty degeneration. [c] +, accumulation of birefractive crystals; −, no crystals. [d] $\chi^2 = 69.04$; d.f. = 3; $P < 0.001$.

stones in this group than in the other ones. This difference is, however, not only nonsignificant but largely illusory, for if only the animals in which gallstones were found are considered, the amount of concretions was even larger in this group than in the other ones.

Table I further shows that fatty degeneration of the liver occurred in a large number of animals in all treatment groups. On the other hand, esterified cholesterol appearing as biretractive crystals in the liver cells was virtually absent in group II but occurred abundantly in most animals belonging to group III and in all mice of groups I and IV. According to the χ^2 test, this difference is highly significant. For a more detailed description of the morphological changes the reader is referred to a previous study on gallstone formation in mice (Bergman, Bogren, Lindelöf & van der Linden, 1966). Apart from the difference in the occurrence of birefractive needle-shaped crystals in the liver cells, histological examination revealed no group differences.

Finally, the serum cholesterol levels showed very large variations. Even so, the mean for group II was considerably lower than that of the other groups.

DISCUSSION

In studies on the effect of different bile acids on cholesterol levels and synthesis rates in mice, Beher and coworkers found that cholic and hyodeoxycholic acid had opposing effects. Cholic acid was found to increase liver and serum cholesterol levels and decrease cholesterol synthesis, whereas hyodeoxycholic acid decreased liver and serum cholesterol levels and increased the synthesis rate (Beher, Anthony & Baker, 1959). When cholic and hyodeoxycholic acids were given simultaneously, 0.5% hyodeoxycholic acid was found to prevent the accumulation of cholesterol induced by 0.5% cholic acid (Beher, Baker & Anthony, 1960). Our results are in accordance with these findings. Group I was given a diet supplemented with 0.5% cholic acid, while group II received both 0.5% cholic acid and 0.5% hyodeoxycholic acid (see Table I); these groups differ as regards serum cholesterol level and liver histology. According to the Mann–Whitney U test (Siegel, 1956), the difference in serum cholesterol level is significant at the 5% level. As for liver histology, there is a striking difference in the occurrence of cholesterol crystals. These crystals are absent in all but one animal given 0.5% hyodeoxycholic acid (group II) and present in all animals of group I, not treated with this bile acid. In spite of this marked reverse effect of hyodeoxycholic acid on cholic acid induced cholesterol accumulation, the formation of cholesterol gallstones was unaffected. This is the more surprising as Dam & Christensen (1962) found that the addition of 0.1% hyodeoxycholic acid to a lithogenic diet gives syrian hamsters a high degree of protection against cholesterol gallstone formation. As in earlier studies (Lindelöf & van der Linden, 1965; Bergman, Bogren, Lindelöf & van der Linden, 1966) we are again confronted with a species difference in gallstone formation. A substance protecting one species when added to a certain diet had no effect in another species forming the same kind of gallstone on a different diet.

The findings in group III show that deoxycholic acid differs little from cholic acid in its ability to induce cholesterol accumulation. This

perhaps to be expected in view of the ability of the mouse to reconvert deoxycholic to cholic acid in the liver (Haslewood, 1963). Still, Beher *et al.* (1959) report that deoxycholic acid produced a significant decrease in liver cholesterol level in the mouse and Yamasaki, Shimizu, Yasumizu & Sugihara (1959) that deoxycholic acid depresses plasma cholesterol in the rat. Portman & Bruno (1961) and Howe & Hutchinson (1962), on the other hand, found that deoxycholic acid elevated the blood cholesterol level of the rat, a species with a bile acid pattern closely similar to that of the mouse (Danielsson & Kazeno, 1959). Our results are in accordance with this latter finding. Comparing group III, fed 0.5% deoxycholic acid, with group I, fed 0.5% cholic acid, there are a few cases without liver cholesterol crystals in the former and none in the latter group. This difference is, however, not significant and the serum cholesterol levels are even somewhat higher in the deoxycholic acid group. Finally, the results of group IV are in accordance with what might be expected when two bile acids, which under the conditions of the present experiment stimulate cholesterol accumulation, are given simultaneously. In all these mice there was an abundant deposition of cholesterol in the liver.

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EFFECT OF CHOLESTYRAMINE ON CHOLESTEROL GALLSTONES IN MICE

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Abstract. Mice which, after treatment with a cholesterol cholic acid containing diet, were found at exploratory laparotomy to harbor cholesterol gallstones, were fed the same diet without cholic acid. This sufficed to bring about dissolution of gallstones in some animals. The addition of 3% cholestyramine to this diet did not influence the dissolution of the gallstones. Nor could any influence of cholestyramine on the dissolution of gallstones be detected when mice with gallstones were fed normal laboratory chow with and without cholestyramine. In a third experiment it was found that esterified cholesterol, which accumulates in the liver of mice fed a gallstone-inducing diet, disappears more quickly in mice fed a diet containing cholesterol and cholestyramine than in mice fed the same diet without cholestyramine.

In earlier experiments we studied the effect of cholestyramine on gallstone formation in Syrian hamsters (Bergman & van der Linden, 1966, 1967 *b*). We found that the resin, when added to a gallstone-inducing diet, was able not only to prevent the formation of cholesterol gallstones but also to bring about the dissolution of such stones once formed. As this was the first time that one particular compound could be held responsible for the dissolution of cholesterol gallstones, it seemed of interest to study this compound's effect on cholesterol gallstones in the other species in which such stones can easily be induced, i.e. the mouse. The results of these studies are reported here.

MATERIAL AND METHODS

Experiment I. Male and female white mice weighing 10–12 g were fed commercial laboratory chow,[1] which was first ground and then repelleted after the addition of 1% cholesterol and 0.5% cholic acid. After 4 months on this diet they were subjected to exploratory laparotomy in ether narcosis. Fifty (50) surviving mice, in which the gallbladder had been found to contain gallstones, entered the experiment. They were divided at random into two equal groups of 25 animals. One group was fed laboratory chow supplemented with 1% cholesterol while the other was fed the same diet but also supplemented with 3% cholestyramine. In order to get some impression of the time needed for dissolution, 4 mice of each group were sacrificed after 3 months. At autopsy the gallbladder was removed and opened, permitting its contents to run out on blotting paper, so that not even small concrements would escape detection. The liver, gallbladder, lungs, heart, spleen and kidneys were fixed in 10% formalin solution, embedded in paraffin, sectioned and stained with haematoxylin-eosin and van Gieson's stain. Frozen sections of liver, heart and kidneys were stained with Sudan Black B and also analysed with the polarizing microscope. The histological examinations were performed with the assessor unaware of the animals' group. Pooled samples of serum were obtained and serum cholesterol levels were determined according to a modified Liebermann–Burchard reaction. After 6 months there were 17 survivors in each group. These animals were then sacrificed and investigated as described above.

Experiment II. As in experiment I, male and female white mice, weighing 10–12 g, were fed commercial chow supplemented with 1% cholesterol and 0.5% cholic acid during four months. Twenty (20) mice, which at exploratory laparotomy had been found to harbor gallstones, entered the experiment. They were randomly divided into two equal groups which were fed commercial laboratory chow without supplement and the same diet supplemented with 3% cholestyramine. After 3 months on this diet all animals were sacrificed and investigated as described above.

Experiment III. As in the two previous experiments, mice of both sexes were fed commercial chow supplemented with 1% cholesterol and 0.5% cholic acid during four months. One hundred survivors were divided at random

[1] Obtained from Harald Fors, Holmsund, Sweden.

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into two equal groups. one was fed laboratory chow supplemented with 1% cholesterol, the other was fed the same diet to which 3% cholestyramine was also added. At 7-, 10-, 14-, 18- and 22-day intervals, 10 mice from each group were sacrificed and investigated as in the two earlier experiments.

In addition, frozen sections of the liver were analysed for cholesterol and its esters with histophysical (melting point, solubility) and histochemical methods (osmium tetroxide-α-naphthylamine method and perchloric acid-naphtoquinone method according to Adams (1959, 1961, 1965)).

RESULTS

The results of experiments I and II are given in Table I. It will be seen that gallstones had dissolved in 5 of 17 animals fed during 6 months with chow supplemented with 1% cholesterol, i.e. the same diet on which they had first formed gallstones, except for the omission of cholic acid. The table also shows that the addition of 3% cholestyramine to this diet did not influence the dissolution. Neither did cholestyramine seem to have much influence when it was added to the unsupplemented diet of experiment II. In almost all the mice, the histological examination revealed a slight to moderate fibrosis of the gallbladder wall, with scattered inflammatory cells and with atrophy of the mucous membrane. Apart from this, the histological examination revealed nothing of interest. Serum cholesterol concentrations were somewhat lower in the mice fed cholestyramine, but the differences were not significant.

The results of experiment III are given in Table II. As previously found (Bergman, Bogren, Lindelöf & van der Linden, 1966), the cholesterol cholic acid diet gave a marked accumulation in the liver of birefractive needle-shaped crystals interpreted as esterified cholesterol. This interpretation was now confirmed with new histochemical methods. The table shows that liver histology rapidly returned to normal, both in mice fed a diet supplemented with cholesterol and in those fed a diet supplemented with both cholesterol and cholestyramine. However, in the latter group the birefractive crystals disappeared more quickly. A significant difference between the two groups was found after 14 days. This difference was less clear after 18 days and had disappeared after 22 days, when no more crystals were found in either group. Also the lipids stained with Sudan Black B seemed to disappear somewhat more quickly in the cholestyramine-fed mice, but this difference did not reach the level of significance. Neither could a significant group difference be found in serum cholesterol level.

Table I. *Experiments I and II: Incidence of gallstones in mice sacrificed after different periods on different diets*[a]

Exp.	Diet	3 months +	3 months −	3 months Σ	6 months +	6 months −	6 months Σ
I	1% cholesterol	4	0	4	12	5	17
	1% cholesterol 3% cholestyramine	3	1	4	14	3	17
II	Unsupplemented	6	2	8			
	3% cholestyramine	5	4	9			

a + denotes animals with stones left, − animals without stones

Table II. *Experiment III: Liver histology in mice sacrificed after different periods on different diets*

		"Fatty liver" (Sudan Black B)[a]						Esterified cholesterol as birefractive crystals[b]			
		Cholesterol			Cholesterol Cholestyramine			Cholesterol		Cholesterol – cholestyramine	
Period, d.	N	++	+	−	++	+	−	+	−	+	−
7	10	8	2	0	7	3	0	10	0	9	1
10	10	6	2	2	3	5	2	10	0	9	1
14	10	3	2	5	1	1	8	8	2[c]	2	8[c]
18	10	4	4	2	2	4	4	6	4	1	9
22	10	0	3	7	0	0	10	0	10	0	10

a ++ denotes moderate + small, − no fatty changes.
b + denotes birefractive crystals present, − no crystals
c P = 0.023 (Mainland, Herrera & Sutcliffe, 1956).

In earlier experiments we have found that cholestyramine is able to prevent the formation of cholesterol gallstones in Syrian hamsters and also to dissolve such stones once formed (Bergman & van der Linden, 1966, 1967 *b*). We now wanted to investigate whether cholestyramine has any effect on cholesterol gallstones in mice. The formation of cholesterol gallstones in mice fed certain diets has been described by Tepperman, Caldwell & Tepperman (1964), who presented evidence that this diet's crucial elements for stone formation are cholesterol and cholic acid. If, for instance, mice are fed a diet containing only 1 % cholesterol and no cholic acid, no gallstone formation will ensue. Clearly, the addition of cholestyramine, a bile acid sequestrant, to the diet would have amounted to the same thing, i.e. the elimination of cholic acid, one of the diet's crucial elements. It therefore seemed of little interest to test the ability of cholestyramine to prevent gallstone formation on such a diet. Other questions seemed, however, of interest. Caldwell, Levitsky & Rosenberg (1965) have found that cholesterol stones dissolved when mice harboring such stones were fed laboratory chow, i.e. a diet without either cholesterol or cholic acid. We now wanted to know if dissolution would also take place on a diet containing only one of the ingredients essential for gallstone formation in mice, i.e. cholesterol. In experiment I we therefore gave mice, which on exploratory laparotomy had been found to have gallstones, a diet supplemented only with 1 % cholesterol. The results of experiment I show that the omission of cholic acid from the diet suffices to bring about the dissolution of cholesterol gallstones. They further show that the addition of 3% cholestyramine to the diet does not seem to have any influence on the dissolution of the gallstones and that the dissolution of cholesterol gallstones in mice under these conditions takes place very slowly as compared with the same process in hamsters (Christensen, Dam & Kristensen, 1956; Bergman & van der Linden, 1967 *b*). Also when mice with gallstones were fed laboratory chow without either cholesterol or cholic acid, dissolution of gallstones took place slowly and neither with this diet did cholestyramine seem to have any influence as shown by the results of experiment II. In both experiments I and II, in which mice were sacrificed at least 3 months after the diet was changed from gallstone-inducing to laboratory chow with or without 1% cholesterol liver histology was normal. In other words, the liver changes, which appear under the influence of the cholesterol cholic acid diet, and the most characteristic element of which is the massive accumulation of birefractive cholesterol crystals (Bergman, Bogren, Lindelöf & van der Linden, 1966), disappear much more rapidly than the gallstones do. This finding, that gallstones once formed may still be left months after liver histology has returned to normal, did not come as a surprise. In previous experiments we found that under certain conditions gallstones may even form in mice while liver histology remains normal (Bergman & van der Linden, 1967 *a*).

Experiment III aimed at investigating whether cholestyramine can influence the rapidity with which the liver changes disappear when cholic acid is omitted from the gallstone-inducing diet. Therefore, having fed mice with the cholesterol cholic acid gallstone-inducing diet, we changed to a diet containing 1 % cholesterol with and without 3 % cholestyramine. As seen in Table II, the addition of 3 % cholestyramine results in the more rapid disappearance of birefractive cholesterol crystals from the liver. The difference between mice fed with cholestyramine and those not fed with this resin, though significant, was small. After 3 weeks the animals not treated with cholestyramine had caught up the cholestyramine-fed mice. These findings are in accordance with the report of Beher, Beher & Rao (1966), who in regression studies in mice found that dietary cholestyramine accelerated the mobilization of accumulated liver cholesterol. In their experiment the cholesterol concentration of both the cholestyramine-fed and the untreated mice had decreased to the normal level after 17 days and a difference between these groups was then no longer discernible.

If we compare cholesterol gallstone formation in the two animal models now accessible to study —the hamster and the mouse—we meet a number of distressing divergences. For instance, the mouse does not form gallstones on a diet which is strongly lithogenic for the hamster (Christensen, Prange & Dam, 1962), nor does the hamster form stones when fed the cholesterol cholic acid diet which induces gallstones in the mouse (Bergman & van der Linden). Still, after the results of the present study, it seems possible to discern at least

one point of agreement. This is the role which bile acids seem to play for the dissolution of gallstones. When, by the addition of cholestyramine to the diet, the excretion of bile acids is enhanced, cholesterol gallstones dissolve in hamsters. In mice —though cholestyramine could not be shown to have any influence—the mere exclusion of cholic acid from the gallstone-inducing diet sufficed to bring about dissolution of gallstones.

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REACTION OF THE MONGOLIAN GERBIL TO A CHOLESTEROL-CHOLIC ACID-CONTAINING GALLSTONE INDUCING DIET

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Mongolian gerbils fed chow supplemented with 1 per cent cholesterol and 0.5 per cent cholic acid, developed steatosis of the liver, accumulation of lipids in the reticuloendothelial system, high serum cholesterol levels and accentuated hypoplastic changes of the thyroid gland. In the bile, a marked rise of the cholesterol concentration, a slight rise of the cholic- and deoxycholic- and a decrease of the chenodeoxycholic acid concentration was observed. After 4 months on the diet, cholesterol gallstones were found in the distended gallbladders. Discontinuance of the diet showed the gerbil to be rather unable to free itself from excess cholesterol with a very slow and incomplete return to normal of liver histology. In this respect the gerbil differs from the mouse but also a number of similarities in the reaction of these two species to the diet were noted.

Comparative studies of gallstone formation in different animals may not only supply valuable information about the conditions which should be fulfilled for gallstone formation to occur. They may also help us in selecting certain species as most suitable for further investigations. This was the reason why we studied gallstone formation in yet another species, the mongolian gerbil.

In the course of our experiments, we observed gallstones in the gerbil after prolonged administration of a cholesterol cholic acid supplemented diet. As we in earlier experiments [2] had found that different species react very differently to this diet, we tried to elucidate this kind of gallstone formation by studying the biochemical and morphological changes — their development and regression — in the gerbil.

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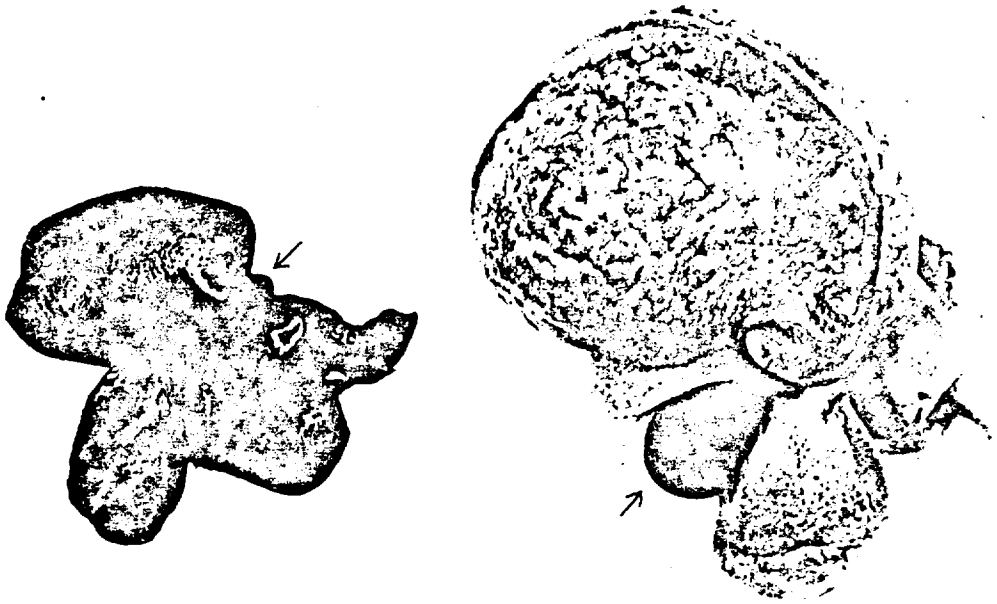
MATERIAL AND METHODS

Experiment 1

Thirty-eight mongolian gerbils of both sexes with a mean weight of 60 g were randomly divided into two groups. All animals were individually caged and had free access to food and water. One group, comprising 30 animals, were fed with chow to which 1 per cent cholesterol and 0.5 per cent cholic acid had been added. The other 8 served as controls. After 4 months the surviving animals were sacrificed after they had been weighed. The gallbladder was incised, permitting the contents to run out on blotting paper for subsequent study under a binocular stereomicroscope. The liver was

[1] Harald Lors, Holmsund, Sweden



g. 1. Liver from normal animal (left) and steatotic liver of cholesterol cholic acid fed animal (right). ote distended gallbladder as compared with normal (see arrows). × 3.5.

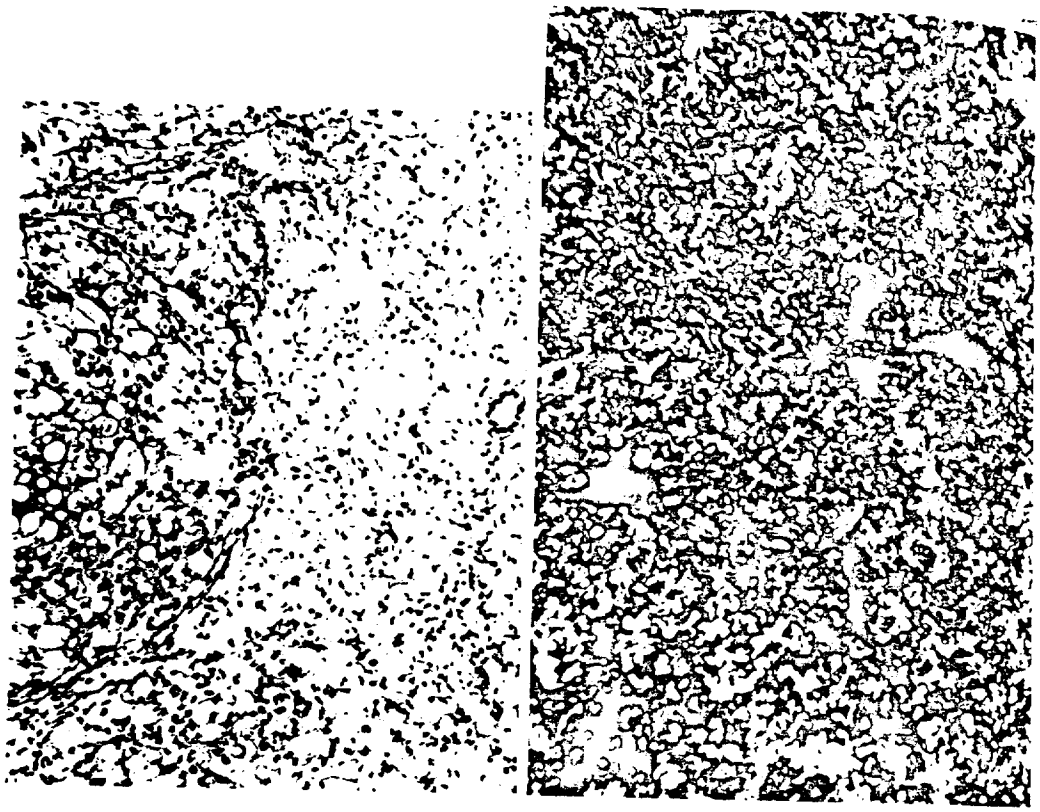
eighed and together with gallbladder, lungs, eart, spleen, lymph nodes, kidneys, adrenals, yroid and pituitary glands fixed in 10 per cent ormalin solution. Paraffin embedded sections were ained with haematoxylin-eosin and van Gieson's ain. Sections of liver and spleen were also stained r haemosiderin with Turnbull's blue method. Fro- n sections of liver, lungs, heart, spleen, lymph des, kidneys and adrenals were stained with harlach-Rot and Sudan Black-B and also ana- ed with the polarizing microscope. In addition, ctions of the thyroid gland were stained with eriodic acid Shiff (PAS) and sections of the itary gland with trichrome-periodic acid Shiff cording to *Pearse* (10). Serum cholesterol levels re determined according to a modified Liebermann-Burchard reaction.

perimi nt 2

One hundred gerbils of both sexes and with a ean weight of 70 g were divided strictly at ran- om into two groups, one of 20 and one of 80 imals. The animals had been fed with normal ow pre-experimentally. All animals of the group twenty were sacrificed after one month on ow. They served as controls. At autopsy the gallbladder was removed and bile was collected for analysis. As the bile samples were small, pooling was necessary. As a rule 2–4 animals contributed to each sample. 0.050 ml of bile was extracted and hydrolyzed according to the method described by *Wolfrathner, Kuttl & Ofner* (16). The free bile acids were separated by thin layer chromatography according to the same method with minor modifications. The spots were quantified by means of a Vitatron Densitometer (U.F.D.). For further details and for a discussion of the precision and accuracy of this method the reader is referred to *Jaul & van der Linden* (9). The cholesterol concentration of the bile was determined after extraction according to a modified Liebermann-Burchard reaction. The lipids were extracted with a mixture of chloroform-methanol (2:1). The extract was filtered and evaporated to near dryness. The residue was redissolved in petroleth er and aliquots were used for determination of lipid phosphorus according to the method of *Ofner, Lorscher & Wolfrathner* (5). The phospholipid content was calculated by multiplying the value for lipid phosphorus by a factor of 25. Liver and spleen were weighed and histological examinations were performed as described in Exp. 1. Finally blood samples were collected and serum cholesterol levels were determined as previously described.

Eighty animals constituted the experimental

[2] Vitatron Dieren, Holland.



a. b.

Figs. 2 a–b. Liver from gerbil fed cholesterol cholic acid diet for 4 months.
a) marked steatosis with cell polymorphism and slight fibrosis. x Gieson × 120.
b) same specimen as in 2 a. Massive accumulation of birefractive cholesterol crystals. Frozen section Polarized light × 50.

group. They were individually caged and had free access to food and water. They were fed during one month with chow supplemented with 1 per cent cholesterol and 0.5 per cent cholic acid. After one month 20 animals were chosen in a strictly random fashion, sacrificed and investigated. For the remaining animals of the experimental group the diet was switched back to unsupplemented chow. One (1), 2 and 3 months later, 20 gerbils chosen at random were sacrificed and examined as described above. In the group of animals sacrificed after 3 months on chow no analysis of blood and bile was performed as the studied variables had already returned to normal.

The histological examinations were performed after the whole experiment had been completed, the assessor being unaware of the group to which the animal belonged.

RESULTS

Experiment I (4 Months Exposure)

After 4 months there were 7 survivors in the test group as compared with 6 among the controls. The mean weight of the surviving test group animals had risen to 75.1 g as compared with 72.8 g of the controls. The mean liver weight of the cholesterol cholic acid fed gerbils was 11.1 g, that of the controls 3.1 g. When subjected to the Mann-Whitney U test (11) this latter difference was highly significant. The liver of the surviving test group animals had the pale yellowish colour typical of extreme steatosis and the gallbladder was extremely dilated (Fig.

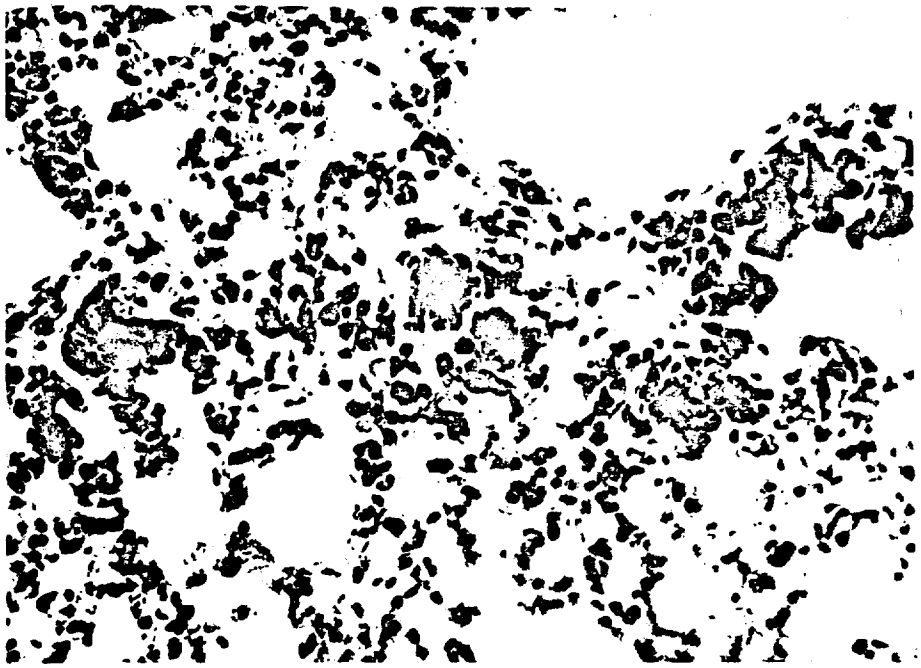


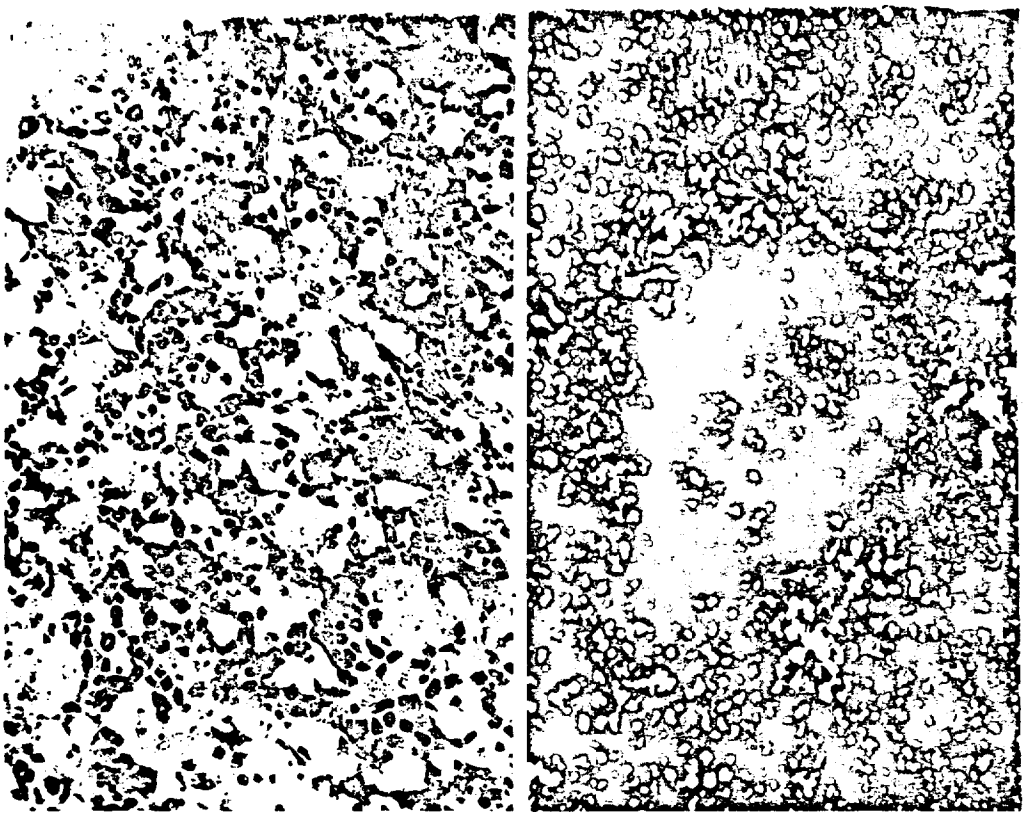
Fig. 3. Lung from gerbil fed cholesterol cholic acid diet for 4 months. Clusters of fat positive alveolar macrophages. Frozen section. Scharlach-Rot × 320.

1). Large accumulations of small whitish gallstones were found in 5 of the 7 survivors. Similar gallstones had been present in most of the animals which had died during the last month of the experiment. Chemical analysis of these stones showed them to consist of cholesterol. No gallstones were present in the controls, nor have such stones ever been observed in dead animals from our stock colony. The spleen was markedly enlarged and ascites was present in all the surviving test group animals.

Histological examination of the liver revealed an extreme fatty degeneration with massive accumulation of intracellular lipid droplets and birefractive cholesterol crystals in the parenchymal and Kupffer cells. The liver cells showed polymorphism and many binuclear cells were present. A slight fibrosis and nodular parenchymal regeneration were observed (Figs. 2 a–b).

The gallbladder had a thin wall with slight fibrosis and atrophy of the mucous membrane. In some animals small mucosal ulcerations and scattered infiltrates of inflammatory cells were found.

A marked accumulation of stainable lipids and birefractive cholesterol crystals were also found in the reticulo-endothelial cells of lungs, spleen and lymph-nodes. In the lungs, clusters of fat-positive alveolar macrophages were found freely in the alveolar lumen or attached to the alveolar wall (Fig. 3). In the spleen, the parenchymatous tissue was in large areas virtually replaced by lipid-containing histiocytes and accumulations of needle-like crystals and granules (Figs. 4 a–b). There was also an increase in Turnbull-positive substance in the spleen of the animals given the supplemented diet. In some cholesterol cholic acid treated animals the kidneys showed slight fatty changes in the loop of Henle and in the proximal convoluted tubules. As for the endocrinal glands the dietary supplement seemed to accentuate the hypoplasia of the thyroid gland found in the normal controls. No difference was found in the anterior pituitary gland while there



a. b.

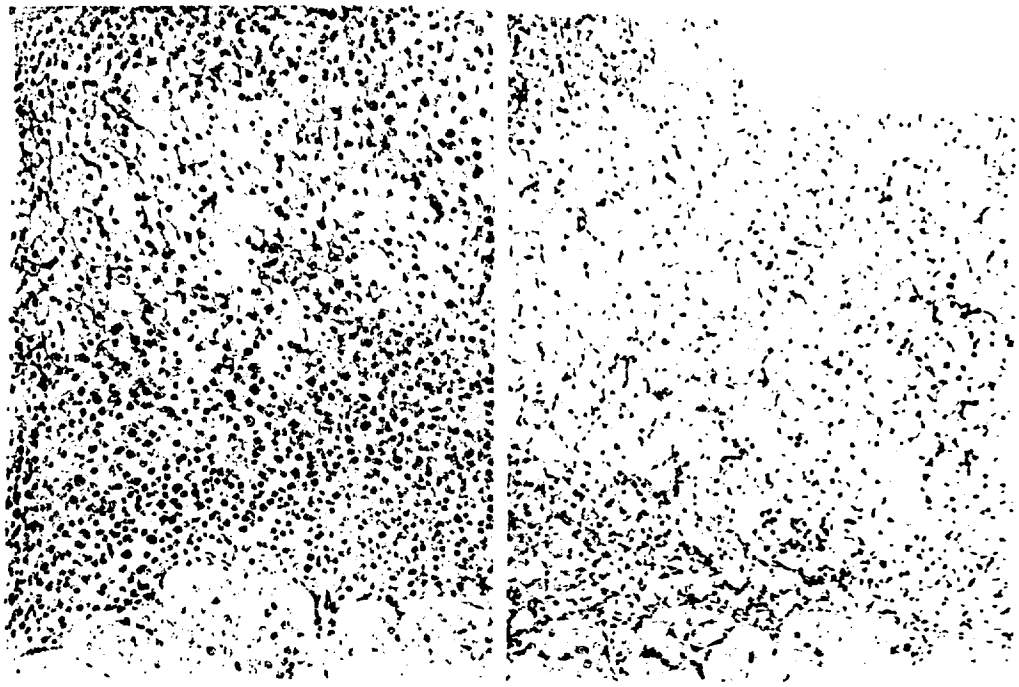
Figs. 4 a b. Spleen from gerbil fed cholesterol cholic acid diet for 4 months.
a) replacement of tissue by lipid containing macrophages. Hx-eosin × 200.
b) accumulation of birefractive cholesterol crystals. In centre, a Malpighian corpuscle with only scattered crystals. Frozen section. Polarized light × 50.

was a marked increase in stainable lipids and cholesterol crystals in all layers of the adrenal cortex of the animals given the supplemented diet (Figs. 5 a–b). No atherosclerotic changes were ever found during the experimental period.

Comparison of the serum cholesterol levels revealed extreme differences with a mean of 867 mg per cent in the test group as compared with one of 104 mg per cent in the controls.

Experiment 2 (Regression after 1 Month Exposure)

Also the gerbils sacrificed after one month on the cholesterol cholic acid supplemented diet showed a conspicuous enlargement of the liver which as a rule had a pale yellowish colour. The histological picture was dominated by the accumulation of intracellular lipid droplets and of birefractive cholesterol crystals in the parenchymal and Kupffer cells. No gallstones were present and the gall bladders were only moderately distended. In the gerbils which were sacrificed one, two and four months after the diet had been switched back to normal, similar changes although of a lesser degree, were observed in the liver. The fatty changes persisted chiefly in the central and midzones of the lobules (Fig. 6). The other histological changes observed after 1 month on the supplemented



a. b.

Figs. 5 a b. Adrenal cortex from gerbil. Hrx-eosin × 150.
a) (chow diet) large lipid-laden cortical cells, particularly in zona fasciculata.
b) (cholesterol cholic acid diet) increased occurrence of lipids in all cortical zones.

diet were of a similar nature but less marked than those observed after 4 months on that diet. They disappeared when the diet was switched back to normal chow. In the thyroid gland a moderate fibrosis was left behind.

After one month on the supplemented diet the mean liver weight had risen from 2.5 g to 3.9 g, a difference which was significant with the Mann-Whitney U test (14). This difference decreased when the diet was switched back to normal. However, even four months later the liver weight still differed significantly from that found in the controls.

After one month on the supplemented diet the serum cholesterol value had risen to nearly the same very high levels as those reached in Exp. 1 after four months (see Fig. 7). Switching the diet back to chow resulted in a rapid decrease of these levels and after 2 months the mean level was about the same as pre-experimentally.

The cholesterol concentration of the liver rose to roughly twice the original value after one month on the supplemented diet (see Fig. 8). Discontinuance of this diet was followed by a gradual decrease, the pre-experimental value being reached after 2 months. As for the bile acid composition, after the supplemented diet had been given during one month we found a significant rise of the cholic acid- and deoxycholic acid concentrations whereas the chenodeoxycholic acid concentration showed a significant decrease. Already one month after the diet had been switched back to chow, these changes had disappeared (see Fig. 9). Finally, no significant differences were observed for phospholipids, the mean value observed in the gerbils sacrificed after one month on the supplemented diet being somewhat higher than that in the other animals.

Fig. 6. Liver from gerbil fed cholesterol cholic acid diet for one month and then chow diet for four months. Deposition of birefractive cholesterol crystals and granules in the central and midzones of the lobuli. Frozen section. Polarized light × 35.

DISCUSSION

The mongolian gerbil is a relatively new but potentially very useful laboratory animal. After the first pairs of the species had been caught in Eastern Mongolia in the midthirties, they have been bred in Japan where deliberate efforts have been made to develop them as an experimental animal. As such they have proved to have rather unique qualities. Most important, they require very little labour and their susceptibility to infection is reported to be low (12).

Attention has been drawn to the gerbil's reaction to the administration of cholesterol in the diet (7). On such a diet they develop high serum and hepatic cholesterol levels (8), *i.e.* a response similar to that found in for instance rabbits and chickens, but in contrast to these latter animals they develop no gross atherosclerotic changes. Data have been brought forward suggesting that the formation of steroid hormones may be a more important pathway in the gerbil's cholesterol metabolism (11). Furthermore the gastro-intestinal absorption of cholesterol seems to be enhanced as compared with the rat (8). As these data seem to indicate that cholesterol metabolism in the gerbils may differ from that in other animals, we thought it of interest to study its reaction to different gallstone provoking diets. We found *i.a.* that a fat-free diet, rich in glucose, which induces gallstones in hamsters, did not have any effect in gerbils. On the other hand, as shown by the results reported here, gallstones can be induced in gerbils by feeding a cholesterol cholic acid containing "atherogenic" diet which induces gallstones in mice (15). Analysis of the bile of gerbils fed the cholesterol cholic acid diet during one month showed the mean cholesterol concentration to be about twice the pre-experimental value. The total bile acid concentration rose only slightly with this diet and so did the phospholipid concentration. As a result, the total bile acid cholesterol ratio and the phospholipid cholesterol ratio fell considerably. This is in agreement with findings in mice which

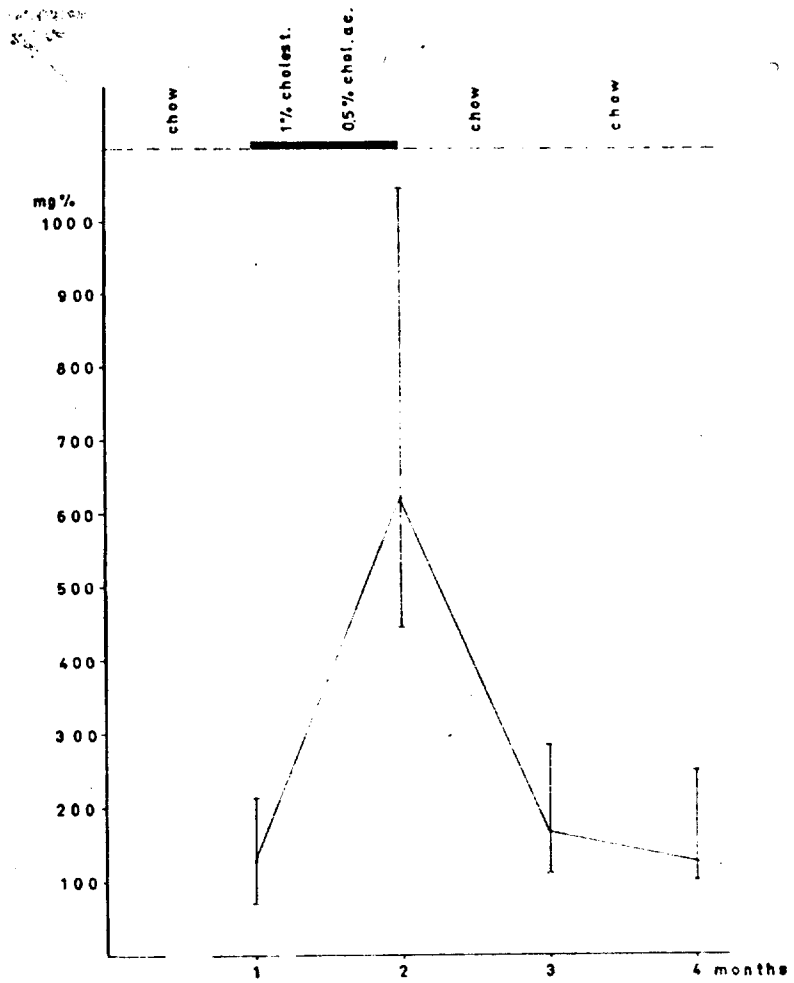


Fig. 7. Serum cholesterol level (mean and range) under different dietary conditions.

stones are formed with the same gallstone inducing diet (4). Similar findings in hamsters when these animals were given a gallstone inducing diet of a totally different composition have been reported (11). The morphological changes in gerbils fed the cholesterol cholic acid containing diet were dominated by steatosis of the liver and accumulation of lipids in the reticuloendothelial system. The changes were even more accentuated than those in hamsters and mice fed the same diet and previously described (2). The accumulation of macrophages in the gerbil's spleen suggests that these cells participate actively in the removal of lipid and cholesterol. The reaction of the gerbil's gallbladder to the cholesterol cholic acid supplemented diet is much like that of the mouse. In both species there is a sometimes very marked distention of the gallbladder with signs of inflammation in some animals. Another point of similarity with the mouse is the reaction of the thyroid and adrenal glands. In both species we found with the atherogenic diet a marked accumulation of stainable lipids in the adrenal cortex and hypoplasia in the thyroid gland. In

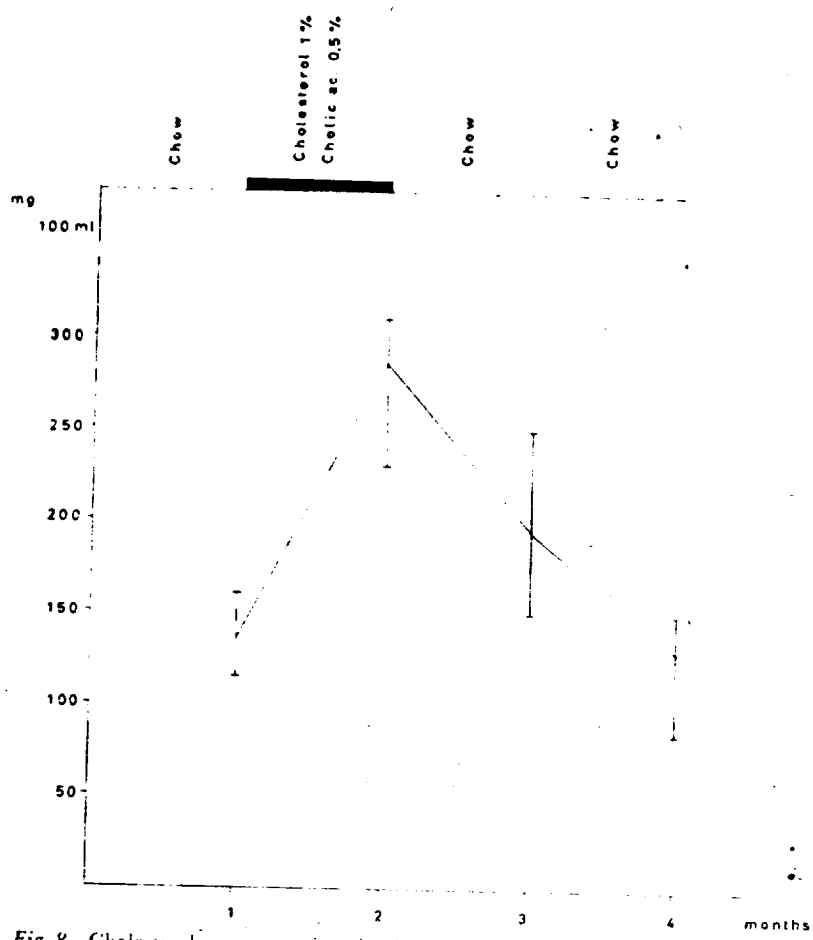


Fig. 8. Cholesterol concentration in bile (mean and range) under different dietary conditions.

the gerbil considerable hypoplasia was also present in control animals but the hypoplastic changes seemed to be more accentuated in the animals fed the supplemented diet. In this latter respect both species react as the rabbit and guinea pig and unlike the hamster and the rat on an atherogenic diet in which cases hyperplasia is found to develop. It has been pointed out that atherosclerotic changes occur only in species in which thyroid hypoplasia develops on an atherogenic diet (3). The gerbil does, however, not follow this pattern. It reacts with thyroid hypoplasia as found here, but is known not to develop atherosclerotic changes. This makes a connexion between these two phenomena less likely.

The serum cholesterol values attained extremely high levels in the gerbil already after one month on the cholesterol cholic acid supplemented diet. The increase observed in the gerbil is much more marked than in mice and hamsters fed with the same diet.

The bile acid pattern of the gerbil was found to be much similar to that of the mouse (6). In the gerbil bile acid consists mainly of cholic acid with only small amounts of chenodeoxycholic- and deoxycholic acids. Just as in the mouse and the hamster, feeding the cholesterol cholic acid diet resulted in a

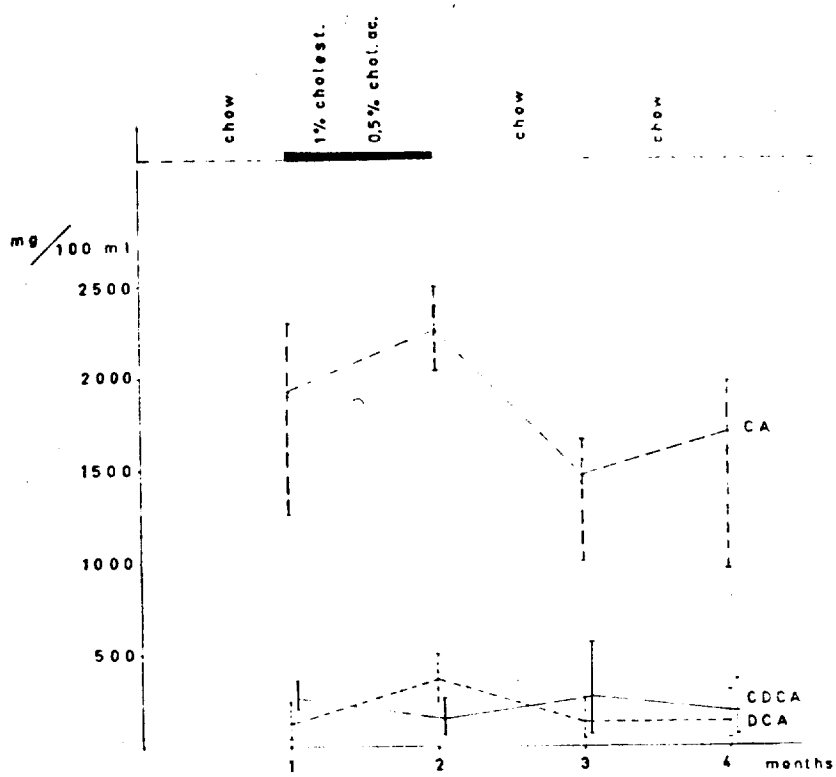


Fig. 9. Concentrations of principal bile acids (means and ranges) under different dietary conditions.

crease of the chenodeoxycholic acid concentration (2). The probable reason is a iminished synthesis of bile acids due to the ntinuous supply of cholic acid via the diet. he deoxycholic acid concentration showed small rise with the diet. In the hamster we und a striking rise while this concentration as unchanged in the mouse. These differnces may reflect specie-differences in the bility to rehydroxylate deoxycholic acid. Just s in the mouse, and in contrast to the hamer, already one month after discontinuance f the diet, the bile acid pattern had returned o normal.

In one respect, however, there is a striking ifference between the gerbil and the mouse. When the diet was discontinued, liver histoogy returned rapidly to normal in the latter pecies (1) whereas it diminished only gradually in the gerbil and was not normal even after 4 months. Although the cholesterol concentration in serum and bile had returned to normal already after 2 months, the histological examination of the liver showed that the gerbil has great difficulty in freeing itself from the excess accumulation of cholesterol. In this respect the gerbil is comparable to the hamster. In this latter species we found in a similar experiment liver histology to be largely unchanged two months after discontinuance of the cholesterol cholic acid containing diet (2).

Apart from this marked difference between the gerbil and the mouse in their ability to rid themselves of the accumulation of excess cholesterol, their reaction to the cholesterol cholic acid containing diet was much the same. It seems likely therefore that gallstone formation observed in the gerbil fed this diet, occurs in the same way as in the

mouse. However, because of its poor tolerance to the diet when it is given during a long period of time, the gerbil seems a less suitable model for study.

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DEVELOPMENT AND REGRESSION OF MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN HAMSTERS AND MICE FED A CHOLESTEROL CHOLIC ACID CONTAINING DIET

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Hamsters and mice were fed during one month with a cholesterol cholic acid containing diet which induces gallstones in mice. In animals on this diet we found, in both species, steatosis of the liver and high serum cholesterol levels. The thyroid gland became hyperplastic in the hamster, but hypoplastic in the mouse. The anterior pituitary gland of the hamster showed an increase of so-called thyroidectomy cells, not found in the mouse. In the adrenal cortex of both species there was an increase in stainable lipids. Discontinuance of the diet resulted in a rapid return to normal of liver morphology, liver weight and serum cholesterol in the mouse, while the hamster appeared to be unable to free itself from the accumulation of cholesterol. In both species the endocrine glandular changes disappeared. As this was also the case with the hyperplastic changes in the hamster's thyroid gland while its liver remained steatotic, the theory that the thyroid hyperplasia is due to stimulation by the increased metabolic needs of he liver, seems less likely. In both species, the supplemented diet resulted in a decrease of the chenodeoxycholic acid concentration of the bile. This is held to be due to a diminished synthesis of this bile acid as a result of the continuous supply of cholic acid to the liver. The biliary deoxycholic acid concentration rose strinkingly in hamsters, but not in mice, probably because the hamster does not possess the ability of the mouse to reconvert deoxycholic to cholic acid. A fall of the dihydroxy/trihydroxy bile acids ratio was noticed in the mouse, in which species gallstones are eventually formed, whereas this ratio rose in the hamster in which they are not. The apparent disagreement between this finding and earlier results in hamsters in which dissolution of gallstones was accompanied by a drastic shift towards cholic acid, was explained when we gave the diet for a longer period and found that the biliary acids/cholesterol ratio fell to much lower values in the mouse than in the hamster. When the diet was discontinued there was an unexplained rise of the chenodeoxycholic acid concentration in the hamster but not in the mouse.

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Among the most intriguing aspects of experimental gallstone formation are the differences which have been found to exist between the species. One animal may react to a certain

while in another there is no effect at all. Such a difference exists for instance, between the hamster and the mouse. A cholesterol cholic acid containing a so-called atherogenic diet induces cholesterol gallstones in the mouse but not in the hamster, a species which otherwise is highly susceptible to the influence of gallstone inducing diets. The present experiments aimed at elucidating this difference about which comparatively little is known. In earlier studies on the dissolution of gallstones we had gained the impression that the two species differ not only in their reaction to the diet itself, but also to its discontinuance. We therefore combined these studies on the development of morphological and biochemical changes under the influence of the cholesterol cholic acid diet with studies concerning their regression when the diet is discontinued.

METHODS AND MATERIAL

Experiment 1a. Young mice of both sexes were used in this experiment, their mean weight at the start being 12 g. They were fed a vitaminized commercial chow chiefly consisting of protein, fat, cellulose and water[1] during one month with free access to water. After this period 25 animals were chosen in a strictly random fashion and sacrificed. At autopsy the gallbladder was removed and the bile was collected for analysis. As the bile samples were, as a rule, rather small, pooling was necessary. On the average 4–6 animals contributed to each analysed sample. 0.050 ml of the bile was extracted and hydrolysed according to the method described by *Wollenweber et al.* (1966). The only modification being that DC Fertigplatten, Kieselgel F 254[2] were used, the free bile acids were separated by thin layer chromatography according to the same method together with cholic acid, chenodeoxycholic acid and deoxycholic acid standards. After spraying with 15 per cent phosphomolybdic acid in ethanol, the plates were heated to 110° C for 10 minutes and the spots were quantified by means of a Vitatron Densitometer, U.F.D. equipped with a 578 mμ filter and compared with standard curves. The Rf values of the bile acids were: cholic acid 0.17; chenodeoxycholic acid 0.42 and deoxycholic acid 0.47. For a discussion of the precision and accuracy of this method the reader is referred to *Junl & van der Linden* (in press).

[1] Harald Fors, Holmsund, Sweden.
[2] Merck, Darmstadt, Western Germany.
[3] Vitatron, Dieren, Holland.

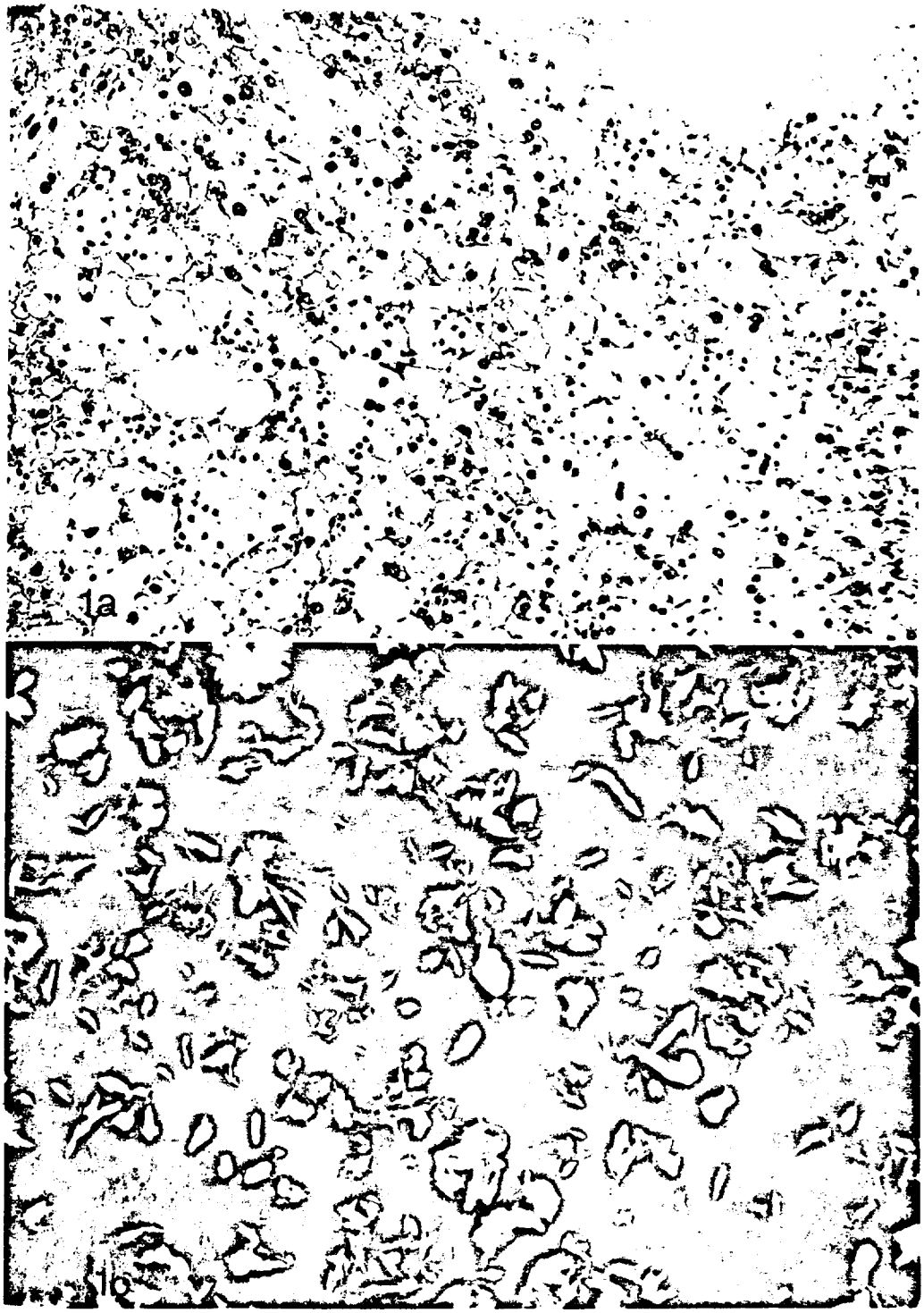
according to a modified Liebermann-Burchard reaction (*Huang et al.* 1961).

The liver was weighed and together with the gallbladder, lungs, heart, spleen kidneys, adrenals, thyroid and pituitary glands fixed in 10 per cent formalin solution. Paraffin-embedded sections were prepared and stained with haematoxylin-eosin and van Gieson's stain. In addition sections of the thyroid gland were stained with periodic acid Sinff (PAS) and sections of the pituitary gland with trichrome-periodic acid Sinff according to *Pearse* (1960). Frozen sections of the liver, lungs, heart, spleen, kidneys and adrenals were stained with Scharlach-Rot and Sudan Black B and also analysed with the polarizing microscope. The histological examinations were performed after the whole experiment had been completed, the assessor being unaware of the group to which the animal belonged. For the purpose of electron microscopy the pituitary glands from five animals in each series were fixed in glutaraldehyde followed by post-fixation in osmium tetroxide solution. The tissue was embedded in Epon. Sections were cut with glassknives on a LKB Ultrotome microtome and double stained with uranyl acetate and lead citrate (*Reynolds* 1963). The sections were examined in a Zeiss EM 9 microscope. In view of the shortness of the experimental period no special search for atherosclerotic changes was made. Blood samples were taken and serum cholesterol levels determined according to the modified Liebermann-Burchard reaction mentioned earlier.

The remaining animals were fed the same diet as before, but supplemented with 1 per cent cholesterol and 0.5 per cent cholic acid. After one month on this diet 25 animals were again chosen at random, sacrificed and examined as described above. For the rest of the animals the diet was switched back to normal chow. After three weeks on normal chow, 25 animals, chosen at random were sacrificed and examined and the same procedure was repeated 5 weeks later.

Experiment 1b. Young hamsters of both sexes with a mean weight of 60 g were used. Just as the mice and under similar experimental conditions, the hamsters were first fed the normal chow diet during one month, after which 15 animals were randomly chosen, sacrificed and examined as described above. The remaining animals were fed chow supplemented with 1 per cent cholesterol and 0.5 per cent cholic acid during one month, after which

Figs. 1a, b. Liver from hamster fed cholesterol cholic acid diet. a. Marked steatosis. Hts-eosin × 140. b. Large aggregates of birefractive needle-like cholesterol crystals. Frozen section. Polarized light × 400.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... mice with sex ... parable to those of the animals of experiment 1a, were used. They were divided strictly at random into two groups. One group of mice, the test group, was fed a diet consisting of normal chow to which 1 per cent cholesterol and 0.5 per cent cholic acid were added, while the others were fed normal chow and served as controls. After 3 months on this diet they were sacrificed. Bile was taken for analysis and investigated as described under experiment 1.

Sixty (60) young hamsters, with sex and age conditions as those of experiment 1b were also divided into two groups and treated in exactly the same way as the mice.

RESULTS

Experiment 1. Both the hamsters and the mice, sacrificed after 1 month on the cholesterol cholic acid supplemented diet showed a conspicuous enlargement of the liver which, as a rule, had the pale yellowish colour typical of extreme steatosis. The histological picture was dominated by a massive accumulation of intracellular lipid droplets and birefractive cholesterol crystal in the parenchymal and Kupfer cells (Figs. 1 a, b). As shown in previous experiments in mice (*Bergman & van der Linden*, 1968), the liver histology in this species returned rapidly to normal when the diet was changed to chow. In the hamster, however, the histological picture remained largely unchanged even after 2 months on normal chow.

These changes were reflected by the alterations of the liver weight/body weight ratio (Fig. 2). In the hamsters, the mean value of this ratio rose from 0.044 before to 0.078 after 1 month on the supplemented diet. Discontinuation of this diet resulted in a gradual fall, and 2 months later the ratio was 0.054. When subjected to the Mann-Whitney U test this value was, however, still significantly higher than the initial one ($P < 0.002$).

In the mice there was also a clear rise in the liver weight/body weight ratio with the supplemented diet (Fig. 2). The mean value

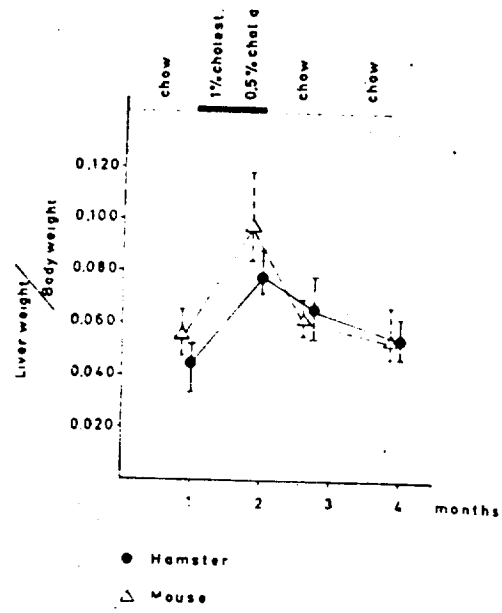

Fig. 2. Liver weight/body weight ratio (means and ranges) in hamsters and mice under different dietary conditions.

rose from 0.056 to 0.099. When the diet was changed to normal the ratio fell rapidly and three weeks later the mean value was 0.061. The difference between this value and the initial ratio was not statistically significant. No further significant decrease was observed after 2 months.

After one month on the supplemented diet the gallbladders were, as a rule, distended with atrophy of the mucous membrane in

Figs. 3a, b. Thyroid gland from hamster. Periodic acid Schiff × 500. a. (Chow diet). Normal picture with spherical follicles lined with cuboidal epithelium and containing well stained colloid. b. (Cholesterol cholic acid diet). Hyperplastic picture with irregular follicles lined with columnar epithelium and containing little or no colloid.

Figs. 4a, b. Thyroid gland from mouse. Periodic acid Schiff × 500. a. (Chow diet). Normal picture. b. (Cholesterol cholic acid diet). Hypoplastic picture with distended follicles lined with flattened epithelium and containing a large amount of well stained colloid.

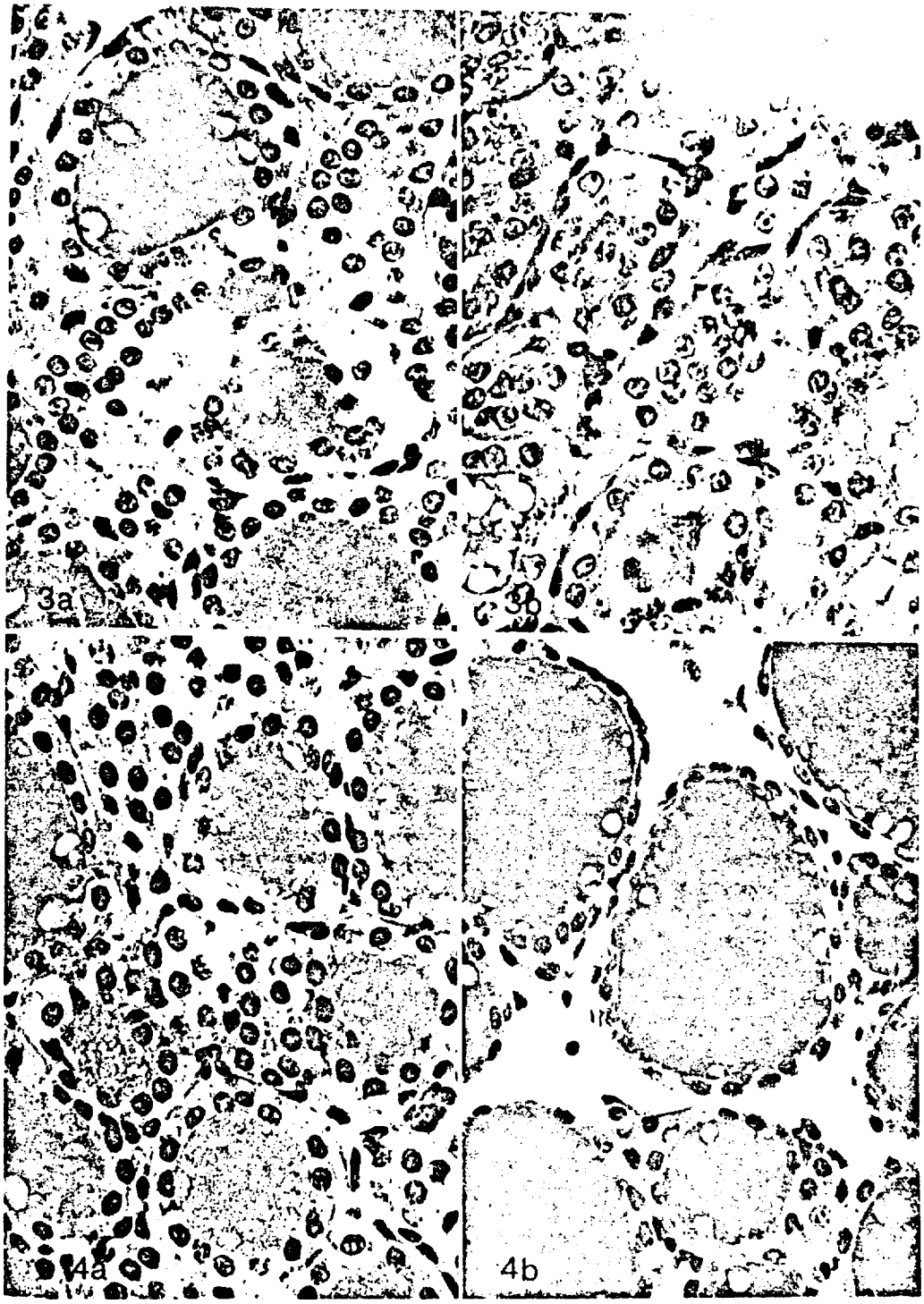
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Fig. 5. Survey picture of anterior pituitary gland from hamster fed cholesterol cholic acid diet. 10 Thyrotropic cell. × 6000.

both species. This distention remained unchanged in hamsters, whereas in mice it disappeared leaving behind a moderate fibrosis with scattered inflammatory cells. No gallstones were found.

Differences between the species also emerged when the endocrine glands were examined after one month on the supplemented diet. In the hamsters the thyroid gland showed hyperplastic changes with tightly packed irregular follicles lined with columnar epithelium and with a reduced amount of colloid in lumen (Figs. 3 a, b). In the mice, on the other hand, the thyroid gland had a resting appearance with dilated follicles filled with strongly PAS positive colloid and lined with flattened epithelium (Figs. 4 a, b). The anterior pituitary gland in the hamsters showed a clear increase in the number of large aldehyde-fuchsin-positive basophils which electron microscopy revealed to be so-called thyroidectomy cells (*Farquhar* & *Rinehart* 1954) (Figs. 5, 6). In the mice the histological picture was entirely normal. Regarding the adrenal glands an increase of birefractive cholesterol crystals was found in the cortex of both species. There was also an increase in stainable lipids which, in the hamsters, were found only in the inner zona reticularis, but in the mice in all layers of the adrenal cortex (Figs. 7 a, b, 8 a, b).

All these histological changes in the endocrine glands were transitory and disappeared

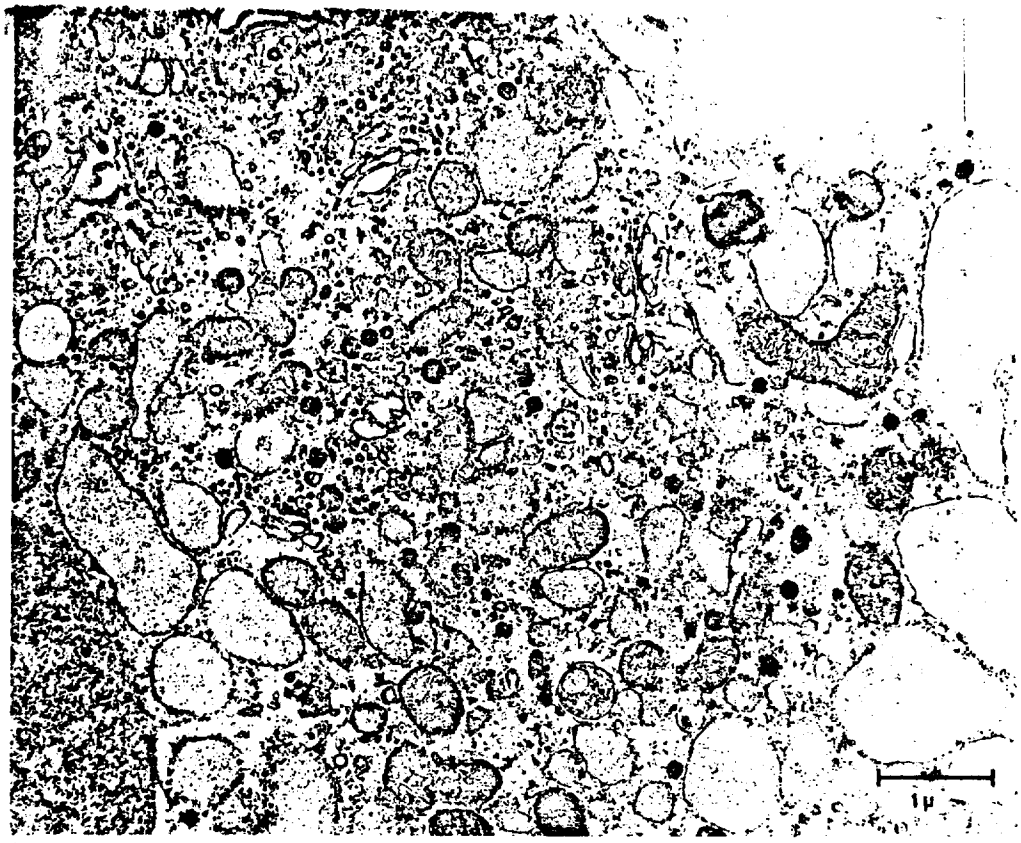


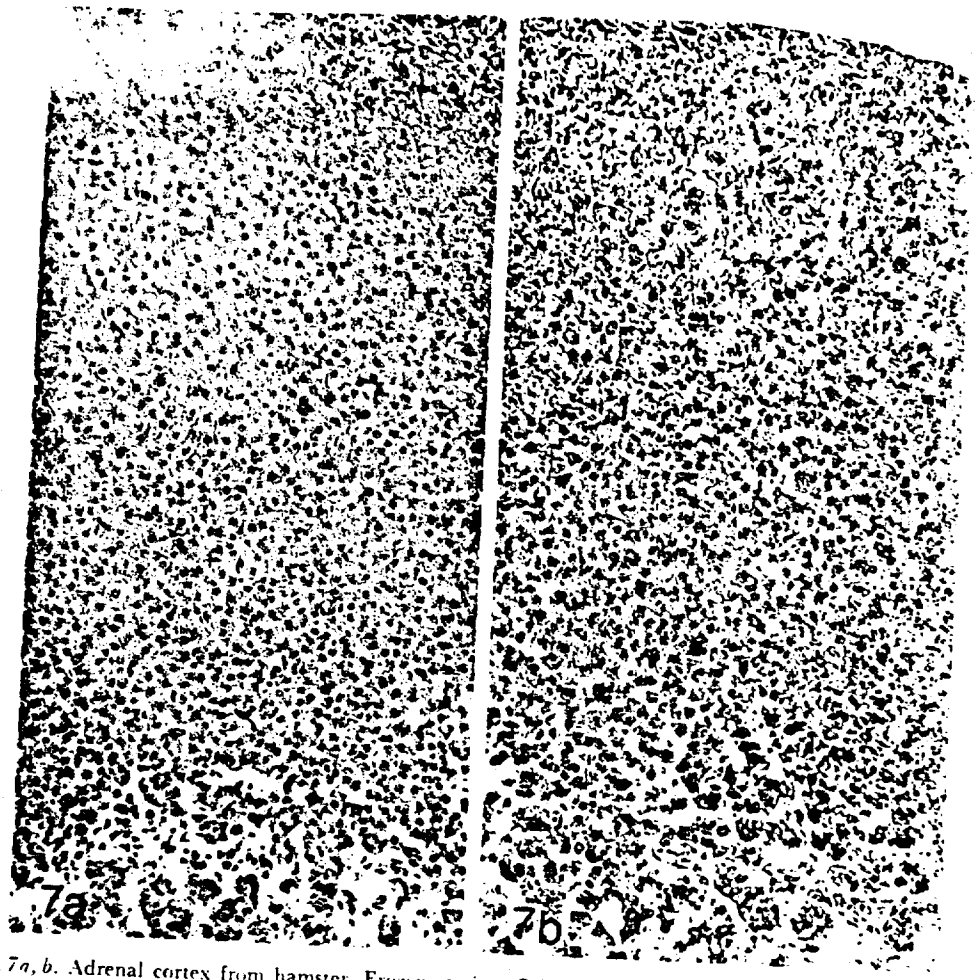
Fig. 6. Detail from the same section as in Fig. 5. Thyrotropic cell showing well developed endoplasmic reticulum with cisternae and few secretory granules. × 19000.

when the diet was changed to chow, their return to normal being, however, more rapid in the mice than in the hamsters.

The histological examination of the other organs revealed no systematic changes.

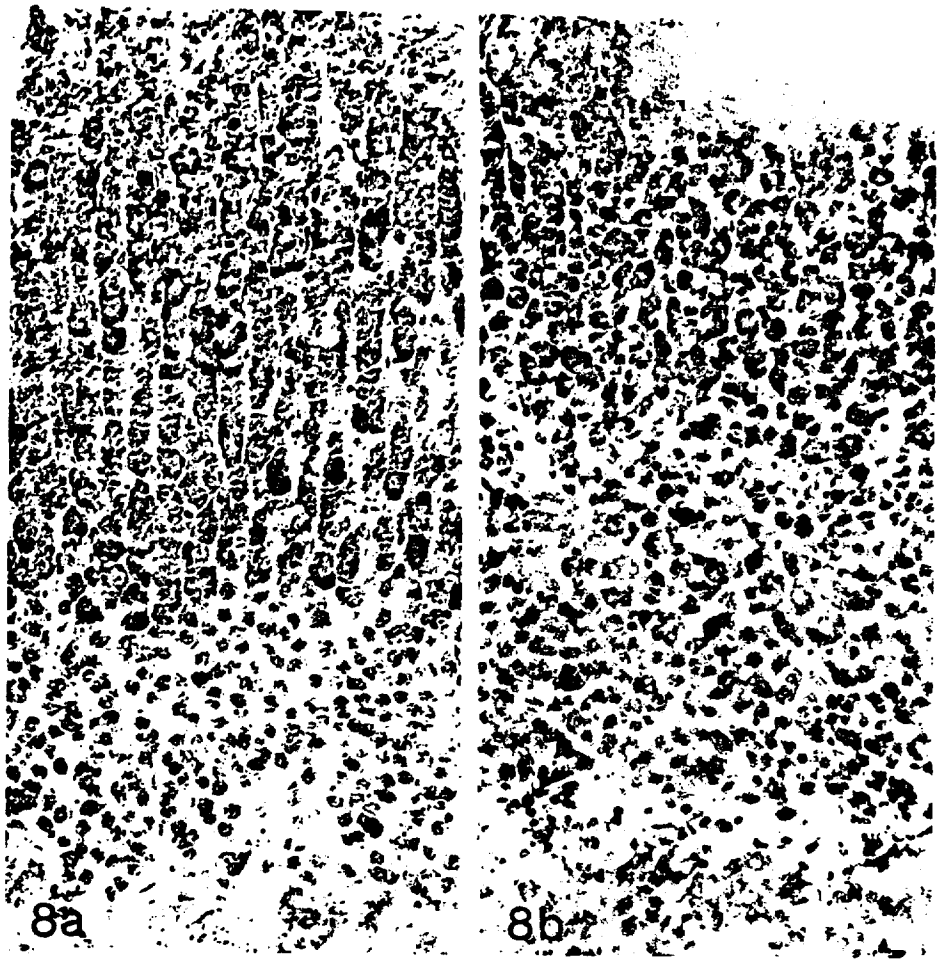
In the hamsters the serum cholesterol levels showed a striking rise after the animals had been fed the cholesterol cholic acid diet (Fig. 9). The mean level rose from 63.9 mg per cent to more than 3 times this value (209.7 mg per cent). But, already 3 weeks after the diet had been changed back to normal chow these levels had fallen significantly again, the mean value then being 159.6 mg per cent. The values after 2 months were still lower. Yet, it is worthy of note that two months after the supplemented diet was discontinued, the serum cholesterol levels were still significantly higher than in the initial samples.

In the mice the rise of the serum cholesterol levels on the supplemented diet, although significant, was far less conspicuous (Fig. 9). The mean level rose from 112.8 to 163.4 mg per cent. After the supplemented diet was changed to normal chow, the levels fell gradually and 2 months later initial values were reached. The analysis of the gallbladder bile revealed a number of significant changes in the bile acid composition. In the hamster (see Fig. 10) the mean value for chenodeoxycholic acid following the supplemented diet was about one third of that before (79 mg 100 ml as compared with 236 mg 100 ml. When subjected to the Mann-Whitney U test (*Sie-*



Figs. 7a, b. Adrenal cortex from hamster. Frozen section. Scharlach Rot × 115. a. Chow diet. Normal picture with hardly any stainable lipid. b. Cholesterol cholic acid diet. Increased occurrence of stainable lipid in the inner zona reticularis.

gel 1956) this difference was highly significant ($U = 0$; $N_1 = N_2 = 5$; $P < 0.01$). The values found for deoxycholic acid, on the other hand, were much higher after the diet than before, with a mean value rising from 260 to 830 mg/100 ml. This difference was also highly significant with the method mentioned above. No significant difference between the cholic acid values was found. The deoxycholic acid values returned rather quickly to normal when chow was given. After three weeks the mean value was already significantly lower (Mann-Whitney $U = 1$, $N_1 = N_2 = 5$, $P = 0.016$), although still clearly higher than before the supplemented diet was given. After 2 months on normal chow the mean value did no longer differ significantly from that of the initial samples. On the other hand, the chenodeoxycholic acid values increased rapidly after the diet was switched back to normal chow. After 3 weeks the mean value was 362 mg/100 ml as compared with 236 mg/100 ml in the first samples. This difference was also significant



Figs. 8a, b. Adrenal cortex from mouse. Frozen section. Scharlach Rot × 260. a. (Chow diet) Stainable lipid particularly in zona fasciculata. b. (Cholesterol cholic acid diet). Increased occurrence of stainable lipids in all cortical zones.

with the Mann-Whitney U test (P = 0.016). After 2 months the values were even higher with a mean of 525 mg/100 ml.

In the mouse (Fig. 11) the gallbladder bile concentrations of cholic and of deoxycholic acid were relatively constant throughout the experiment. The mean value of cholic acid was somewhat lower at the end than at the start of the experiment, but as few analyses were made the level of significance was not reached. As for chenodeoxycholic acid, also in the mouse there was a significant decrease of this bile acid after the supplemented diet had been given. The mean value fell from 193 mg/100 ml to 37 mg/100 ml. Three weeks after the diet had been switched back to chow, the values were again about as high as the initial ones. In contrast to the hamster no further rise of the chenodeoxycholic acid values was observed, the values after 2 months not differing significantly from those after 3 weeks.

As in an earlier study (*Bergman et al.* 1968) the values for the cholesterol concen-

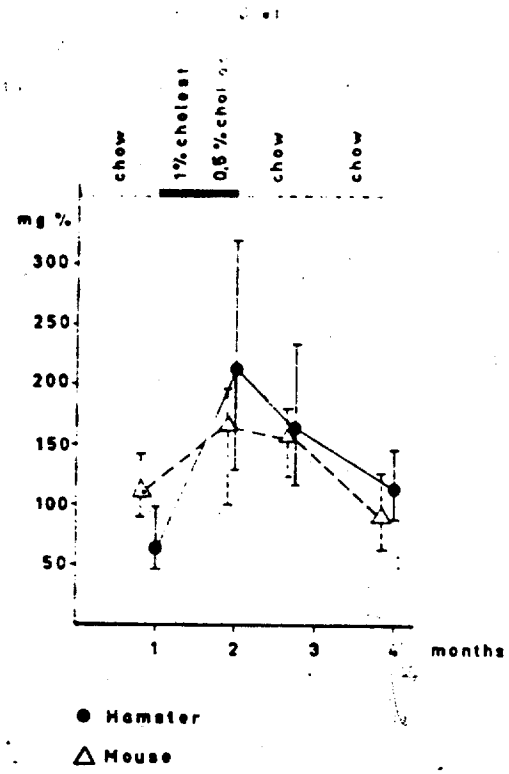


Fig. 9. Serum cholesterol level (means and ranges) in hamsters and mice under different dietary conditions.

tration of the gallbladder bile showed large individual variations. This is probably the reason why we failed to find significant differences. In both the mouse and the hamster a tendency towards higher values on the supplemented diet was noted. After the diet was changed to normal again a decrease was observed which seemed to occur more rapidly in the mice than in the hamsters.

Experiment 2. The results of the gallbladder bile analyses showed biliary bile acid concentrations comparable to those found in experiment 1 in animals fed the supplemented diet during 1 month. In this experiment significant group differences in the cholesterol concentration of the bile were found. In the hamster the mean concentration rose significantly from a mean value of 45.7 mg/100 ml up to 84.1 mg/100 ml after supplementation with cholic acid. In the mouse the mean concentration rose even higher, judged on the basis of similar control values. In this latter animal the mean concentration after 3 months was 150.3 mg/100 ml i.e. nearly twice as high as in the hamster. The difference between these two species was statistically highly significant ($P \leq 0.001$). Consequently the bile acid cholesterol ratios were 2–3 times as high in the hamster as in the mouse. No significant sex differences were found either in experiment 1 or in experiment 2.

DISCUSSION

A large variety of animals has been used in studies of the effect of atherogenic diets containing cholesterol alone, or in combination with bile acids. While all the investigated species seem to react to these diets with steatosis of the liver, although its degree may vary, the morphological reaction of other organs and especially of the endocrine glands show large differences. For instance, on a

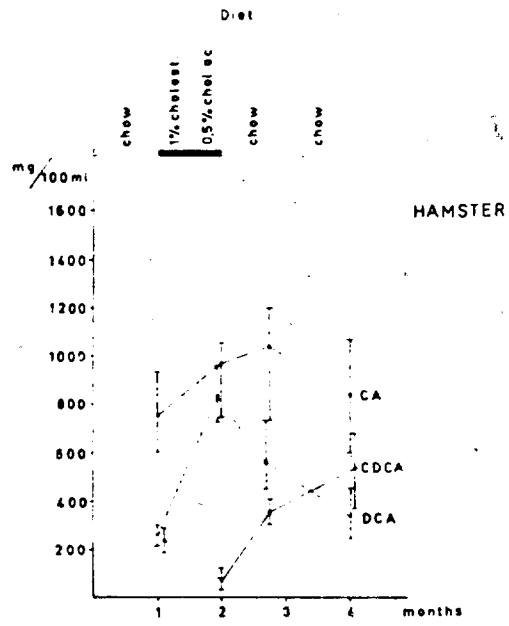


Fig. 10. Bile acid concentrations (means and ranges) in gallbladder bile of hamsters under different dietary conditions. CA = Cholic acid, CDCA = Chenodeoxycholic acid, DCA = Deoxycholic acid.

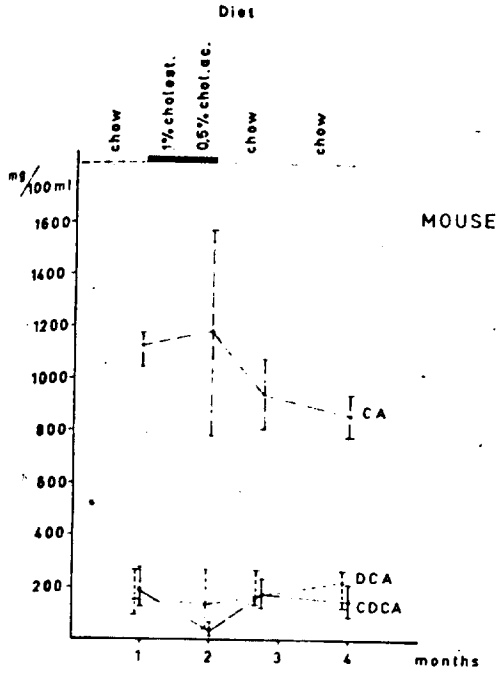


Fig. 11. Bile acid concentrations (means and ranges) in gallbladder bile of mice under different dietary conditions. CA = Cholic acid, CDCA = Chenodeoxycholic acid, DCA = Deoxycholic acid.

cholesterol diet the thyroid gland becomes hypoplastic in appearance in rabbits and guinea pigs, whereas it becomes hyperplastic in hamsters and rats (*Bernick et al.* 1962).

As expected we found in the hamster as well as in the mouse steatosis of the liver and a rise of the serum cholesterol levels when they were fed a cholesterol cholic acid containing diet. When the diet was changed back to normal a significant difference between the species appeared. In the mouse, liver morphology, liver weight and serum cholesterol level returned rapidly to normal. The hamster, on the other hand, appeared to be unable to free itself from the accumulation of cholesterol.

The morphological changes in the endocrine glands especially those in the thyroid gland followed a different pattern in the two species studied. This latter gland became hyperplastic in the hamster and hypoplastic in the mouse. In this respect the hamster is comparable to the r[illegible] rabbit and guinea pig. Differences of this kind have been held responsible for specie-differences in the susceptibility to atherosclerosis. Probably connected to these differences in thyroid gland morphology are the changes found in the anterior pituitary gland. In the hamster, but not in the mouse, there was an increase in the number of basophils as previously described by *Bernick* & *Patek* (1961). These cells could now be identified at the ultrastructural level as so-called thyroidectomy cells which are generally assumed to be related to an increased production of T. S. H. The reaction of the adrenal cortex showed more similarity. In both species there was an increase in stainable lipids.

All these morphological changes in the endocrine glands had returned to normal in both species after two months. *Bernick* & *Patek* (1961) hold that the hyperplasia of the thyroid gland as seen in the hamster and the rat is due to a stimulation caused by the increased metabolic needs of the steatotic liver. This seems now unlikely: two months after the diet had been changed back to chow, the thyroid gland was normal while the liver was still steatotic.

Significant changes in the bile acid composition of the bile were found. In both species, feeding of the supplemented diet resulted in a fall of the chenodeoxycholic acid concentration. The most likely explanation is a decrease in the synthesis of this bile acid, due to the continuous supply of large amounts of cholic acid via the portal blood to the liver. *Bergström* & *Danielsson* (1958) have demonstrated that, in a bile fistula rat in which from the second day onward there is a 10–20 fold increase in the synthesis of bile acids (*Eriksson* 1957), the injection of taurochenodeoxycholate into the intestine results in an appreciable decrease in the formation of cholic acid. It seems likely that a corresponding decrease in the formation of chenodeoxycholic acid occurs when cholic acid is given with the diet.

As f[illegible]

a stressing difference between the hamster and the mouse was found. In the hamster there was a conspicuous increase in the concentration of this secondary bile acid while in the mouse the concentration was unaltered. The most likely explanation is that the hamster does not possess the ability of the mouse (*Danielsson & Kazano*, 1959) to reconvert deoxycholic to cholic acid in the liver at least not under the conditions of this experiment. When the cholesterol cholic acid supplemented diet was discontinued, another difference between the two species emerged. In the mouse the chenodeoxycholic acid concentration returned to normal after 3 weeks. In the hamster, on the other hand, the concentration rose to values, much higher than those attained before the supplemented diet was given. The reason for this rebound phenomenon in the hamster is unclear. It is possible that it is connected in some way with this species' inability to free itself rapidly from an accumulation of cholesterol.

It appears from the results of experiment 1 that, in the hamster, the cholesterol cholic acid diet results in a clear rise of the dihydroxy trihydroxy bile acid ratio of the bile. The decrease of chenodeoxycholic acid is more than compensated by the increase in deoxycholic acid. In the mouse, on the other hand, this ratio, already low, falls to still lower values. Although in model synthetic solutions, glyco- and tauro-deoxycholate have been reported to solubilize significantly more cholesterol than other bile salts (*Niederhiser & Roth* 1968), it is held by others that gallstone formation is associated with a shift towards dihydroxy bile acids (*Burnett* 1965). They reason that the more polar dihydroxy bile acids tend to form larger micelles with less surface charge (*Bouchier & Freston* 1968). *Haslewood* (1967) has drawn attention to the differences in bile acid composition in mammals on different diets. Carnivores, living on cholesterol rich food, have mainly cholic acid in contrast to herbivores. Man's liability to gallstone formation may thus be connected with the fact that man eats partly like a carnivore although he is equipped with a herbivore's bile acids *i.e.* a good proportion of dihydroxy bile acids (*Haslewood* 1967). Experimental evidence supporting these theoretical speculations was brought forward when we found that cholesterol gallstones in hamsters can be dissolved by the addition of an anion exchange resin to an otherwise lithogenic diet (*Bergman & van der Linden* 1967) and that such a supplement to the diet brings about a drastic shift from chenodeoxycholic acid towards cholic acid (*Bergman et al.* 1968). We hesitated, however, to assume a direct causal relationship between these two phenomena, partly because a similar shift in the bile acid composition was not observed by *Prange et al.* (1962) in hamsters fed gallstone inducing and -preventing diets. Recent experience seems to indicate that at least this argument hardly holds true. The mode of action of Dam's antilithogenic diet is probably not comparable to that of the anion exchanger. When hamsters are injected with C^{14} cholic acid, the excretion of radioactivity via the faeces is as high on the gallstone inducing as on the antilithogenic diet (*Halvorsen & van der Linden*). Cholesterol gallstone formation can be prevented in hamsters in very different ways *e.g.* with the antilithogenic diet, anion exchangers, or as we recently have found with AY9944, a cholesterol biosynthesis inhibitor (*Bergman et al.*, to be publ.). As the number of antilithogenic agents increases, it seems more and more unlikely that they could have a common mode of action, or even that their effect could be linked to similar changes in the bile.

However this may be, the results of experiment 1 – a shift towards cholic acid in the mouse in which, using this diet, gallstones are formed, and a shift towards dihydroxy bile acids in the hamster in which they are not – seem to contradict the earlier mentioned theories. The results of experiment 2 show that this is not necessarily so. The analysis of a large number of bile samples taken in animals which were fed the cholesterol cholic acid diet during three months *i.e.* nearly the period after which gallstones start to appear

in mice, revealed another significant difference between the species. In mice the biliary cholesterol concentration rose to significantly higher values than in hamsters. This resulted in very different bile acid/cholesterol ratios with a mean value of 8.81 in mice as compared with 23.02 in hamsters. Although we are well aware of the fact that other bile constituents, not studied here, may also play an important role, we feel that this finding alone suffices to explain the apparent contradiction mentioned above.

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BILE ACIDS: FORMATION AND METABOLISM

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THE first unequivocal demonstration of the direct transformation of cholesterol into bile acids was done by Bloch, Berg and Rittenberg (1943). After administration of deuterated cholesterol to a dog, they found that the cholic acid contained a significant concentration of deuterium. When ^{14}C-labelled cholesterol became available these reactions were studied in greater detail in a number of laboratories.

It was then found that the cholesterol ring system was metabolically inert in the sense that none of the carbon isotope appeared in the expired carbon dioxide of the animal when the isotope was contained in ring *A*, whereas it did when the label was in the terminal *iso*propyl group. Furthermore, generally only 10–20 per cent of the isotope was slowly excreted as neutral steroidal compounds whereas the remainder was contained in acidic compounds identified as bile acids and various metabolic products.

The early work has been reviewed several times and will not be itemized here (cf. Bergström, 1955; Bergström and Borgström, 1956; Gould, 1958).

Instead we will discuss recent results on the reactions of the formation and metabolism of bile acids in man, rat, rabbit and pig and some quantitative aspects of the cholesterol-bile acid metabolism. The main bile acids present in these animals are listed in Fig. 1.

The main bile acid of the rat is cholic acid (80 per cent) and most of the remainder is chenodeoxycholic acid with minor amounts of other metabolites, all conjugated with taurine. The thick lines in Fig. 2 show the metabolic reactions that have been demonstrated by administering labelled

compounds to rats. The crossed thin lines show reactions that do not occur. It is thus clear that the dihydroxy acid, chenodeoxycholic acid, is not a precursor of cholic acid. Two other trihydroxy acids (3α : 6β : 7α- and 3α : 6β : 7β-trihydroxycholanic acid) are instead formed to a minor extent (Hsia *et al.*, 1957, 1958).

The hydroxylations on the ring system have apparently to take place before the side chain is oxidized, otherwise cholic acid is not formed, i.e. the "12α-hydroxylase" does not work when the side chain has been oxidized—3α : 7α-dihydroxycoprostane yielded both chenodeoxycholic acid and cholic

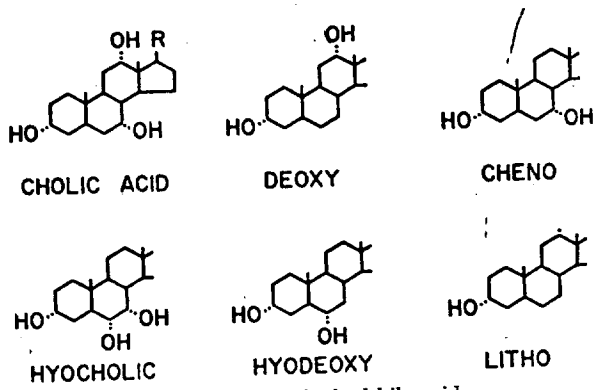

FIG. 1. Some principal bile acids.

acid, but chenodeoxycholic acid or the corresponding C_{28} acid did not yield any cholic acid. The rate-determining step might very well be the first hydroxylation at $C_{(7)}$ as all the 7α-hydroxylated compounds in the left column in Fig. 2 are transformed into bile acids much more rapidly than cholesterol itself. The 7α-hydroxylation of deoxycholic acid to cholic acid seems to be a reaction specific for the rat—it does not occur in rabbit or in man.

The stereospecific course of the introduction of the 7-hydroxyl into cholesterol has been studied with cholesterol stereospecifically labelled with tritium in position 7α or 7β (Bergström *et al.*, 1958). It has been found that the 7α-hy-

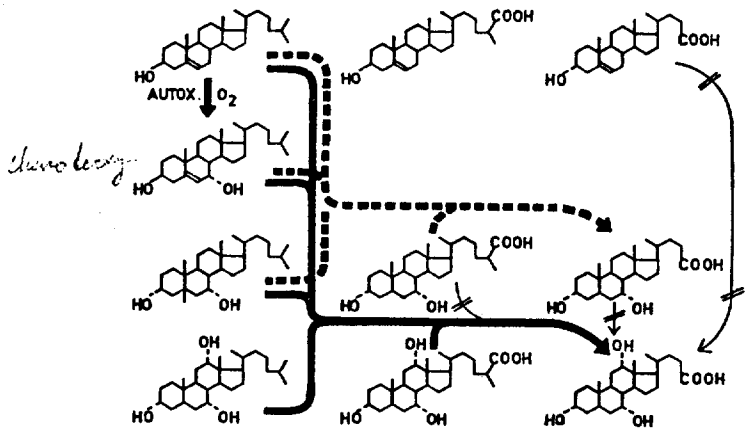

FIG. 2. Some metabolic reactions in the rat.

droxyl of cholic acid specifically replaces the 7α-hydrogen of cholesterol, leaving the 7β-hydrogen intact (Fig. 3).

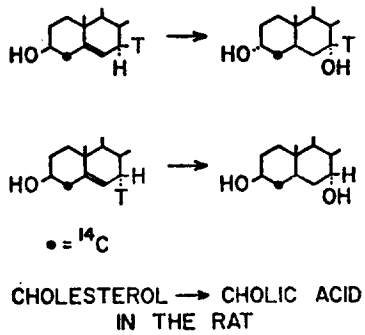

FIG. 3. Stereochemistry of 7α-hydroxylation in the conversion of cholesterol to cholic acid in the rat.

Normally taurocholate and taurochenodeoxycholate make up 90–95 per cent of bile acids in rat bile and a chromatogram gives the simple curve shown in Fig. 4. When the acids

present in the faeces of similar rats are chromatographed a very complicated picture appears (Fig. 5). Practically all conjugates have been split and a large number of metabolites appear. Norman and Sjövall (1958*a*, *b*) have recently concluded a study of the products formed and the main results as to the cholic acid metabolites are summarized in Fig. 6. One of the main reactions is the elimination of the hydroxyl

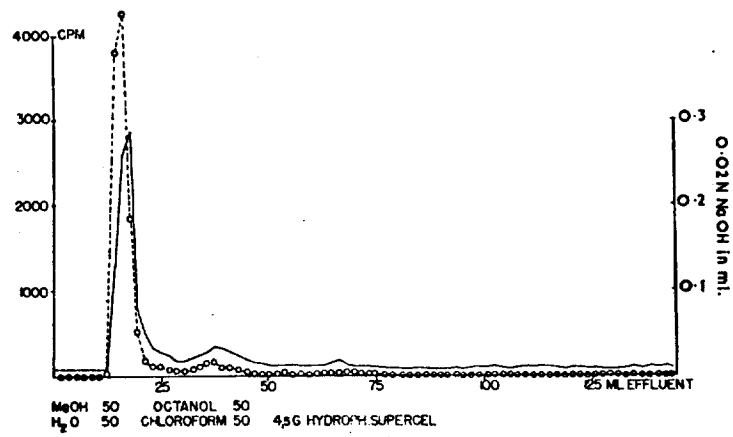

FIG. 4. Chromatogram of unhydrolysed bile acids nine days after intraperitoneal administration of 1 mg. [4-^{14}C]cholesterol to rat 121. The bile fistula was made on the eighth day after addition of labelled cholesterol.

group at position 7, yielding deoxycholic acid which is subsequently oxidized to 12-ketolithocholic acid. Another is the dehydrogenation of the 7-hydroxyl to the 7-ketone that is then partially reduced to the 7β-isomer. The two columns at the right in Fig. 6 show a rough estimate of the proportions of the different acids in two rats of the same colony, indicating the expected great variability of these reactions caused by the intestinal flora. In the rat liver the deoxycholic acid formed by the action of the intestinal flora is then rehydroxylated to cholic acid. Recent experiments with doubly-labelled cholic acid (7β-^{3}H : 24-^{14}C), in which these reactions have

been studied are discussed below (p. 191). When studying the turnover of the cholic acid pool in this animal this factor has to be taken into account.

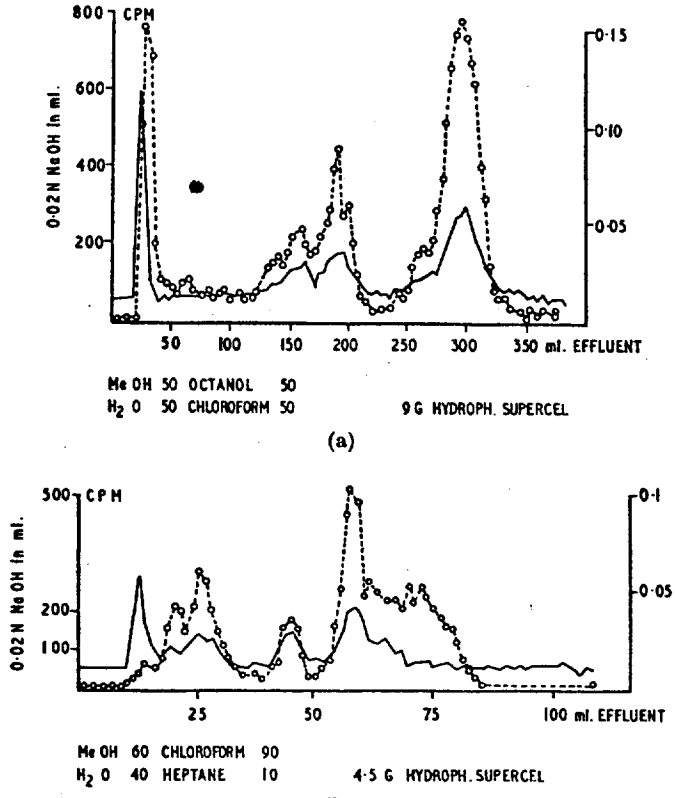

FIG. 5. Chromatograms of bile acids in faeces of rat 121 (cf. Fig. 4).
(a) Unhydrolysed faecal bile acids; seventh day after addition of labelled cholesterol.
(b) Less hydrophilic acids from unhydrolysed faecal extract from same day.

That these reactions are indeed caused by the intestinal micro-organisms has been shown both by the administration of antibiotics to normal rats (Lindstedt and Norman,

1956*b*) and in germ-free rats (Gustafsson *et al.*, 1957). In neither case do changes occur during the intestinal passage and the faeces contain the same bile salts as the bile (cf. Fig. 4).

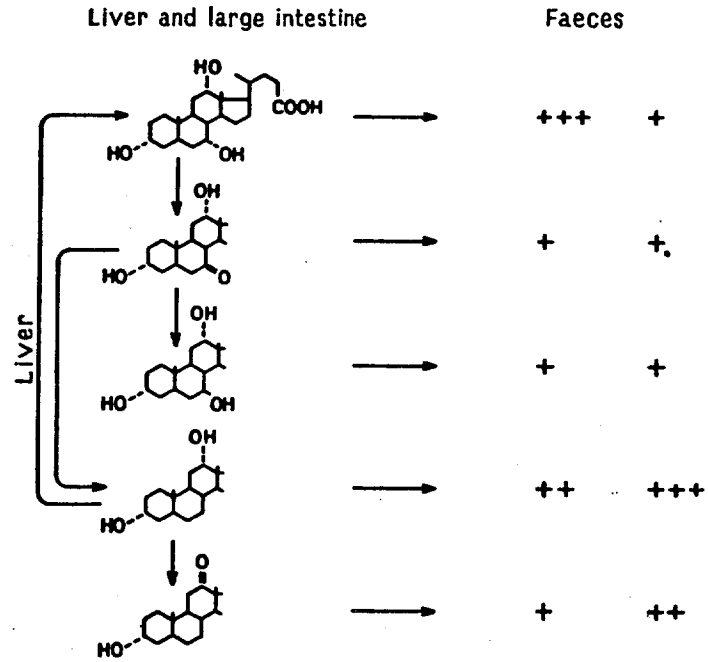

FIG. 6. Summary of results on cholic acid metabolites in the faeces of normal rats (Norman and Sjövall, 1958*a*). The two columns at the right show a rough estimate of the proportions of the different acids in two rats of the same colony.

Another striking example of the influence of the intestinal flora is found in the rabbit. Rabbit bile from the gall bladder contains almost pure deoxycholic acid conjugated with glycine. Lindstedt and Sjövall (1957) found, however, that when a bile fistula is made in this animal, the deoxycholic acid rapidly disappears and is replaced by cholic acid in the fistula bile. Likewise, if labelled cholic acid is given to an

intact rabbit, labelled deoxycholic acid is formed from it in the gut. However, it is not rehydroxylated to cholic acid in the liver, in contradistinction to what happens in the rat. Thus there seem to be intestinal micro-organisms universally present in rabbits that cause what could have been thought to be the results of a special property of the rabbit liver cells.

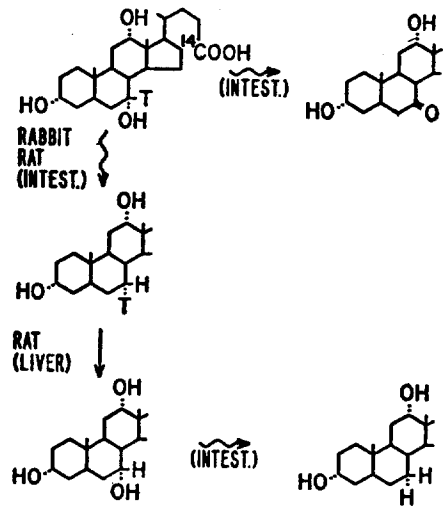

FIG. 7. Stereochemistry of the metabolism of cholic acid in the rabbit and the rat.

The mechanism of the elimination of the 7α-hydroxyl group has been studied by Bergström, Lindstedt and Samuelsson (1958) using cholic acid labelled in the 7β-position with tritium and at $C_{(24)}$ with ^{14}C (cf. Fig. 7). This acid was administered to intact rabbits in which bile fistulas were made after various intervals. It was found that a formation of deoxycholic acid occurred in which the ratio of $^{3}H/^{14}C$ was unchanged. These results thus indicate that a dehydrogenation to the 7-ketone does not occur during this transformation, but a dehydration to an unsaturated compound

followed by reduction of the double bond might have occurred. These results are also compatible with a direct elimination of the hydroxyl group.

The steric position of the tritium label appeared, however, to have been changed to the 7α-position. When this deoxycholic acid was injected into fistula rats, it gave rise to cholic acid that only retained the carbon label.

In view of recent experiments by Corey and co-workers (1958), Hayano and co-workers (1958), and the above-mentioned experiments on the transformation of 7-labelled cholesterol into cholic acid, the indications are that the label had been brought into the 7α-position during the transformation of cholic acid into deoxycholic acid by the intestinal organisms of the rabbit. In accordance with these results it was found that when the doubly-labelled cholic acid was given to rats cholic acid isolated from a subsequently-made bile fistula had lost progressively more tritium the later the fistula was made. If the cholic acid was injected directly into the caecum, however, the faeces contained deoxycholic acid with a relatively high tritium content, i.e. the intestinal micro-organisms produce deoxycholic acid without losing the 7β-hydrogen both in the rabbit and in the rat, but during the enterohepatic circulation in the latter animal this hydrogen (then in the 7α-position) is lost in the 7α-hydroxylation in the liver (Fig. 7) (Bergström, Lindstedt and Samuelsson, 1958). Norman (unpublished) has been able to reproduce these reactions *in vitro* with intestinal micro-organisms.

Similar findings have also been made in humans, in whom the normal gall bladder bile contains cholic, deoxycholic and chenodeoxycholic acids (Wootton and Wiggins, 1953). Deoxycholic acid present in the body seems, however, to be entirely of microbiological origin in the gut and it is not rehydroxylated to cholic acid in the liver (Lindstedt, 1957*a*). It is absent from bile of total fistulas that have functioned for a day or two and also from the bile of newborn babies, in which cases only cholic and chenodeoxycholic acids are present (Encrantz and Sjövall, 1957).

Thus, in the pig, a 7α-hydroxyl present in the primary bile acid is eliminated by intestinal micro-organisms, leaving the 3α : 6α-dihydroxy acid, while in the rat, the rabbit and in man, the 3α : 12α-dihydroxy acid is left. The characteristic feature of the hydroxylating system of the pig is that the third hydroxyl is introduced at 6α on the *B* ring, instead of the usual 12α-position on the *C* ring, but the common feature is the 7α-hydroxyl that might be removed secondarily.

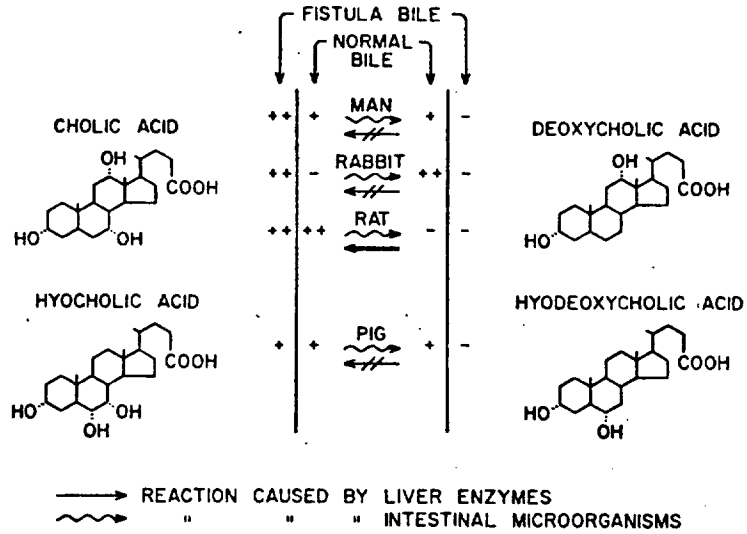

FIG. 9. Principal bile acids in fistula bile and in gall bladder bile.

A summary of the composition of the main bile acids in the cases discussed is shown in Fig. 9 (a) in fistula bile and (b) in gall bladder bile with intact enterohepatic circulation, when the intestinal micro-organism could have caused the secondary changes.

Quantitative aspects of bile acid metabolism

In the normal rat a half-life of about 2–3 days has been found for any carbon-labelled bile acid that is studied—a pool size of 20–25 mg. in a 200 g. rat corresponds to a daily

production of 5–6 mg. bile acids per day. In a bile fistula rat, however, the picture is entirely different. An example from Eriksson's work (1957*a*) is shown in Fig. 10. Most of the bile salts present in the rat have been excreted through the fistula after 12 hours (left part), but already from the second day an

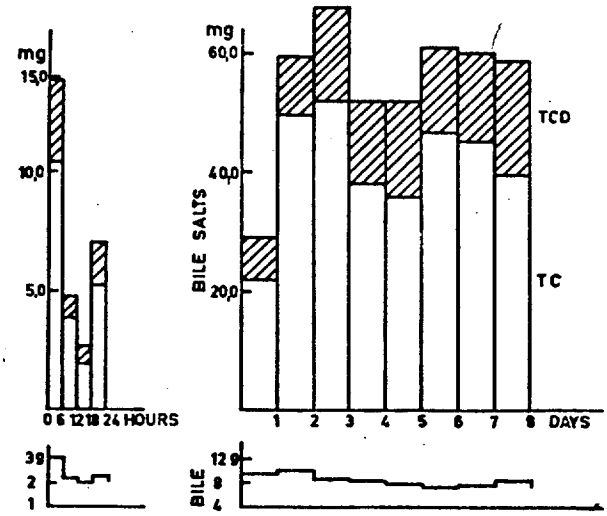

FIG. 10. Excretion of bile acids (upper diagrams) and bile (lower diagrams) in bile fistula rat (260 g.).

Values for the excretion of taurocholate (TC) and taurochenodeoxycholate (TCD) are expressed as mg./6 hr. during the first day (left upper diagram) and as mg./24 hr. during eight days (right upper diagram).

increased synthesis results in the daily formation and excretion of 40–50 mg. of bile acids per day, or ten times the normal daily synthesis. In spite of the fact that about three times the amount of cholesterol present in the liver is transformed into bile acids daily and there is a corresponding increase in the cholesterol synthesis in the liver, no change in the blood cholesterol was observed.

Under these conditions the normal enterohepatic circulation is broken and no bile acids reach the liver via the portal blood.

In recent experiments (Bergström and Danielsson, 1958*a*, *b*) we have shown that the continuous infusion of 5–10 mg. of sodium taurochenodeoxycholate per hour into the lower part of the bile duct of a fistula rat brought the cholic acid synthesis down into the normal region. These experiments are exemplified schematically in Fig. 11.

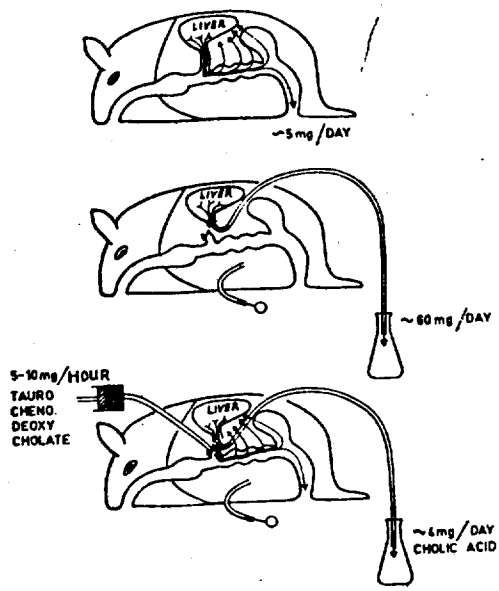

FIG. 11. Schematic results exemplifying the continuous intestinal infusion of sodium taurochenodeoxycholate when injected into the lower part of the bile duct of bile fistula rats.

During these experiments the actual concentration of bile salts in the portal blood probably only reaches 1–2 mg. per cent, but the bile acids are so efficiently absorbed that the concentration in the general circulation is probably less than 0·1 mg./100 ml. (Sjövall, unpublished).

These results are also of interest in connexion with the atherogenic diets containing free cholic acid that have been used in several studies.

Generally the addition of cholic acid used has been one or two per cent, i.e. if the rat eats 10 g. of food per day this corresponds to an average hourly intake of 5–10 mg. This addition can thus have a direct influence on the rate of bile acid formation. The free cholic acid in the diet is also conjugated and that results in a large demand on the organic sulphur compounds present in the body. A more or less severe sulphur deficiency might occur—another factor of importance for producing atherosclerosis in experimental

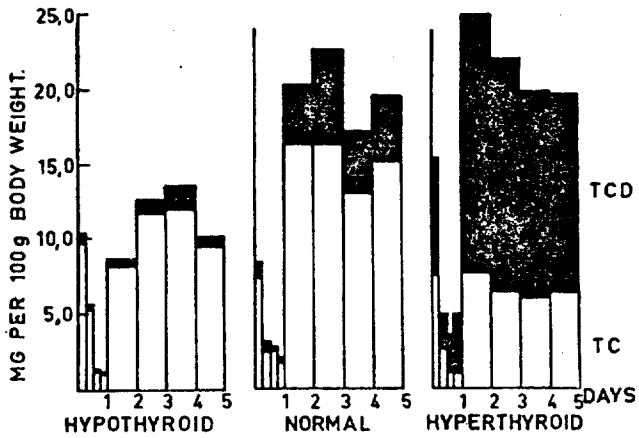

FIG. 12. Excretion of bile acids in hypothyroid, normal and hyperthyroid bile fistula rats.
TC = taurocholate. TCD = taurochenodeoxycholate.

animals. In order to study the influence of bile acids *per se* they should be added to the diet as taurine conjugates.

There are furthermore quite pronounced changes in the bile acid picture in the hyper- and hypothyroid states. Some data published by Eriksson (1957*b*) are shown in Fig. 12. In the normal rats there is the usual mixture of about 80 per cent cholic acid and 15 per cent chenodeoxycholic acid. In the hypothyroid rats there is practically only cholic acid present, whereas in the hyperthyroid state there is more chenodeoxy- than cholic acid. A possible explanation is obviously

that the side-chain degradation is speeded up in relation to the 12-hydroxylation in the latter case, whereas the reverse is true in the former case. The results illustrate anyhow the necessity of analysing for all the different bile acids present in order to get a quantitative picture—if only the cholic acid

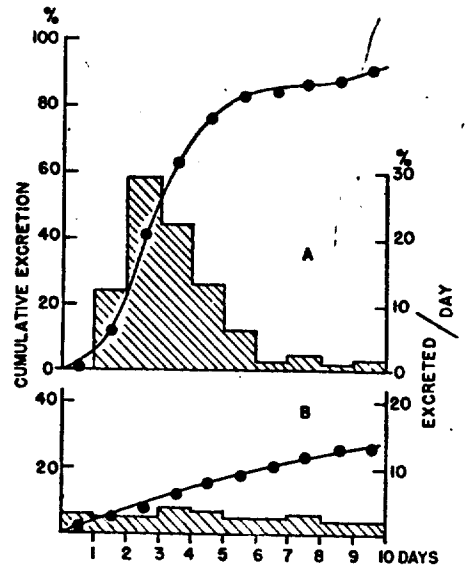

FIG. 13. Excretion of labelled material in faeces after intraperitoneal injection of [24-^{14}C]cholic acid into a normal rat (A) and one treated chemotherapeutically (B).

had been followed in this example, the results would have been quite misleading.

Another difference between these groups is that the normal and hypothyroid rats excrete 1–2 mg. of cholesterol in the bile fistula per day, whereas the hyperthyroid rats excrete about 6 mg., as observed earlier by Rosenman, Byers and Friedman (1952).

If we now turn again to the quantitative aspects of bile acid metabolism in normal rats we see it was found by Lindstedt and Norman (1956*a*) that the half-life of the different

common bile acids was about two days. The technique is simply to give a tracer dose of carbon-labelled bile acid by mouth and follow the rate of excretion of the isotope with the faeces. They also found that the rate was very markedly decreased, with half-life times of ten days, if the rats were treated with antibiotics at the same time (Fig. 13).

To study this question further we are collaborating with Dr. Bengt Gustafsson in studying various aspects of the steroid and bile acid metabolism in the germ-free rats of the

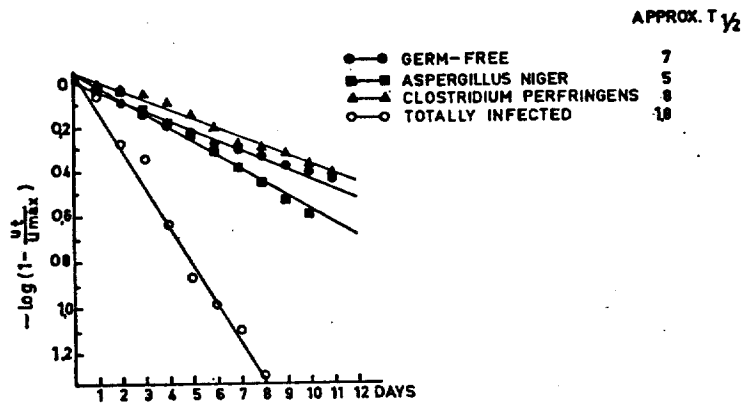

FIG. 14. Elimination of cholic acid by a rat during periods with different conditions in the intestine (semilogarithmic plot).

colony that he has reared at Lund. These animals appear normal in all respects except that there is a marked distension of the caecum.

The four experiments shown in Fig. 14 and 15 have been performed on the same germ-free rat and the data are recorded in the usual semilogarithmic plot in Fig. 14. In the germ-free condition as well as after infection with *Aspergillus niger* and *Clostridium perfringens* there is no significant change in the rate of excretion. After total infection there was, however, an immediate return to the normal half-life.

The chromatograms of the labelled compounds present in the faeces of that rat during these different periods are shown

in Fig. 15. Under germ-free conditions and after infection with *Aspergillus niger* the chromatograms are identical with the picture in the bile, i.e. no changes take place during the passage through the intestinal tract. When *Clostridium perfringens* was present, the taurine conjugates had been split

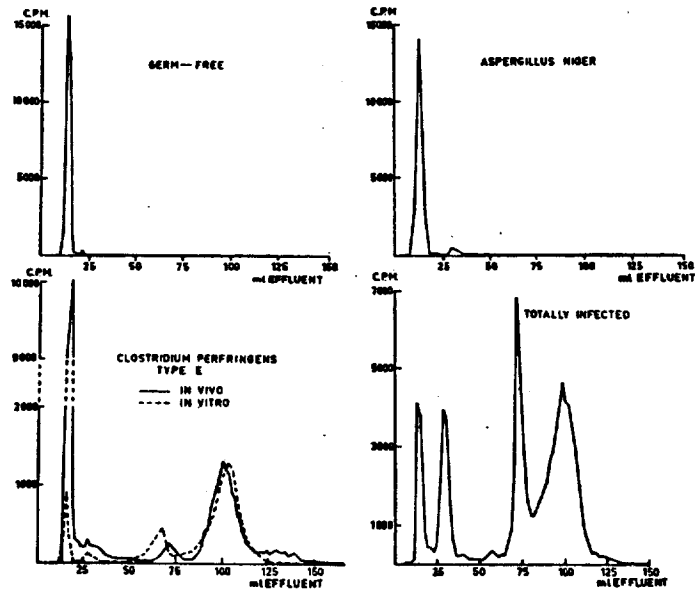

FIG. 15. Chromatograms of faecal acids excreted during periods with different conditions in the intestine.

to a large extent, yielding free cholic acid. After total infection most of the conjugates had been split, but furthermore the cholic acid had been transferred into a number of other metabolites.

The composition of the intestinal flora has thus a pronounced influence and possible changes of it have also to be considered when dietary changes are made.

In man the main bile acids are cholic, chenodeoxycholic and deoxycholic acid, which undergo secondary changes to varying degrees in the intestine. In order to determine the total

amount present and its turnover, Lindstedt (1957*b*) has used the following approach.

A tracer dose of ^{14}C-labelled cholic acid is administered by mouth. Subsequently every second or third day small samples of pure bile are collected via a thin plastic duodenal tube. When the tube has reached the duodenum, one mg. of cholecystokinin (obtained from Professor E. Jorpes, Stockholm) is given intravenously, resulting in an immediate contraction of the gall bladder. The small sample of bile that is taken up is hydrolysed, pure cholic acid isolated and its specific activity determined. The relative amounts of the other bile acids are determined at the same time with the aid of Sjövall's quantitative methods (1955).

In eight male students the total bile acid pool was found to be 3·58 g. (1·88 g.–4·97 g.). The cholic acid part of this pool was 1·38 g., with a half-life of 2·8 days, which corresponds to a daily production of 0·36 g. To obtain the amount of cholesterol degraded to bile acids per day one must, however, take the chenodeoxycholic acid into account, thus reaching a figure of about 0·7 g. day.

If the cholesterol present in the liver and the blood is estimated at 12–15 g., these results agree reasonably well with the half-life of eight days found by London and Rittenberg (1950).

These studies are now being extended to different pathological cases. However, there is still no exact data available on the amount of endogenous steroids excreted on a normal triglyceride-containing diet entirely free of steroids.

In Fig. 16 there is, finally, a summary of the turnover of cholesterol in man. The cholesterol coming in with the food is generally less than half a gram, but with the bile at least 1 g. and apparently often about 2 g. of cholesterol is secreted into the intestine apart from the steroids of other intestinal juices and sloughed mucosal cells. Most of the former cholesterol is, however, reabsorbed, and transported via the lymph as chylomicrons that are then predominantly absorbed into the liver cells. It is thus apparent that the exogenous cholesterol

of the diet is less than the cholesterol introduced into the intestine via the bile, etc. Furthermore about 0·7 g. of cholesterol is degraded to bile acids per day.

Clearly the process of intestinal absorption is of prime importance for the cholesterol metabolism—an impairment

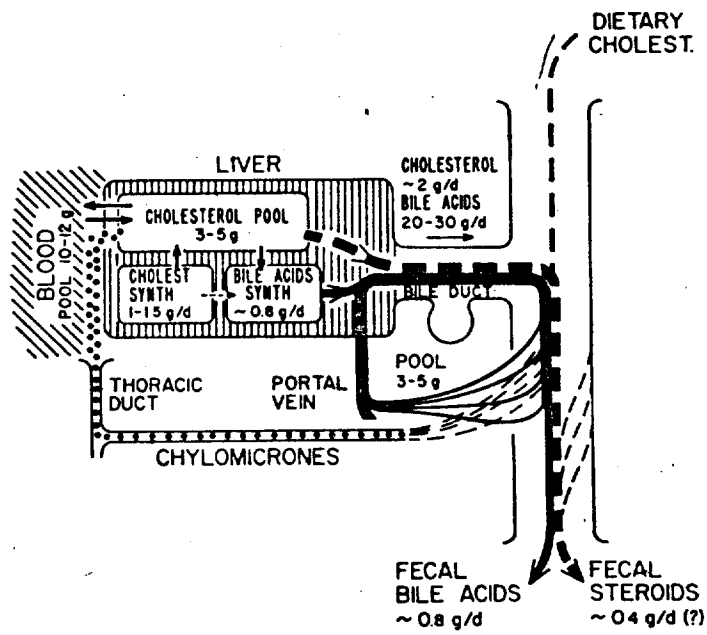

FIG. 16. Summary of the turnover of cholesterol in man.

of the absorption can rapidly lead to the loss of considerable amounts of cholesterol. The question is then to what extent and how rapidly this loss stimulates to increased endogenous synthesis and/or changes in the rate of bile acid formation or excretion.

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DISCUSSION

Bloch: Would you be willing to generalize and say that there are really no intrinsic species differences in the chemistry of the bile acids, but that they are all secondary and due to differences in the intestinal flora of the various species?

Bergström: No, but you might have a working hypothesis that the degradation of cholesterol is started with a 7α-hydroxylation. In rat, rabbit and man a 12α-hydroxylation follows, but this enzyme system seems to be totally lacking in the pig, which instead has a 6α-hydroxylase system in the liver. Furthermore when deoxycholic acid has been formed by bacterial action in the first three cases a rehydroxylation to cholic acid can only take place in the rat liver.

Bloch: But these differences are definitely not the result of bacterial action?

Bergström: No. They occur also in fistula animals, when the newly formed bile is excreted directly from the liver.

Haslewood: Would you be prepared to say that all the deoxycholic acid in all the species you have examined is a bacterial artifact? Is there any possibility that any is endogenous?

Bergström: We have no indication that there is any direct formation of the acid. If you give labelled cholic acid to man, and follow the specific activities in cholic acid and deoxycholic acid, you get the normal precursor-product curve.

Glover: How does the 3-hydroxy group change from the β to the α orientation? Does the change take place after the 7-hydroxy group is inserted? Do you get first a 3-keto compound and another reduction, or is there some other mechanism?

Bergström: If there is a 3α-tritium label on the cholesterol it is completely lost during its transformation to cholic acid. But we have not found out whether that is reduced *in situ* or not.

Coon: Can you say whether all of the steroid hydroxylations require TPNH and oxygen? Does the oxidation of the side chain involve a similar mechanism?

Bergström: The only one we have worked on to any extent is the 7α-hydroxylase system. That certainly is the usual TPNH oxygen-requiring reaction. We have not worked on the oxidation of the side chain. We did make this cholesterol with an extra methyl group and that oxidized to a dimethyl acid.

Bloch: When you spoke about the model compound, αα-dimethylstearic acid, you mentioned ω-oxidation, but you indicated that two carbons were eliminated. Was this a ω or a ω−1 oxidation?

Bergström: It was presumably a ω-oxidation followed by six β-oxidations.

Bloch: Then the initial oxidative attack is on the terminal carbon atom, but no intermediates have been isolated?

Bergström: That is correct.

Birch: I am very interested in this remark on the size of the side chain, and I take it that no bile acids other than the C_{24} compounds have been discovered. We have one from Australian koala bears which from equivalent weight determinations seems to have 22 carbon atoms. But I must admit we have not found out what the structure is, and it is not easy to work with.

Bergström: It might start out with a sterol with an extra carbon atom in the side chain. For example, if you have homocholic acid

you get a C_{23} acid formed. The animal may be eating some unusual sterol.

Haslewood: I think it is only fair to say that the Japanese have found some very odd steroids in amphibian bile, containing, I think, 25 carbon atoms.

Birch: The koala bear of course lives on the leaves from one species of gum tree. So it could certainly absorb something peculiar in its diet.

Coon: We were stimulated by Prof. Bergström's important work on αα-dimethylpalmitate to study the ω-oxidation of αα-dimethyloctanoic acid. This ^{14}C-labelled compound when fed to animals or incubated with liver slices gives a keto acid. Miss H. Den was able to recrystallize the dinitrophenylhydrazone of the product to constant activity and to identify it by carbon, hydrogen and nitrogen analyses. The keto acid gives a positive iodoform reaction and is presumably 2 : 2-dimethyl-7-oxo-octanoic acid. Presumably the formation of this compound indicates that the oxidative attack occurs on the ω − 1 carbon atom of the fatty acid.

Glover: In the bile acid side chain, the methyl group is in the γ position. Does that inhibit a further β-oxidation step?

Bergström: The C_{27} coprocholanic acids contain an α-methyl group and they lose the three terminal carbons. That is the first step. The resulting C_{24} γ-methyl acid is not degraded whereas I believe straight-chain analogues are oxidized further. The structure of the remainder of the molecule thus has an influence on the β-oxidation system.

Glover: It would seem possible for another two carbons to be split off. We have fed experimental rats with homologues of vitamin A with two additional carbons, and these two carbons are easily split off again.

Bloch: With vitamin A you have the double bonds to assist you.

Klyne: Is there any indication that the C_{27}-C_{24} degradation may be an intermediate in the further degradation and complete removal of the side chain?

Bergström: I have not seen anything further about Gurin's work on the direct formation of pregnane derivatives from cholesterol.

Boyd: In rats on a sterol-free diet we found that 200–300 mg./day of taurocholic acid had to be given before the serum cholesterol levels were elevated. We found that at lower dosage levels (less than 100 mg./day) *in vitro* hepatic cholesterol synthesis was depressed by taurocholate, which suggests that it is possible to dissociate the effects on synthesis from the serum cholesterol-elevating effect.

When you investigated the amount of chenodeoxycholic acid secreted in the bile did you make any observations on thyroidectomized as opposed to thiouracil-treated animals?

Bergström: No. Unfortunately the thyroidectomized rats died when Dr. Eriksson made the fistulas.

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EXPERIMENTAL LITHIASIS DUE TO EXCESS CHOLIC ACID IN MICE

by

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Summary

Cholic acid cause cholesterol stones and gall-bladder lesions in mice, even when administered alone in a normal diet, at a concentration of 0.5 % to 30 mice, and 1% in 20 mice, without the addition of cholesterol. Cholesterol alone is not lithogenic, even when added at a concentration of 8%. Compared or associated with sodium dehydrocholate, cholic acid has a less constant and less intense lithogenic effect. However, on the other hand, it causes more intense lesions in the gall-bladder and the liver.

These unexpected results differ from previous publications for the reasons discussed. It would be expedient to revise the pathogeny of lithiasis which attributes an important rose to the deficiency of biliary salts in relation to the cholesterol in the gall-bladder bile. As an experimental model of the human disease, lithiasis due to excess cholic acid offers the advantage of being induced by a natural substance, and the disadvantage of being inconstant. Clofibrate, added at a concentration of 0.5%, possesses a protective effect against lithiasis and vesicular lesions.

Cholic acid is reputed to be incapable of inducing a vesicular lithiasis in the mouse if it is administered alone at a concentration of 0.5% in the diet: in order to obtain calculi, it would be necessary to add some cholesterol at 1% to it (Tepperman et al., 1964 (23) and 1965 (22)). Nonetheless, thanks to a sufficiently sensitive technique, we have been able to observe that cholic acid at 0.5% is of itself lithogenic. This fact deserves to be indicated because, in the pathogenesis of human lithiasis, the primary biliary acids, of which cholic acid is the principal one, are reputed to be deficient in relation to cholesterol, rather than surplus (Grigaut, 1913 (13), Small, 1968 (20)).

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* Work realized with the collaboration of Mme M. Damianakis. Presented October 3, 1969 at the Research Forum of the National French Society of Gastro-Enterology.

Methods

The mice were young female Swiss nullparas. They received a manufactured food similar to that of Tepperman, described in our previous publication (Besancon et al., 1966), and given in the form of compressed unbaked tablets. The litter consisted of wood shavings.

At first we tried cholic acid in association with the dehydrocholate, each at a concentration of 0.25%. Later, we administered it alone, at 0.5% and at 1%.

Comparatively, we tested cholesterol alone at the high dose of 8%.

The tables indicate the duration of the experiments and the number of mice.

In the autopsy, the gall-bladders were put into formol intact, after ligation of the cystic canal. Several hours or several days later, and placed on a microscopic slide. The drop of vesicular content ins immediately examined under polarized light. Lithiasis is confirmed if there are any birefractive crystals of a large size (0.15 to 0.6 mm), either isolated or collected into calculi. This method is explained extensively in articles 7 and 8, and in a book in progress. The same is true of the methods of study of vesicular and hepatic lesions, those of chromatography and histochemistry. It has been verified that the time the organs are kept in the formol lends no error to the diagnosis of lithiasis.

The preventive effect of clofibrate (chlorophenoxyisobutyrate) was examined by adding it at a concentration of 0.5% to a diet supplemented by cholic acid at 0.5%. Autopsies were performed on nine mice at the end of 4 months, and on nine at the end of 7 months.

Results

The results are recapitulated in tables I through IV. The tables comparatively record the condition of the test mice, and the results which we had previously obtained with the Tepperman diet (23) (cholesterol + cholic acid) and with the dehydrocholate alone at 0.5% and 0.25%.

1. Cholic acid alone at 0.5% (tables I and II). -- Mortality rate is negligible (1 out of 20).

The lithogenic effect is manifested by the appearance of large birefractive crystals of cholesterol in the bile before 2 months, and of stones before 4 months (fig. 1). Their chemical constitution is verified by a Schultz staining done on the slide. Lithiasis affects 11 mice of 19 at the end of 4 months, and 5 out of 6 at the end of 8 months, while dehydrocholate produces 17 cases of lithiasis out of 17 mice in 4 months.

The dessiccation of the bile, whether lithiasic or not, induces the formation of numerous crystals, in the form of rectangles or oriflammes, different from the needle-form crystals observed in the dessiccated bile of the mice subjected to dehydrocholate.

TABLE I

1. Régime	2. Témoins	Acidecholique 0,5 %	Acidecholique 1 %	Acidecholique 0,5 % + cholestérol 1 %	Cholestérol 8 %	Déhydrocholate 0,5 %	Déhydrocholate 0,25 %	Acide chol. 0,25 % + Déhyd. 0,25 %
a. Nombre de souris	42	33	19	11	16	100	20	20
Bile à 4 mois 3.								
Calculs b.	0	9/19	0	8/8	0/13	13/17	1/15	7/15
Grands cristaux c.	0	11/19	8/10	8/8	0/13	17/17	7/15	1/5
Lésions vésicule à 4 mois 4.								
Hyperplasie muqueuse d.	0	0 à ++	++	++	0	0 à +++	++	++
Épithél. déform. e.	0	0 à ++	++	++	+	++	+	++
Épaissie f.	0	0 à ++	++	++	+	0 à ++	++	++
Lipides g.	0	++	+	+++	+	±	±	++
Stries lipides h.	0	+++	+++	++	+	0 à ++	++	++
Lipophages isolés i.	0	++	±	++	+	0	±	±
Amas lipophages j.	0	++	0	++	0	0	+	+
Lésions foie à 4 mois 5.								
Poids/corps k.	4,2	4,8	8,0	9,0	4,3	4,8	4,2	4,7
Lipides l.	0	±	+	++	+	±	0	±
Cristaux m.	±	++	+++	++	++	++	+	++
Lipides Küppfer n.	0	++	++	++	±	++	++	0
Cancer o.	0	0	0	0	0	0	0	0

Λ. Les fractions indiquent le nombre des souris atteintes du symptôme rapporté à l'effectif du lot au bout de 4 mois. Les « calculs » étant formés de « grands cristaux » agrégés, les souris atteintes de « calculs » sont comptées aussi parmi celles atteintes de « cristaux ».

Recapitulation of the lithiases and associated lesions induced by cholic acid, cholesterol and dehydrocholate, alone or associated.

Key:
1. = diet 2. = animals 3. = bile at 4 months
4. = vesicular lesions at 4 months
5. = lesions of liver at 4 months

a. = number of mice
b. = stones
c. = large crystals
d. = mucous hyperplasia
e. = epithelial deformation
f. = thickness
g. = lipides
h. = lipide streaks
i. = isolated lipophagies
j. = lipophagic clusters
k. = body weight l. = lipides m. = crystals n. = Kupfer lipides
o. = cancer
Λ. = The fractions indicate the number of mice affected with symptoms related to the actual number of the group at the end of 4 months. The "calculi" being formed of groups of "large crystals", the mice showing "calculi" are also counted among those with "crystals".

TABLE II

Régime 1.	Acide cholique 0,5 p.100			Acide cholique 1 p. 100		
Durée régime . . . a.	2 mois	4 mois	8 mois	2 mois	4 mois	8 mois
N° souris . . . b.	1835-39 2196-98	2065-69 2074-70,2319-28	2304-09	2265-69	2489-98	2808-10
Poids moyen (g) . . c.	31	31	32	27	31	32
Bile 2.						
Calculs . . . . d.	0	8/19	1/6	0	0	0
Grands cristaux . . e.	2/8	11/19	5/6	2/5	8/10	0
Lésions vésicule 3.						
Hyperplasie muqueuse . f.	±	0 à ++	+	+	++	±
Épithél. déform. . . g.	++	0 à ++	++	++	++	+
Épaissie . . . . h.	+	0 à ++	++	+	++	±
Lipides . . . . i.	++	++	+	±	+	+
Stries lipides . . . j.	+++	++	++	++	+++	+
Lipophages . . . k.	++	++	++	++	±	++
Amas lipophages . . . l.	1/5	5/9	3/4	3/4	0	1/3
Lésions foie 4.						
Poids/corps p. 100 . . m.	6,5	4,8	5,3	9,1	8,0	9,5
Lipides . . . . n.	±	±	+	+	+	
Cristaux . . . . o.	++ A,R (*)	++ A,R	++ A,R	+++ A	+++ R,A	
Lipides Küpffer . . p.	±	++	++		±	
Cancer . . . . q.	0	0	0	0	0	0

(*) Les abréviations A et R signifient que les cristaux sont en aiguilles (A), ou en réseaux ou amas (R).

Cholic acid at 0.5 % and 1 %: effects on the mouse.

Key:
1.= diet 2.= bile 3.= gall-bladder lesions 4.= liver lesions

a.= duration of diet
b.= number of mice
c.= average weight
d.= stones
e.= large crystals
f.= mucous hyperplasia
g.= epithelial deformation
h.= thickness
i.= lipides
j.= lipide streaks
k.= liporhagies
l.= lipophagic clusters
m.= body weight %
n.= lipides
o.= crystals
p.= Kupfer lipides
q.= cancer

(*)The abbreviations A and R indicate whether the crystals are needle-formed (A), or in nets or clusters (R).

Because of their small size (20 to 30μ), the crystals created by the desiccation are easily distinguishable from the large crystals (150 to 600μ), which are observed at once upon the incision of the lithiasic gall-bladders. These latter are of identical aspect, whether the mice were subjected to cholic acid or to dehydrocholate.

Fig. 1 -- Gall stones and birefractive crystals in the gall-bladder of a mouse fed a diet supplemented with 1% cholic acid for four months.

The vesicular lesions (fig. 2) are precocious and intense. The mucous reveals a design of hyperplasic crests, and the epithelial cells become cylindrical and even digitiform.

Fig. 2 -- 1% cholic acid for four months. Frozen sections of the gall-bladder stained by Sudan III, and observed in half-extinguished polarized light. Lesions consist of: a thickened wall, hyperplastic mucosal folds, lipide overload (in black in the photograph), and birefractive bodies of cholesterol, most of which are contained in either isolated or agglutinated lipophages.

The chorion is precociously thickened and inflammatory. The lipidic overload, in the form of sudanophilic, non-birefractive drops and streaks, is important. These streaks intersect on the gall-bladder fragments observed in transparency. They are sometimes slightly moniliform. In the sections, they are located in the chorion, the muscular, and beneath the serous. They possibly occupy the lymphatic network.

Small birefractive crystals of esterized and free cholesterol are observed in the frozen sections, especially if they have not been subjected to the action of a lysochrome such as Sudan, and especially in the zones where the chorion is thickened. These crystals can be found on all the planes of the wall.

The appearance of these crystals seems to precede the fluxion of the lipophages, and their evolution is regressive. It is probably that at least a part of them are phagocyted by the lipophages.

The macrophagias (lipophages) are abundant at the end of 2 months, and frequently collect into agglutinations charged with sudanophilic lipides and birefractive crystals of cholesterol. The frequency of these agglutinations reaches 5 out of 9 at the end of 4 months. Their relation to the lesions of the fresh gall-bladder is discussed in a separate publication.

At the end of 2 months, the liver weight has clearly increased. It later decreases. Its aspect is pale, yet under the microscope, the charge of sudanophile lipides appears moderate. There is an abundant and precocious overload of birefractive crystals, in the form of extra-cellular needles, in nets of clusters, in the parenchyma of the liver (fig. 3). Numerous Kupfer cells charged with lipides are spread throughout the liver. Some agglutinated lipophages around the portal spaces may contain birefractive crystals.

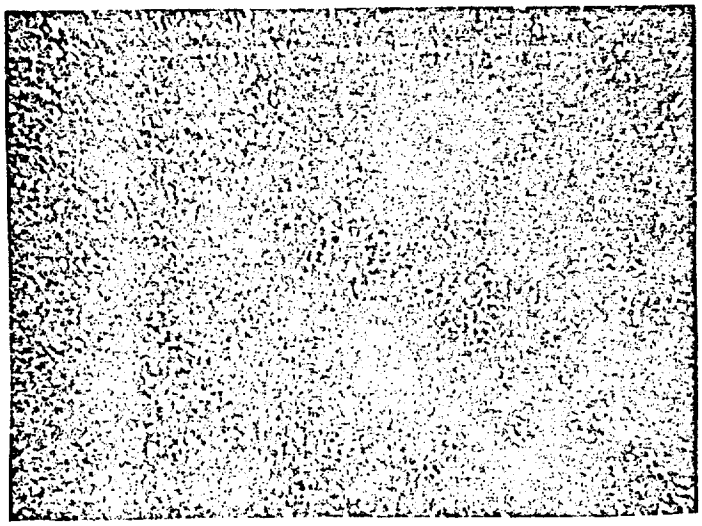

Fig. 3 -- 0.5% cholic acid for 2 months. Frozen section of formolin fixed liver stained lightly with hematin and mounted in Apathy's syrup. Overload of birefractive crystals, histochemically identified as esterized cholesterol. x 78.

2. Chromatography of the lipides. -- The chromatographs in thin layers (Philippe Laudat) show that the increase of cholesterol in the bile is accomplished to the benefit of the free fraction, and not the esterized fraction. On the other hand, in the vesicular wall, the increase of cholesterol is to the benefit of the esterized fraction.

In the chromatographs of the liver, the increase of cholesterol is for the profit of the esterized fraction. The triglycerides appear to be reduced, and the fatty acids undergo few modifications.

These qualitative data are confirmed by the doses, explaindd in a separate publication. In the liver, while the cholesterol increases, the trigylcerides and the phospholipides decrease.

3. Histochemistry of cholesterol. -- Precipitation by digitonine in an alcoholic environment shows the cholesterol in the form of birefractive bodies in two forms: on the one hand, small free crystals in all the layers of the vesicular wall; on the other hand, birefractive bodies included in the lipophagic macrophagias. The cholesterol is authenticated by the reaction of Schultz. Under the effect of Oil Red O, this cholesterol is revealed to be in an esterized form.

In the liver, histochemistry confirms that the birefractive crystals are indeed of cholesterol, especially esterized, and its topography is in the forms of crystalline needles spread over the entire parenchyma. In contrast to the lipophages of the gall-bladder, the soudanophile Kupfer cells do not incorporate cholesterol. Neither cholesterol nor lipophages are seen in the intrahepatic biliary ducts.

4. Cholic acid alone at 1% (tables I and II). -- The results were as follows: more constant and early lithiasis, 8 out of 10 in 4 months, but only in the form of crystals in tablets. Extreme intensity of all the vesicular and hepatic lesions, but the gall-bladder lesions appear regressive to a large extent, as does the lithiasis.

5. Cholic acid at 0.5 % + cholesterol 1% (tables I and III). The results are those obtained by Tepperman, which we have previously confirmed.

The addition of cholesterol to the cholic acid yields: a more precocious and intense lithogenic effect; 100% stones in 3 months, in our experiments, and even in 2 months according to Tepperman (1969), identical vesicular lesions, a hepatic steatosis. The weight of the liver in relation to the weight of the body rises to 9.5%, as compared to 4.2% or 6.5% with cholic acid alone.

6. Cholesterol alone at 2% (tables I and III). -- Despite this high concentration, no lithogenic effect is produced. The moderateness of the inflammatory lesions of the gall bladder is noted. The lipidic overload is average. The overload of the liver of birefractive crystals of cholesterol, esterized and free, is as intense as in the mice rendered lithiasic by the dehydrocholate, and this overload of soudanophile lipides is clearly more intense.

TABLE III

Régime	Cholestérol 8 %	Cholestérol 1 %	Cholestérol 1 % + acide cholique 0,5 % (3 ou 4 mois)
N° souris	2357-59, 2347-56		
Poids moyen (g)	36	49	32
Bile			
Calculs	0	0	11/11
Grands cristaux	0	non étudiés	non étudiés
Lésions vésicule			
Hyperplasie muqueuse	0	0	++
Épithél. déform.	+	0	++
Épaissie	+	0	++
Lipides	+	0	++
Stries lipides	+	0	++
Lipophages	+	0	+
Amas lipophages	0	0	+
Lésions foie			
Poids/corps p. 100	4,3	4,3	9
Lipides	++	0	++
Cristaux	++ R, A	non étudiés	
Lipides Küppfer	±	*d°*	
Cancer	0	0	0

Effects of cholesterol introduced in various concentrations, alone or associated with cholic acid, for 4 months.

For key see tables I and II.

7. Dehydrocholate at the reduced dose of 0.25%, isolated or associated with cholic acid at 0.25% (tables I and IV). -- When these agents are associated the lithiasis is clearly more inconstant and backward than with dehyerocholate alone at 0.5%, while the lesions are more intense. They are as intense as with cholic acid at 0.5%.

8. Preventive treatment with clofibrate (table V). -- Introduced in the same dose as cholic acid (0.5%) for 4 months, chlorophenoxyisobutyrate has a nearly absolute preventive effect on lithiasis of the gall-bladder: 1 mouse out of 9 is lithiasic, as compared to 11 out of 19 without clofibrate. At the end of 7 months, 3 mice out of 9 are lithiasic.

The mice protected against lithiasis are equally protected by the clofibrate against most of the lesions of the biliary vesicle (fig. 4). On the other hand, in mice affected with lithiasis in spite of the clofibrate, the inflammatory lesions may be important, as well as the proliferations of lipophages, while the lipidic overload is inhibited.

The clofibrate favors the hypertrophy of the liver. It is ineffective, or only slightly effective, in the prevention of the surcharge in the liver of birefractive crystals of cholesterol (fig. 5). These crystals frequently take filamentous form in

TABLE IV

Régime	Déhydrocholate 0,25 p. 100	DHC + ac. cholique à 0,25 p. 100
Durée régime	4 mois	4 mois
N° souris	1888-92, 1928-37	1883-87, 1918-27
Poids moyen (g)	34	31
Bile		
Calculs	1/15	7/15
Grands cristaux	7/15	9/15
Lésions vésicule		
Hyperplasie muqueuse	++	++
Épithél. déform.	+	++
Épaissie.	++	++
Lipides	±	++
Stries lipidiques	++	++
Lipophages	±	±
Amas lipophages	5/15	6/15
Lésions foie		
Poids/corps p. 100	4,2	4,7
Lipides	N	±
Cristaux	+ R	
Lipides Küppfer	++	
Cancer	0	0

Dehydrocholate at 0.25%, isolated or associated with cholic acid at 0.25%.

TABLE V

	Souris non traitées Acide cholique seul		Souris traitées Clofibrate + Ac. cholique	
Durée régime (mois)	4	8	4	7
Poids moyen (g)	31	32	26	30
Bile				
Calculs	8/19	1/6	0	1/9
Grands cristaux	11/19	5/6	1/9	3/9
Lésions vésicule				
Hyperplasie muqueuse	0 à ++	+	0	0 à ++
Épithél. défor.	0 à ++	++	0 à ++	0 à ++
Épaissie	0 à ++	+	±	0 à ++
Lipides	++	++	0	0
Stries lipidiques	+++	++	0	0
Lipophages	++	++	0	++
Amas lipophages	5/9	3/4	2/3	0
Lésions foie				
Poids/Corps p. 100	4,8	5,3	6,4	6,3
Lipides	±	+	0	±
Cristaux	++ A, R	++ A, R	+++	++
Lipides Küppfer	++	++	±	+
Cancer	0	0	0	0

Preventive effect of clofibrate at 0.1% on the lithiasis and lesions induced by cholic acid at 0.1%.

For keys see tables I and II.

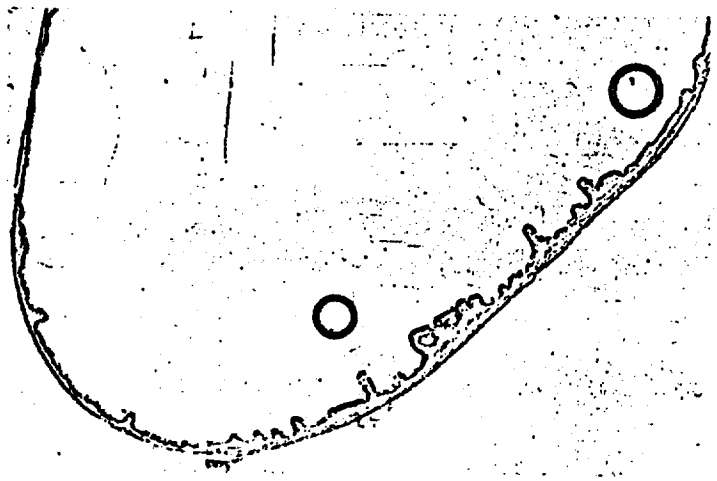

Fig. 4 -- 0.5% clofibrate and 0.5% cholic acid for 4 months. Frozen section of the gall-bladder, stained with Sudan III. Moderate degree of thickening, inflammation and lipide overload. The clofibrate not only prevents gall-stones, but also lesions of the gall-bladder.

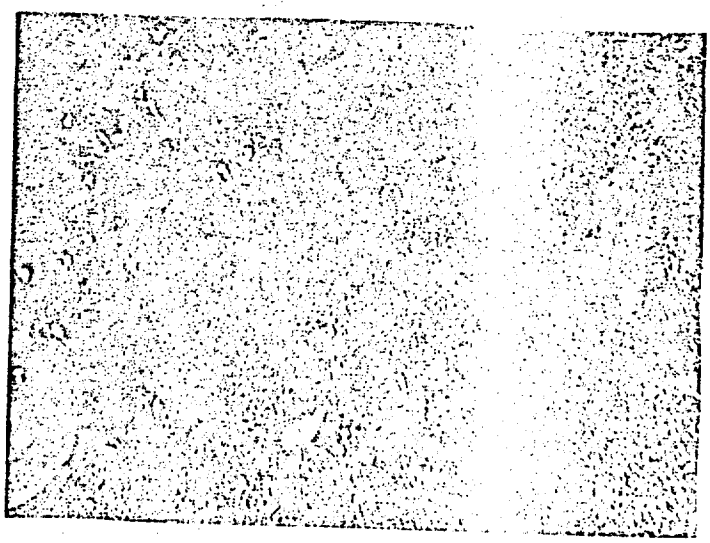

Fig. 5 --0.5% clofibrate and 0.5% cholic acid for 4 months. Frozen section of the liver, stained by hematein and mounted in Apathy's syrup. Failure of clofibrate to prevent birefractive crystals of cholesterol in the liver.

the mice thus treated. Clofibrate prevents the soudanophile surcharge of the Kupfer cells.

Discussion

1. The lithogenic effect of cholic acid appears never to have been indicated, and our results seem to be in contradiction with those of Tepperman et al., 1964(23) and 1965 (22), for these authors did not obtain lithiasis with cholic acid employed alone at 0.5%. This divergence might be explained by the following reasons:

a) Our method of observation of the vesicular content under polarized light is much more sensitive than inspection on a filter.

b) Fitness for lithiasis of the gall-bladder is unequal in various varieties of mice. For example, the pure line of C3HeB brown mice is unsusceptible to lithiasis, as we indicate in a separate publication.

c) The litters of our mice are wood shavings which they nibble. It is known that the production of cholic acid is increased in the rat by the addition of indigestible foods to the diet (Portman, 1960, cited by Bergstrom and Danielsson, 1965 (5)). Jay Tepperman, whom we have consulted, indicated to us that on the one hand, his mice have no litter, and on the other, that he saw stones occasionally with cholic acid alone.

Cholic acid does not favor lithiasis of the gall-bladder induced in the hamster by a diet without fats (Dam et al., 1962 (12)).

2. The lithogenic effect of cholic acid is not constant, in contrast to that of dehydrocholate, at a concentration of 0.5% for four months: 11 out of 19 and 17 out of 17. In general, as many lithiases are obtained with dehyrocholate at 0.25% as with cholic acid at 0.5%.

3. We found no mention of lesions of chronic cholecystitis induced by cholic acid in the literature. These lesions seemed remarkable to us by reason of their constancy, their precocity, and their intensity. They are similar to those which are induced by dehydrocholate, but more intense.

4. Under the microscope, the hepatic lesions appear to be comparable to those which are induced by dehydrocholate. Cholic acid possesses no inflammatory hepatic effect, as does lithocholic acid as described by Carey et al., 1964 (9).

Quantitatively, insofar as it is expressed by the weight of the liver divided by the weight of the body, the hepatic overload of lipides (cholesterol and triglycerides) is more intense than with dehydrocholate, but less intense than with the Tepperman diet (cholesterol + cholic acid).

This overload is in accord with certain previous publications: cholic acid increases the hepatic concentration of cholesterol (Hummel, 1929 (17). The mechanism is analyzed by Beher

et al., 1958 (2), 1959 (3, 4) in the rat: the catabolism of the cholesterol seems to be retarded by the effect of the mass: its hepatic rate increases by 50% in the liver, in esterized form as well as in free form. In consequence, the synthesis itself of cholesterol with radiomarked acetate or mevalonate is checked.

5. Relations between lithiasis and the lesions of chronic cholecystitis. -- All the lesions described are frequently present in the absence of lithiasis. Therefore, they are not secondary to lithiasis. Inversely, with cholic acid, it is exceptional to observe lithiasis without lesions. Considered separately, this experiments offer the possibility that lithiasis would be secondary to the lesions. Other results, in the process of being published, suggest that the role of the lesions in lithogenesis is solely adjuvant.

6. Does cholic acid provide a good experimental model of human cholesterol gall stones, through its effects or its mechanism?

In its effects, it seems to us to be less interesting than dehydrocholate, because the gall stones are not as constant, even when much higher and much more toxic doses are used, and because cholic acid increases the weight of the liver besides.

Can it be maintained that the mechanism of action of cholic acid deviates less in the human disease, because this acid is natural and is a former of micellas, in contrast to dehydrocholate? Nonetheless, this "natural" character deserves to be discussed: a) in the mouse, because it has ω-muricholic acid (Haslewood, 1965 (14 and 15)), which is trihydroxylated in 3α 6β 7α instead of 3α 7β 12α as is cholic acid; in reality, it is the latter which predominates. b) Knowing that cholestanol is synthesized by the intestinal flora (Haravolas et al., 1965 (18)), and that cholestanol in excess is lithogenic (Cook, 1954 (11)), one might ask whether cholic acid does not favor the intestinal absorption of cholestanol, which would be the actual lithogenic agent. This does not seem probable, for lithiasis due to cholestanol is composed of allodeoxycholic acid (Hoffmann et al., 1964 (16)), in contrast to the lithiasis due to cholic acid, which consists of cholesterol.

7. The pathogeny of lithiasis will be discussed in a separate publication, in order to take into account the entirety of the experimental facts concerning the gall-bladder content, the parietal alterations, the hepatic bile, the metabolic disturbances, and experimental therapy. We will limit ourselves here to a few remarks.

A deficiency of biliary acids in relation to cholesterol is often invoked as the principal factor in human lithiasis, since Grigaut (1913 (13)) and Chauffard (1914 (10)): this is the thesis of "cholaligenic insufficiency of the liver".

It was recently supported by the diagrams of Small and Dervichian, 1966, 1967, 1968 (21, 19, 20), representing in a systematic fashion the physical state of mixtures comparable to

to the bile, and by the isotopic studies of Vlahcevic et al., (1969 (25)): the pool of the biliary acids and notably of cholic acid is insufficient in lithiasic patients, not due to insufficient production, but due to the continuation of their half-life. It is remarkable that the excess of cholic acid in food, in our mice, like the deficiency in biliary cholic acid in the human, both induce gall stones of cholesterol.

One of the hypotheses which might explain the pathogenesis of lithiasis induced by dehydrocholate rests on the incapacity of this modified biliary salt to form micellas: it is substituted in the bile for natural biliary salts, which results in an insufficiency of micellas. This hypothesis does not quite agree with the similarity between the lithogenic effects of dehydrocholate and cholic acid, when this latter is capable of forming micellas.

8. The excess of alimentary cholesterol does not cause the formation of crystals of cholesterol in the bile in the mouse, in contrast to the related observations in the rabbit, made by Anitschkow et al., (1913 (1)).

9. Clofibrate has a preventive effect against lithiasis and lesions of the gall-bladder, but this preventive effect appears less perfect against the lithiasis induced by dehydrocholate. The curative effect was not examined herein.

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THE SITE OF ABSORPTION OF CONJUGATED BILE SALTS IN MAN

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Ingested triglyceride is hydrolyzed to lower glycerides and fatty acid in the small intestinal lumen by pancreatic lipase during fat digestion.[1] Evidence has been presented recently[2] that the bile salts in intestinal content act to dissolve the fatty acid and lower glycerides into a water-clear micellar solution, from which the absorption of lipid occurs.

Although the solvent or "hydrotropic" action of bile salt solutions was well documented by the turn of the century,[3] it was only rather recently understood that bile salts were detergents,[4, 5] and that, as all detergents, they aggregate in water to form micelles.[4, 7] Such micellar solutions can dissolve water insoluble materials, and this phenomenon is termed "solubilization."[8, 9] Verzár *et al.*[10] demonstrated the solubilization of fatty acids by bile salt solutions, but realized that the amount of bile salt needed to dissolve the fatty acid deriving from the hydrolysis of the triglyceride present in a normal meal was greater than that which could be excreted during digestion, even allowing for recirculation.

Subsequent quantitative work on the solvent properties of micellar bile salt solutions[2, 11, 12] as well as reliable estimates of the bile salt pool size and its turnover rate in man[13] have confirmed Verzár's opinion. As a result, two views on the function of bile salts during fat digestion and absorption have been popular. Either the bile salts serve as a transport mechanism, dissolving the fatty acids and partial glycerides and carrying them up to the mucosal cell (possibly entering the cell) but being released immediately to the surface of the cell or the lumen;[10, 14] or the bile salts serve to emulsify the ingested lipid and its hydrolytic products, and as a part of the surface of the oil droplet, pass in particulate form into the mucosal cell and are simultaneously absorbed.[15]

We report here three types of intubation studies in man, concerned with a comparison of the site of absorption of conjugated bile salts with that of fat. In initial experiments, a test meal containing radioactive bile salt and a nonabsorbable reference compound was fed, and the extent of absorption of bile salt compared with that of fat at different intestinal locations. Because the experimental results could only be explained by a rapid recirculation of bile salts, additional experiments were done to determine the recirculation time of bile salts when injected into the intestinal lumen at different sites. Finally, studies were performed with a double lumen balloon, in which different regions of the small intestine were perfused with a micellar solution containing bile salt, fatty acid, and monoglyceride, and the extent of absorption of each determined.

The results of all of these experiments are in agreement and seem to indicate that in man, fat absorption normally occurs in the proximal intestine, whereas net bile salt absorption occurs in the distal small intestine. It was also shown that lipid can be readily absorbed throughout the small intestine from a micellar solution.

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Experimental Procedure

In the absorption and recirculation experiments healthy male and female medical students, aged 19 to 23 years, were used as subjects. In the double lumen experiments 3 patients who had undergone total pancreatectomy for cancer of the pancreas were used.

Comparison of the site of absorption of fat with that of sodium taurodeoxycholate and sodium taurocholate. The intubation technique of Blankenhorn *et al.*[16] was used, as described by Borgström[17, 18] and Borgström and Dahlqvist.[19] Subjects were intubated with polyvinyl tubing, advised to ingest only water overnight, then fed a test meal of fat, carbohydrate, and protein, containing 10 μc. of S^{35} radioactivity as sodium S^{35} taurodeoxycholate and sodium S^{35} taurocholate (about 20 μmoles or 10 mg. of bile salt) and polyethylene glycol. Intestinal content was collected in ice at 15- or 30-minute intervals, and aliquots were taken for analysis. Samples not immediately analyzed were stored at −18° C. until analysis was performed. At the end of the collection period, the location of the tip of the tube was established by x-ray, and the tube then removed. As the tube occasionally curled on itself extensively in the stomach, localization by x-ray was considered mandatory.

The test meal contained sucrose, 65.3 gm.; corn oil, 23.2 gm.; dried egg white, 21.1 gm.; polyethylene glycol, 4.0 gm.; water, 295 gm.; and an egg yolk. The corn oil was almost pure triglyceride by thin layer chromatography on silicic acid.[20] For preparation of the test meal, the egg white, protein, sucrose, and S^{35} taurine conjugated bile salts were dissolved individually in small volumes of water, then blended with each other and the egg yolk with an Ultra-turrax homogenizer (manufactured by Janke and Kunkel, Staufen, Germany). The corn oil was added slowly with continuous homogenization; a fairly stable emulsion was obtained.

Determination of the recirculation time of taurine conjugated bile salts. Intubated subjects were fed the test meal prepared as described but without the S^{35} taurine conjugates and polyethylene glycol. About 20 minutes after its ingestion, when a generous flow of intestinal content was flowing from the tube, 5 to 10 μc. of S^{35} sodium taurodeoxycholate and sodium taurocholate was injected into the intestinal lumen *via* the tubing. The tubing was flushed several times with saline, then clamped. Ten minutes later, 8-minute collections were begun, and continued for 2 hours or until the flow of intestinal content had ceased.

Comparison of the absorption of fatty acid and 1-monoglyceride with that of sodium C^{14}-glycochenodeoxycholate from a micellar solution during intestinal perfusion. A bile salt solution was composed to simulate human intestinal content in terms of dihydroxy- and trihydroxy-bile acid content. To this was added sodium C^{14}-glycochenodeoxycholate. 1-C^{14}-oleic acid, glycerol-1-monooleate (9,10-H^3-). The resultant micellar solution was water clear and stable at room temperature; its composition is listed in table 1.

From the first opening of a double lumen tube 100 ml. of this solution was infused over a 20-minute period. Samples were taken from the second hole, 30 cm. from the first, for analysis, the difference between the values representing the absorption in the gut segment between the holes, when corrected for dilution, as assessed by the polyethylene glycol concentration figures.[21] Three

TABLE 1

Composition of micellar solution used in the perfusion experiments

The solution was prepared in phosphate buffer pH 6.3, 0.15 M in Na^+ containing polyethylene glycol 1200 mg. per liter.

	mmoles per liter	Radioactivity
Sodium glycocholate	2.5	
Sodium taurocholate	2.5	
Sodium taurodeoxycholate	5.0	
Sodium glycochenodeoxycholate		4 μc. (C^{14} glycine
Oleic acid	5.0	8 μc. (1-C^{14} oleic acid)
Glycerol-1-monooleate	2.5	50 μc. (9,10H^3)

such experiments were performed at different intestinal levels to determine whether absorption of micellar lipid does occur in man and whether bile salt absorption occurs simultaneously. This experimental technique was suggested to the authors by Franz Ingelfinger, who generously made available two such tubes.

The 3 patients studied had been totally pancreatectomized and no lipolysis of monoolein was anticipated. In patients A. E. and N. V. a Billroth type II anastomosis had been performed: in the third patient, I. A., the pylorus was intact and a Billroth type I operation had been performed with a duodenojejunostomy after resection of approximately 30 cm. of duodenum-jejunum. The levels sampled as calculated from the operation data and x-ray during intubation corresponded to the parts of the small intestine that had been 100, 150, and 115 cm. from the nostrils before the operation.

Compounds used. Nonradioactive conjugated bile salts were synthesized by the method of Norman[22] from purified cholic, deoxycholic, or chenodeoxycholic acid, as described.[22] The purity of the bile salts was assayed by thin layer chromatography on silicic acid.[24, 25]

1-C^{14}-oleic acid was purchased from Isotopes Specialties, Burbank, Calif. and used without further purification. Its specific activity was 7 μc. per μmole.

Glycerol-1-monooleate (9,10-H^3-) was synthesized according to standard procedures with oleic acid (9,10-H^3-) purchased from Amersham, England.

Glycerol-1-monooleate (carrier) was a gift from Distillation Products, Rochester, N. Y. It had class purity, but not homologue purity; however, homologue purity was unnecessary for the experiments. Its composition has been described in detail elsewhere.[12]

Sodium S^{35}-taurodeoxycholate and sodium S^{35}-taurocholate. A mixture of S^{35}-labeled sodium taurodeoxycholate and S^{35}-labeled sodium taurocholate—henceforth termed S^{35}-taurine conjugates—was synthesized from S^{35}-taurine (Radiochemical Center, Amersham, England) and an equimolar mixture of cholic and deoxycholic acid by the method of Norman.[22] A 70 per cent excess of free acid was used. After the liberation of carbon dioxide, the reaction mixture was concentrated, without warming, on a rotary evaporator, diluted with water, adjusted to pH 10 with 1 N NaOH, and extracted twice with diethyl ether-petroleum ether (1:1, by volume) to remove the tri-*n*-butylamine. After acidification to pH 5, the solution was evaporated. The residue was then refluxed in 95 per cent ethanol and filtered.

The excess free bile acid was eliminated by ion exchange chromatography. After adsorption of the reaction mixture in 80 per cent ethanol to an anion exchange column (Dowex 1-X2, OH-form) the free acid was eluted with 0.2 N ethanolic formic acid. The S^{35} taurine conjugates were then displaced from the column with 0.5 N ethanolic HCl. After neutralization and evaporation of the ethanol, the reaction product was dissolved in methanol to remove the majority of salt present. After the methanol was evaporated, the residue was subjected to a three stage counter-current distribution between *n*-butanol and 1 N HCl, the lower acidic phase containing carrier taurine. 95 per cent of the radioactivity was present in the first two butanol phases, and the remainder rather evenly distributed in the aqueous acidic phases. The first two butanol phases were pooled, neutralized, evaporated, and the residue dissolved in methanol, and filtered.

The final product contained about 0.5 μc. of S^{35} radioactivity per μmole of conjugated bile salt and no free bile acid or taurine by thin layer chromatography.[24] It contained two spots by thin layer chromatography, representing sodium taurodeoxycholate and sodium taurocholate. Its composition considered in terms of dihydroxy- and trihydroxy- components corresponded approximately to the taurine conjugates present in human small intestinal content.[26] For administration, an aliquot of the original preparation in methanol was transferred to a test tube, blown to dryness, and dissolved in water.

A simpler method for preparing pure taurine conjugated bile acids has since been developed.[23]

Sodium-C^{14}-glycochenodeoxycholate was synthesized according to Norman[22] as described[23] from C^{14}-glycine (Radiochemical Center, Amersham, England) and chromatographically pure chenodeoxycholic acid.[23] Because this synthesis always gives reaction products containing more polar impurities containing glycine,[23] the reaction mixture was resolved by preparative thin layer chromatography, with a layer 2 mm. in thickness of Silica Gel G (E. Merck AG, Darmstadt, Germany) and a solvent system for conjugated bile acids.[24] The glycochenodeoxycholic acid band was located by spraying with an ethanolic solution of iodine. After the iodine had been sublimed by vacuum, the adsorbent was scraped off and the glycochenodeoxycholic acid eluted from it with methanol. The final product was pure chromatographically.

Analytical procedures. Extraction of lipid from

intestinal content. A cross current scheme was used, based upon the extraction scheme described by Blankenhorn and Ahrens.[27] The sample of intestinal content was adjusted to pH 6 to 7, if necessary, with phosphate buffer, pH 6.5. To 1 volume of intestinal content in a separatory funnel were added 3 volumes of ether-heptane-ethanol (1:1:1, by volume). After shaking, the upper phase was aspirated with a capillary pipette attached to a vacuum trap arrangement, so that the upper phase was collected in a round bottom flask. The remaining lower phase was extracted 2 additional times with preequilibrated upper phase of diethyl ether-heptane-ethanol-water (1:1:1:1, by volume). The upper phases were aspirated similarly, and the combined extracts were evaporated in a rotary evaporator.

Control experiments had shown such an extraction procedure to remove all of the glycerides and fatty acids, but none of the bile salts. A fraction of the phospholipids is extracted. If the lower phase is acidified to pH 3, and such a cross current scheme is used, glycine conjugated bile salts pass into the upper phase. However, counter current distribution (3 stages) at pH 3 gives a complete separation of fatty acids and glycerides from all bile salts. The cross current procedure as described is more convenient, however. Chloroform-methanol extraction schemes cannot be used, as a significant fraction of bile salt enters the chloroform phase.

Saponification and titration of intestinal lipid. The lipid residue obtained from the extraction procedure was saponified with a 1:1, volume per volume, mixture of petroleum ether and alcoholic KOH (4 gm. KOH is dissolved in 4 ml. of water and 96 ml. of absolute ethanol is added) for 24 hours at room temperature. The resulting fatty acids were extracted with a 3 stage counter current distribution of 50 per cent alcohol and petroleum ether in which the second and third aqueous phases were acidified. Thin layer chromatography of the upper phase in the first stage showed only cholesterol to be present, indicating the saponification was complete.

The second and third petroleum ether phases containing the fatty acid were pooled, dried with sodium sulfate, and evaporated. The fatty acid was dissolved in absolute alcohol, and titrated with 0.1 N methanolic KOH.

Extraction of S^{35}-taurine conjugated bile salts. Two extraction procedures were used; the second is the recommended procedure. The first was derived from that of Norman[28] and was used on intestinal content samples from which the lipids had been extracted as described. To the sample, already in 50 per cent ethanol, were added 4 to 6 volumes of 95 per cent ethanol, and the sample was refluxed for [illegible] filtered through filter paper; the paper was rinsed repeatedly with hot 95 per cent ethanol. The filtrate was blown to near dryness on a water bath and dissolved in 10 ml. of water. 2 ml. of 6 N HCl was added, followed by 12 ml. of *n*-butanol. After repeated inversion, the butanol phase (total volume 14.4 ml.) was removed and centrifuged. A 10 ml. aliquot of this phase was transferred to an Erlenmeyer flask and evaporated to a brown syrup on a water bath with a jet of air. The residue was dissolved in methanol -0.3 N NH_4OH (1:1, by volume) to a volume of 5 ml. or 10 ml. in a volumetric flask. Aliquots were taken from this solution for plating.

Counting of the 1 N HCl phase and control experiments indicated a single butanol extraction was sufficient to extract at least 95 per cent of the S^{35} sodium taurodeoxycholate and taurocholate radioactivity. The recovery of S^{35} taurine conjugate when added to intestinal content was 93 per cent in two experiments. The experimental results indicated almost complete recovery of radioactivity in samples of intestinal content from which no bile salt absorption had taken place. However, the procedure was inconvenient and involved. The filtration of the alcoholic suspension was difficult. The final residue was brown and dissolved incompletely in methanol aqueous ammonia.

The second procedure was tested extensively in control experiments. It was much more satisfactory than the butanol extraction procedure described, but recovered only the taurine conjugates, as the glycine conjugates were lost in the protein precipitation step. The final extracts for counting were virtually colorless.

Five milliliters of intestinal content or 10 ml. lower phase (which had originally contained 5 ml. of intestinal content) was placed in a 200 ml. Erlenmeyer flask, and the pH adjusted to 8 (pH paper) with NaOH. To the sample were serially added the following, with thorough mixing after each addition: 5 ml. of absolute ethanol (this was omitted if the sample was a lower phase and, therefore, already in 50 per cent ethanol); 5 ml. $Ba(OH)_2$; 5 ml. 10 per cent weight per volume $ZnSO_4$; and 30 ml. of absolute ethanol. The $Ba(OH)_2$ and $ZnSO_4$ were prepared as described by Somogyi.[29]

The resultant mixture was heated over a boiling water bath for at least 15 minutes and then blown down on a water bath to a volume of about 30 ml. The suspension was transferred quantitatively with rinses of absolute ethanol to calibrated polypropylene centrifuge tubes, ethanol being added to a final volume of 40 ml. After centrifugation, 25 ml. of the clear supernate

was permitted to run down the flask. This solvent mixture caused the sample to spread evenly and rapidly across the nonsiliconized surface. It evaporated slowly, in several hours, leaving the sample deposited in what appeared to be a thin, even layer.

blown to near dryness on a water bath. It was then dissolved in 50 per cent ethanol and transferred quantitatively to a graduated glass stoppered test tube, the final volume being not more than 5 ml. If the original sample had been rich in triglyceride, a few oil droplets might be present on the surface of the 50 per cent ethanol phase, in which case it was extracted twice with an equal volume of diethyl ether-heptane (1:1, by volume). Aliquots of the solution were taken for counting; the solution could be blown down to an even smaller volume if desired. It was occasionally necessary to centrifuge this final alcoholic solution briefly for clarification, but no radioactivity was lost in the procedure. It was advisable to dilute the formula with 2 volumes of water before carrying out the above procedure, because of its high protein content.

Extraction of sodium C^{14}-glycochenodeoxycholate. The bile salt, in its acid or unionized form, is the least polar of all of the common conjugates and can be extracted into ethyl acetate from an acidified aqueous solution.[30]

The lipids were extracted from intestinal content, as described. The alcohol of the lower phase was largely removed by a brief concentration on a rotary evaporator. The phase was then acidified to pH 3 with HCl, and extracted three times with equal volumes of ethyl acetate. The ethyl acetate phases were pooled, washed once with water, then dried with sodium sulfate, filtered, and the filtrate evaporated. The residue was dissolved in 10 ml. 0.1 N aqueous NaOH and aliquots taken for counting.

Control experiments showed that less than 1 per cent of sodium C^{14}-glycochenodeoxycholate is extracted into the ether-petroleum ether phase when the lower phase is at pH 6.3. The radioactivity was completely extracted by three extractions with ethyl acetate after acidification of the lower phase to pH 3, and no radioactivity was lost to a single water washing.

Determination of radioactivity. In the experiments described in Experimental Procedure radioactivity was determined on aluminium planchets in a Frieseke-Hoepfner FH51 gas flow counter. Samples were counted to a standard error of counting of less than 5 per cent.

The samples in ammonia-methanol or 50 per cent ethanol were applied to the planchets with Carlsberg-Levy pipettes. The spread of the solvent was confined to a uniform circular area by a ring of silicone grease inside the edge of the planchet. After the sample was applied and before evaporation had occurred, it was flooded with methanol-isobutanol (1:4, by volume).

The samples were corrected for self absorption by plating an identical aliquot containing an internal standard of negligible mass. The efficiency of the counting was 60 to 90 per cent relative to a sample of infinite thinness. The validity of this plating and quench correction procedure was proved by obtaining the same results per milliliter sample if aliquots of different size were plated. Recently an extensive study showing the usefulness and validity of this method has been published.[31]

In the perfusion experiments, radioactivity was determined by liquid scintillation in an automatic Packard spectrometer. The lipids were counted in toluene and the bile acids in the dioxane solvent mixture devised by Bray.[32]

Polyethylene glycol determinations. Polyethylene glycol was determined turbidometrically by the method of Hydén;[32] the determinations were kindly performed by A. Dahlqvist. Because there had been reports[31] of substances in intestinal content interfering with the determination, two groups of control experiments were performed. For 4 subjects, the test meal was composed without polyethylene glycol. Polyethylene glycol determinations on the intestinal content samples from these subjects gave completely negative values. For several other subjects, a known amount of polyethylene glycol was added to the samples of intestinal content. Recovery was 105 per cent with a standard deviation of 10 per cent ($n = 11$).[33] Calculation of the per cent of absorption was made using the formula

$$\text{Absorption (\%)} = 100\left[1 - \frac{S_i PEG_m}{S_m PEG_i}\right]$$

where S_i is the concentration of fat or bile salt radioactivity in the intestinal content sample, S_m is the concentration of fat or bile salt radioactivity in the meal. PEG_i is the polyethylene glycol concentration in the intestinal content sample; PEG_m, in the meal.[19]

Results and Discussion

In figure 1 are plotted the absorption figures for fat and S^{35} taurine conjugated bile salts calculated from the different intestinal samples. These figures reflect the change in the fat or S^{35} to polyethylene glycol concentration ratio from that of the

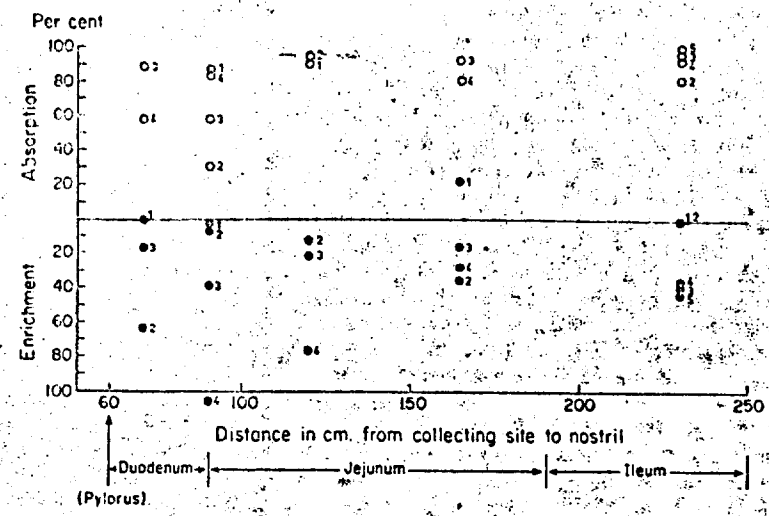

FIG. 1. Extent of absorption and enrichment of fat (○) and bile acids (●) at different levels of the human small intestine. The *figures* beside the *dots* refer to the order of collection of the samples.

test meal. Each point represents a 30-minute collection and indicates the amount of lipid or S^{35} taurine conjugated bile salt already absorbed from that sample.

The figures for fat absorption show extensive absorption in the proximal small intestine. These figures are in agreement with those obtained previously with this technique.[17] It has recently been shown that although some separation of fat and the reference substance occurs in the stomach,[36] this does not significantly affect the results.[37]

The bile salt figures show no absorption, with the exception of one sample. To the contrary, they show enrichment, *i.e.*, the samples of intestinal content contained a higher S^{35} to polyethylene glycol ratio than did the test meal fed. The most obvious explanation for this enrichment in radioactivity is the recirculation of S^{35} taurine conjugated bile salt, such that portions of the test meal passing the sphincter of Oddi are enriched with bile containing S^{35} activity, but no polyethylene glycol. The time course of the enrichment figures are shown in figure 1; in most cases, the percentage of enrichment increased with time.

When it was realized that the S^{35} taurine conjugated bile salt figures were complicated by recirculation, the recirculation experiments were planned. The time of reappearance of radioactivity in these experiments should be independent of the site of its injection, as the total length of intestine traversed is the same for any injection site. In figure 2 are shown the results from the recirculation experiments. As the amount of radioactivity injected was not the same and corrections were not made for dilution, the absolute values of radioactivity have no meaning. The shapes of the curves probably correlate roughly with the amount of radioactivity recirculated as variations in dilution are generally of smaller magnitude than the changes observed in radioactivity. It is apparent that radioactivity appeared within 20 to 30 minutes and was continuing to increase at 1 hour. The curves are somewhat similar despite the injection points being 130 cm. apart. The reappearance of radioactivity when the sample was injected in the distal ileum (230 cm.) indicates that absorption of bile salt can occur at this location or at a more distal site. These figures are in harmony with a previous estimate that the bile salt pool normally circulates twice during the digestion of a meal.[17]

The figures for S^{35} taurine conjugated bile salt absorption in figure 1 are, therefore, the algebraic sum of absorption and enrichment. The recirculation data suggest that little recirculation occurs during the first

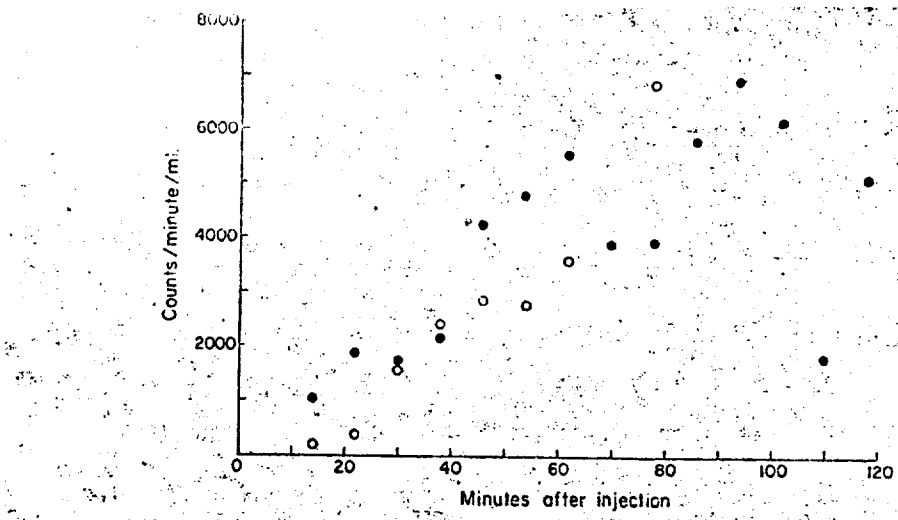

FIG. 2. Activity of intestinal content collected after the injection of a single dose of S^{35}-taurodeoxycholate into the intestine at levels of 100 cm. (●) and 230 cm. (○) from the nostrils. For further explanation see text.

minutes and it might therefore be thought that the first 30-minute collection would be a valid index of bile salt absorption. These samples showed little change in the S^{35} to polyethylene glycol ratio from that of the test meal. The observation that samples collected from the distal jejunum showed enrichment must mean that not all bile salt is absorbed at a more proximal site. For if bile salt behaved as lipid and was completely absorbed by the proximal jejunum, then samples collected in the distal jejunum would never contain radioactivity despite the rate or magnitude of recirculation.

Therefore, the absorption and recirculation experiments show that at least some bile salt is absorbed at a location distal to that where almost complete fat absorption has occurred, and that absorption of bile salts can occur in the distal jejenum or at a more distal site.

The results of the perfusion experiments are shown in table 2. These data provide unequivocal evidence that lipid can be absorbed from a micellar solution in man. The interpretation of this kind of experiment, however, is also complicated by admixing of the perfused solution with nonlabeled lipids and bile salts and at least in experiment N. V. by recirculated bile salt.

TABLE 2

Absorption figures from the three perfusion experiments

The levels of collection have been calculated to correspond to the distance nostril—site of collection before operation. The injection of the samples was performed 30 cm. more proximal.

Experiment	Level	Per cent absorbed		
		Fatty acid	Monoolein	Bile salt
	cm.			
A. E.	110	26	46	18
I. A.	115	12	37	7
N. V.	150	75	58	33 (enriched)

This is obvious from the specific activities of the lipids of the collected samples which in all cases were considerably lower than those of the perfused solution. In all 3 cases, the samples collected were definitely bile colored. The polyethylene glycol values of these samples showed that the solution perfused had been diluted approximately 3- to 5-fold during the passage through the 30 cm. of intestine between its entry and the site of collection.

The data obtained in the present investigation do not necessarily mean that the bile

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BILE ACIDS: A CLASS OF ANOREXIGENIC DRUGS. George Bray* and Thomas Gallagher, Jr.* (spon: Morris Friedkin). Tufts-New England Medical Center Hosps.,* Boston, Mass.

Our experiments in obese subjects have shown that bile acids reduce appetite. In the present studies 20 bile acids and derivatives were tested with intraperitoneal instillation of 100 μmoles/kg in rats trained to eat in 6 hours. Deoxycholic (DC) and chenodeoxycholic (CDC) acids were the most potent inhibitors of feeding. Food intake after one dose of DC or CDC was depressed for 3 days. Conjugation of DC and CDC with taurine or glycine did not reduce potency, however, esterification of C-24 abolished activity. Changing the position or total number of hydroxyl groups in the 5-β cholanic acid molecule altered relative potency. DC and CDC prevented esterification of palmitate by intestinal slices in vitro, but cholic and lithocholic acid did not. Feeding 0.15% of the major bile acids ad lib retarded weight gain in all groups by 14 days, but after 28 days there was no histologic evidence of damage to intestinal mucosa or liver. In conclusion: Bile acids may cause the anorexia of some disease states and this class of compound may have a physiologic role in regulation of appetite.

Lancet I (7551) : 1066-7 , 1968

SUPPRESSION OF APPETITE BY BILE ACIDS

Summary Chenodeoxycholic and deoxycholic acid decrease desire for food in grossly obese patients on a free diet. The effect, which was evident in two comparison studies, suggests both a pathophysiological role and a therapeutic use for these bile acids.

INTRODUCTION

Harris et al.[4] conclusively demonstrated in 1947 that D-amphetamine reduced the food intake of dogs and of volunteers. Since then this drug and its many derivatives have been widely used in the treatment of obesity. With few exceptions[5-7] subsequent anorexigenic drugs have been sympathomimetic amines which have as their principal side-effect stimulation of the central nervous system.[8] The search for safe drugs which reduce appetite has been advanced by the spontaneous observation by some of our patients that certain bile acids reduced the desire for food.

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TABLE I—CLINICAL DATA ON OBESE PATIENTS

Patient no.	Sex	Age (yr.)	Weight (lb.)	Response
*Study 1:**				
1	M	21	440	Suppression
2	M	28	352	
3	F	30	228	
4	F	39	347	
5	F	37	275	No effect
6	F	36	236	
Study 2:†				
2	M	28	352	
3	F	30	238	
7	F	48	281	
8	F	25	314	
9	M	35	440	

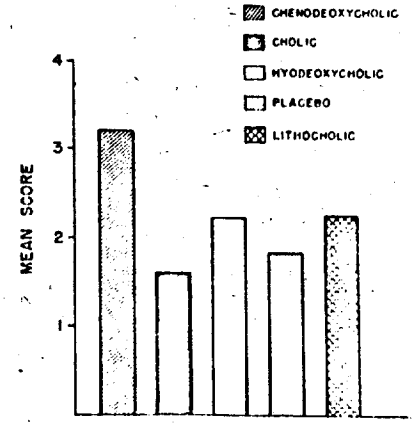
* Alternate deoxycholic acid and placebo.
† Latin square, double blind.

METHODS AND MATERIALS

Two studies were performed (table I).

In the first study deoxycholic acid, 1200 mg. daily in three divided doses, or a corn-starch placebo were alternated at two-week intervals over a three-month period. Each patient was instructed to eat at least 1800 calories of a high-fat diet, and during a careful explanation of the experimental nature of the studies the probability was stressed that steatorrhœa could occur in one alternating interval. Biweekly interviews were conducted with double-blind administration of the capsules for the succeeding two-week period. Six patients continued for the entire three-month period, and two of these were also receiving triiodothyronine throughout the study.

In the second study a double-blind 5 × 5 latin-square design was used to compare four additional bile acids (cholic acid, lithocholic acid, chenodeoxycholic acid, and hyodeoxycholic acid) and a placebo. Three of these patients were receiving triiodothyronine at a constant dose throughout the study, and no specific diet was prescribed during this trial with the free outpatients. Patient no. 8 was studied in the clinical study unit of the New England Medical Center Hospitals where she was provided with more food than she could eat. The quantity of food actually consumed was recorded each day by the dietitian, who had no knowledge of what medication the patient was receiving. The bile acid was given in 400 mg. capsules three times daily for six out of seven days. Each patient ranked the effect of each week's pill on his appetite using a scale of one to



Mean score for the bile acids and placebo in the study using a 5×5 latin-square design.

The maximum mean score would be 5 and the mean minimum score would be 1. Chenodeoxycholic acid was significantly more effective than cholic acid by Student's t test ($P < 0.05$).

TABLE II—EFFECT OF BILE ACIDS ON PATIENT NO. 8

Bile acid	Calorie intake (calories) *	White blood-cells (per c.mm.)	Biochemical tests †					
			Blood-urea-nitrogen (mg./100 ml.)	Bilirubin (mg./100 ml.)	S.G.P.T. (units/100 ml.)	Calcium (mg./100 ml.)	Cholesterol (mg./100 ml.)	Potassium (mg./100 ml.)
Deoxycholic	4446 ± 106	10,400	20	0·1	20	9·4	154	4·9
Chenodeoxycholic	4633 ± 129	7700	17	0·1	..	9·4	183	4·8
Placebo	5900 ± 276	7250	19	0·2	10	10·2	175	5·0
Cholic	5979 ± 192	8500	17	0·2	20	9·5	..	4·8
Hyodeoxycholic	6094 ± 163	9350	18	0·2	10	10·0	..	4·5

* Mean ± S.E.M. for seven days.
† Biochemical tests were done on the morning after the sixth day on each preparation.

ge with "one" meaning no effect and "five" meaning highly effective. Intermediate values were assigned in relation to the ranking by the patient. This ranking was used to score the trial.

Materials

Deoxycholic acid (3α, 12α-dihydroxycholanic acid) was purchased from 'Calbiochem'. Chenodeoxycholic acid (3α, 7α-dihydroxycholanic acid), hyodeoxycholic acid (3α, 6α-dihydroxycholanic acid), and lithocholic acid (3α-hydroxycholanic acid) were purchased from K and K Laboratories. Cholic acid (3α, 7α, 12α-trihydroxycholanic acid) was purchased from Eastman Organic Chemicals. The bile acids were used as received and were prepared in gelatin capsules containing 100 mg. of one of the bile acids.

RESULTS

The first study was designed to test whether deoxycholic acid would induce steatorrhœa when fed to obese patients. During the three months of alternating placebo and deoxycholic acid, four of the six patients spontaneously reported that appetite was reduced part of the time (table I). In retrospect it was clear that this coincided with administration of deoxycholic acid. Two patients, however, did not experience a change in appetite on this dose of deoxycholic acid, even when we told them that the other patients had noted a reduction in the desire for food.

The second study was a double-blind test of four other bile acids using a 5 × 5 latin-square design. The scores for each bile acid are presented in the accompanying figure where the maximum possible score would be 5 and the minimum score 1. In this small study, placebo and cholic acid were indistinguishable. Chenodeoxycholic acid, however, reduced appetite in three of the five patients and was significantly more effective than cholic acid ($P < 0·05$) when compared by Student's t test. Hyodeoxycholic acid and lithocholic acids were intermediate between cholic and chenodeoxycholic acids and were not significantly different from either. In patient no. 8 (table II) food intake rose sharply in the period following chenodeoxycholic acid, and the patient reported to the dietitian that her appetite had increased. Blood tests on this patient are representative, and show no changes from the treatment.

DISCUSSION

These data suggest that certain bile acids may suppress appetite in man. The important correlation of appetite suppression with weight reduction could not be made in either study because of the short periods of observation and the deliberate choice not to restrict or control food intake. However, in animal studies bile acids reduced weight gain and decreased food intake. In experiments to be reported in detail elsewhere,[9] a large number of bile acids and their derivatives were fed or injected into rats, and some bile acids have been fed to volunteers in hospital under carefully controlled conditions. No untoward changes in chemical tests of hepatic, renal, or haematopoietic function were found after feeding bile acids for two to twenty weeks. Examination by light microscopy of the upper and lower intestine of rats fed all the bile acids used in the present study showed no histological changes. Similar examination of peroral intestinal biopsy specimens of obese patients after two, six, and twenty weeks of treatment with bile acids showed no changes from control.[10]

In rats the greatest inhibition of food intake was produced by deoxycholic acid and chenodeoxycholic acid or their taurine and glycine conjugates. Cholic acid and lithocholic acid were less effective, while their taurine and glycine salts were ineffective in the test system used. These observations in the rat parallel those noted in table I and the figure and raise the possibility that bile acids may have a physiological role in the control of appetite. The anorexia of patients with some forms of liver disease may also be explained by raised levels of bile acids in their blood.

We thank Dr. E. B. Astwood for his continuing support and encouragement, and Dr. T. Colton, of the Harvard Medical School, for his help with statistical evaluation.

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9. Bray, G. A., Gallagher, T. F., Jr. *Fedn Proc. Fedn Am. Socs exp. Biol.* April, 1968.

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Weight Gain and Intestinal Histology of Rats Fed Cholic, Lithocholic, Hyodeoxycholic, Chenodeoxycholic, and Deoxycholic Acids* (33515)

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Studies from this laboratory showed that bile acids inhibit food intake in rats (1) and reduce appetite in man (2), but the mechanism for this effect is unknown. One possibility is that they have a direct effect on the intestinal mucosa with the eventual production of histologic alterations. To test this, we investigated the effect of five bile acids on the reesterification of fatty acids by slices of intestinal mucosa and examined the histology of intestine from rats fed bile acids for 4 weeks.

Methods and Materials. Esterification of oleate-1-^{14}C was determined as described by Dawson and Isselbacher (3). Two feeding experiments were performed:

I. Six groups of 7 male Gofmoor rats weighing 55–70 g were placed in stainless steel wire bottom cages and fed a semisynthetic diet (group I) or a diet fortified with 1.5 g of one of the 5 bile acids for each kg of diet. Body weight of each rat was recorded two to three times weekly and at the end of 26 days, the rats were anesthetized with ether and bled from the abdominal aorta. The intestine, liver, heart, gonads, and thyroid were dissected free, weighed, and sections of heart, liver, stomach with duodenum, and ileum with colon were fixed in both 10% formalin and Zenker's solution. Histological sections from the fixed tissues were prepared and stained with hematoxylin and eosin.

II. Two groups of 6 male rats, with mean weights of 244 and 250 g, were fed a semisynthetic diet, with or without 0.15% deoxycholic acid, for a period of 120 days with frequent measurement of body weight.

Materials. Cholic acid (3α,7α,-12α-trihydroxycholanic acid) was obtained from Eastman Organic Chemicals. Deoxycholic acid (3α,12α-dihydroxycholanic acid) was purchased from Calbiochem. Lithocholic acid (3α-hydroxycholanic acid) and chenodeoxycholic acid (3α, 7α-dihydroxycholanic acid) were purchased from K and K Laboratories and hyodeoxycholic acid (3α,6α-dihydroxycholanic acid) was purchased from Mann Research Laboratories. In the feeding experiments the bile acids were used as supplied, but for the experiments on reesterification they were recrystallized from ethylacetate (chenodeoxycholic acid) or 70% ethanol.

Results. Esterification. Lithocholic and cholic acid were without effect on the incorporation of oleate into lipid by slices of rat intestine at any concentration from 0.01 to 10 m*M*. While chenodeoxycholic acid, hyodeoxycholic acid, and deoxycholic acid all significantly reduced the incorporation of oleic-1-^{14}C into lipid by intestinal slices at 10 m*M* (Fig. 1), hyodeoxycholic acid and deoxycholic acid also reduced esterification at 1 m*M*. At lower concentrations none of the bile acids diminished esterification, or reduced the up-

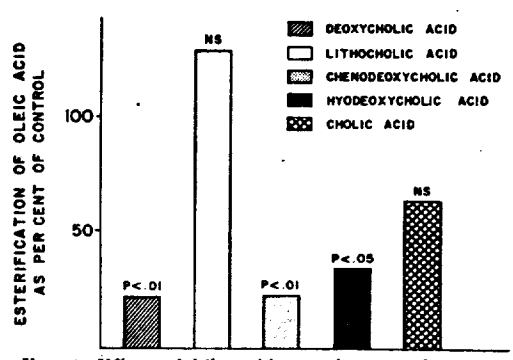

FIG. 1. Effect of bile acids on the esterification of oleate-14C by intestinal slices of the rat. Each bar is the mean of eight replicates incubated with a 10 m*M* concentration of bile acid as described in the text. Three bile acids significantly decreased esterification and two did not.

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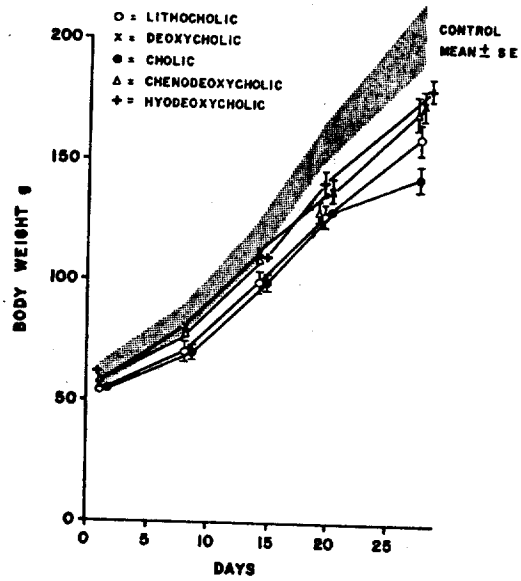

FIG. 2. Effect of bile acids on weight gain of rats. Male albino rats were fed a diet with 0.15% of bile acid. The mean± 2 SE are shown for the control group ($n = 7$) but only the mean for the other 5 groups.

take of radioactive oleic acid by the intestinal slices.

Feeding experiments. The mean body weight of the six groups of rats used in the first feeding experiments were identical at the beginning of the experiment. After the first week, the rats on lithocholic and cholic acids weighed significantly less (Fig. 2). By the end of 3 weeks, the mean weight of all rats receiving bile acids was reduced. The weight of the liver per 100 g of body weight was reduced in rats receiving cholic acid, but the other bile acids had no effect on the weight of the liver (Table I). The weight of the heart per 100 g of body weight was significantly increased in the rats fed cholic and lithocholic acids, but not by the other bile acids. The weight of the gonads and thyroid were unaffected by any of the bile acids. Histologic sections of heart, liver, duodenum, ileum, and colon were examined by Dr. Ronald Hunt, Veterinary pathologist at the New England Regional Primate Center, who was unable to find any evidence of alteration in the histology of these organs as a result of feeding bile acids.

Deoxycholic acid, 0.15%, was fed to six rats for 120 days and the rate of weight gain was slowed for the first 30 days, but there was no detectable effect of the bile acid during the remaining 90 days.

Discussion. Deoxycholic acid is widely used by biochemists to solubilize membranes and it was shown by several groups (3–5) to reduce esterification of fatty acids by intestinal slices *in vitro*. Our data confirm this effect at 1 and 10 m*M* concentrations and show in addition, that at 10 m*M* concentrations, chenodeoxycholic acid and hyodeoxycholic acid also inhibit esterification. Lithocholic and cholic acid had no effect on esterification at any concentration up to 10 m*M*. Since all five bile acids were comparably effective in reducing weight gain, this would suggest that the effect of the dihydroxy bile acids on esterification are not directly related to the mechanism of reduced food intake.

Dawson and Isselbacher (3) showed that when intestinal slices from rats were incu-

TABLE I. Effect of Bile Acids on the Body Weight and the Weight of the Heart and Liver.[a]

Bile acid	No. of animals	Body wt.	Liver (g)	Wt. (g/100 g of body wt.)	Heart (mg)	Wt. (mg/100 g of body wt.)
None	5	205 ± 6[b]	8.3 ± 0.3	4.05 ± 0.15	809 ± 52	390 ± 25
Lithocholic	7	161 ± 6	6.3 ± 0.3	3.91 ± 0.16	740 ± 21	460 ± 13
Deoxycholic	5	165 ± 7	6.9 ± 0.3	4.18 ± 0.24	700 ± 10	420 ± 10
Cholic	7	144 ± 5	5.1 ± 0.2	3.52 ± 0.19	653 ± 18	450 ± 12
Chenodeoxycholic	7	172 ± 7	6.5 ± 0.4	3.75 ± 0.25	717 ± 24	420 ± 14
Hyodeoxycholic	7	181 ± 5	7.2 ± 0.4	3.98 ± 0.20	818 ± 74	450 ± 15

[a] Body and organ weights were measured after 26 days on the bile acids.
[b] Mean ± SEM.

bated with 1 to 5 m*M* deoxycholate, histological damage ensued. Fry and Staffeldt (6) observed histological changes within 8 days in the mucosa of mice fed 2% deoxycholate, but these changes had almost completely resolved after 38 days. Ductular cell hyperplasia of the liver can be readily produced by feeding lithocholic acid to chickens or rabbits (7, 8) and production of similar lesions was observed in rats fed high concentrations of lithocholic acid (9, 10). In our experiments none of the bile acids altered hepatic architecture or produced detectable histological changes in the intestine. This confirms the recent findings of Dawson (11) who failed to produce histological changes in the gut of rats fed deoxycholate *in vivo*. Yet our diet, containing 0.15% of bile acid significantly reduced the rate of weight gain implying that the effect on weight gain occurs without detectable histological alterations.

Retarded weight gain has been reported previously (12, 13) and Visek *et al.* (13) observed that each 0.1% increment in the concentration of deoxycholic acid in the diet produced a 10% reduction in weight gain. We have demonstrated that the parenteral injection of chenodeoxycholic acid and deoxycholic acid acutely reduce food intake in the rat (1) again making it unlikely that a toxic effect on the intestinal mucosa was involved.

Summary. Deoxycholic, hyodeoxycholic, and chenodeoxycholic acid at 1 or 10 m*M* inhibited esterification of oleate *in vitro*, by slices of intestinal mucosa, but lithocholic and cholic acid did not. All five bile acids at a concentration of 0.15% in the diet reduced weight gain over a 30-day period, but none of them produced any distologic alteration in the intestinal mucosa.

The authors thank Dr. E. B. Astwood for his generous support, and Dr. Ronald Hunt for his expert assistance in reviewing the histologic sections. Mrs. Nancy Fortin and Miss Andrea Siegal rendered expert technical assistance.

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THE BINDING OF CHOLIC ACID AND ITS TAURINE CONJUGATE TO SERUM PROTEINS

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SUMMARY

By the method of steady-state gel filtration, cholic and taurocholic acids have been shown to be strongly bound by plasma albumin. Cholic acid is more avidly bound than its taurine conjugate. There is probably one primary binding site per albumin molecule, and approximately 7 secondary sites of weaker affinity. Plasma has a very high binding capacity for bile acids and the binding shows rapid equilibration.

In the stagnant loop syndrome, protein binding of unconjugated bile acids may be a factor contributing to their high concentration in plasma.

INTRODUCTION

The concentrations of the individual bile acids in normal serum have recently been ascertained[1]. In health, only conjugated acids are detectable; but in intestinal malabsorption due to the stagnant loop syndrome, high concentrations of unconjugated bile acids are also found[2]. The mechanism of this abnormality is of some interest. Intestinal absorption of bacterially deconjugated bile acids might be responsible; if so it remains necessary to account for their presence in peripheral blood, in the face of their low water solubility and of the avid uptake by the liver of unconjugated cholic acid, in normal circumstances[3]. A possible explanation is the selective protein binding of bile acids in plasma.

The binding of several bile acids to plasma proteins was shown in 1957 by Rudman and Kendall, using equilibrium dialysis of bile acids in protein solution. These experiments were made at bile acid concentrations approximately 50–100 times those present in normal plasma; they used 1% solutions of Cohn-fractionated proteins and the binding was observed at 5°. They found albumin to have the greatest avidity for bile acids, though some globulin fractions, too, bound deoxycholic acid under these conditions. The molar binding ratios of free and conjugated bile acids were found to be similar. Because of the high bile acid concentrations in these experiments the findings may have been influenced by the properties of secondary binding sites.

We have measured bile acid–protein interactions in whole serum at 37°, by the method of steady-state gel filtration. We have found the binding of cholic acid to be much tighter than that of taurocholic acid, and believe this to be a factor in maintaining the high concentrations of unconjugated cholic acid found in peripheral blood in the stagnant loop syndrome. All the binding of both acids to human serum may be accounted for by albumin, with relatively fast binding kinetics.

METHODS

Total concentrations of cholic acid and taurocholic acid were measured by extraction, chromatography and fluorimetry as previously described[1].

The binding of these acids to serum proteins was measured by steady-state gel filtration[5]. The principle is that a large sample of equilibrium mixture is passed continuously into a gel filtration column until a steady state is reached. In the steady state, faster moving bile acid–protein complexes and free protein migrate in equilibrium with slower moving free bile acid. The concentrations of complexes and free bile acid are then estimated by displacement.

Water-jacketed columns of Sephadex G-50, 0.9 by 20 cm (except where indicated) were maintained at 37°. They were packed, washed and eluted with 0.15 M sodium phosphate buffer, pH 7.4. All samples applied to the columns were equilibrated with tracer amounts (400–700 ng) of the appropriate [^{14}C]bile acid for 30 min at 37° under 7% CO_2 before gel filtration. The pH of column effluents was at all times between 7.4 and 7.7. by the micro-Astrup procedure. Completeness of equilibration on the column was shown by constancy of the elution profile of serum samples containing each bile acid at elution rates ranging from 0.3 to 0.8 ml/min; the elution rate used for quantitative work was 0.55 ml/min. Ten ml samples of serum of 4% w/v human serum albumin in phosphate buffer were applied to the columns for quantitative work, after determination of the bile acid content. The bile acid concentration in the effluent was followed by ^{14}C counting of 1-ml fractions. Effluent protein concentrations were measured by the method of Lowry *et al.*[6]. A typical steady-state elution profile is shown in Fig. 1; this was an experiment with albumin and taurocholic

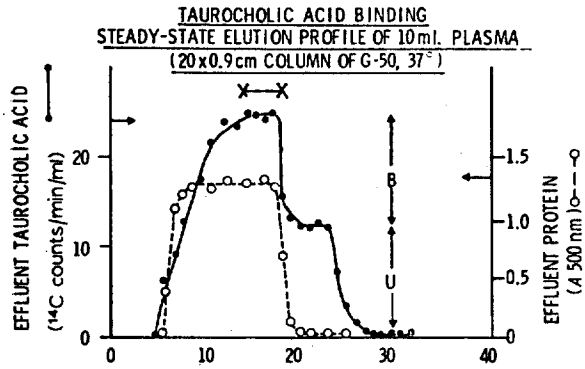

Fig. 1. Steady-state gel filtration of taurocholic acid in 4% (w/v) human albumin, in 0.15 M sodium phosphate (pH 7.4) at 37°. ●—● = effluent taurocholic acid concentration, ○------○ = effluent protein concentration. x--x denotes equilibrium region. 10 ml sample. Sephadex G-50

acid. The concentrations of bound and unbound taurocholic acid are indicated as B and U respectively.

Four principal conditions require satisfaction before steady-state gel filtration can be accepted as a valid method of measuring binding of a particular ligand; all four were satisfied for cholic and taurocholic acids. First, a truly steady state was achieved, for the profile was independent of elution rate. The profile was also independent of sample size, identical concentration plateaux being observed with samples of 10, 15, 20 and 30 ml; this is further evidence of attainment of equilibrium. Second, a single sharp trailing boundary of protein-bound radioactivity was always observed to be coincident with a single sharp trailing boundary of protein, indicating equality of migration rate of the complex and the free protein. Third, the slopes of the boundaries were independent of bile acid concentration, indicating that the elution volume of bile acids is not concentration dependent. It is necessary to show this since concentration dependence of elution volume may falsify the observed unbound concentration[7]. Fourth, there was no absorption of cholic or taurocholic acids to the columns; recovery of ^{14}C was close to 100% and the elution volume of both acids in serum was the same as the salt volume of the column, as is the case with neutral steroids[8].

The human serum albumin used was obtained from Behringwerke (RHA 05) and used without further purification. The serum used was a large pool obtained from several normal subjects in the fasting state. [^{14}C]Cholic acid and [^{14}C]taurocholic acid were obtained from New England Nuclear Corporation. Stable cholic and taurocholic acids were obtained from Maybridge Research Chemicals. Their chromatographic purity was confirmed by thin-layer chromatography.

RESULTS

Rapidly equilibrating binding of bile acids to serum

Fig. 2 shows the results of simple gel filtration of cholic and taurocholic acids in human serum. In the experiment shown in Fig. 2(a), a small serum sample (5 ml) was labelled by equilibration with [^{14}C]taurolcholic acid at 37° for 30 min under 7% CO_2, and subjected to gel filtration under the conditions shown in the legend. The taurocholic acid was strongly protein bound, for 45% of the applied taurocholic acid was eluted with the proteins. This degree of protein binding is rather more than would be predicted from Rudman and Kendall's[4] data; calculation from values in their Table II shows 17.3% of taurocholic acid was bound to protein in their system.

The shape of the elution profile suggests change of the binding equilibrium on the column, because taurocholic acid free from protein is present in the trough between the two peaks. The taurocholic acid in the trough (shaded) was eluted before the free taurocholic acid peak, because it was released from complex with protein at various levels in the column. This implies rapid equilibrium change on the column and is characteristic of a rapidly equilibrating binding system. A detailed interpretation of this phenomenon of "peak skewing" in a very similar elution profiele (cortisol binding) is given elsewhere[5].

Fig. 2(b) shows the result of a similar experiment with cholic acid. The binding of cholic acid was even stronger than that of taurocholic acid; over 90% of the cholic acid emerged from the column with protein (*cf.* the value of 65% of cholic acid bound to protein which can be computed from Rudman and Kendall's data). Moreover the

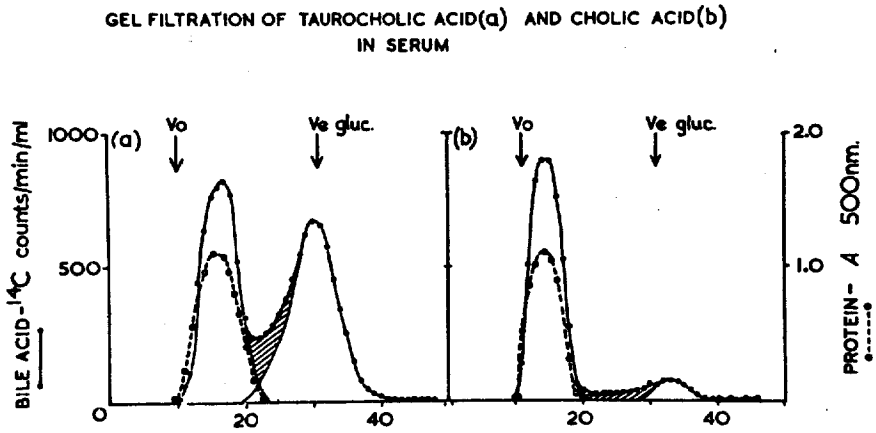

Fig. 2. Non-steady-state gel filtration of [^{14}C]-taurocholic acid (a) and [^{14}C]-cholic acid (b) in 5 ml serum; pH 7.4 at 37°. Column G-50 24 × 1.1 cm. Vo = column void volume; Ve gluc. = elution volume of glucose. ●—● = effluent ^{14}C concentration, ○------○ = effluent protein concentration. Boundaries of shaded area constructed as mirror images of leading and trailing boundaries.

elution profile is of similar type to that found with taurocholic acid, again indicating equilibrium change on the column.

Because of this evidence for equilibrium change, the binding of cholic and taurocholic acids to whole human serum and to albumin was studied at equilibrium and at various bile acid concentrations.

Cholic and taurocholic acid binding to serum proteins at equilibrium

The binding of cholic acid was studied in human serum and in 4% human albumin solution. [^{14}C]Cholic acid (400–700 ng) was equilibrated with fasting serum or with 4% (w/v) human albumin in 0.15 M phosphate buffer (pH 7.4) for 30 min at 37°, at various cholic acid concentrations from about 0.19 μM to 3 mM (over 1000 times physiological), and 10-ml samples were subjected to steady-state gel filtration at 37°. (For comparison, the normal serum concentration of taurocholic acid is 0.12–0.72 μM, and the normal serum concentration of cholic acid is below the limit of sensitivity

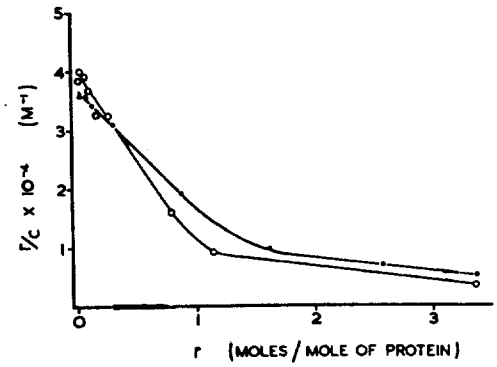

Fig. 3. Binding of cholic acid in fasting human serum (closed circles) and in 4% (w/v) human albumin in phosphate buffer (open circles). 37°. r = moles of steroid bound per albumin molecule. c = unbound steroid (M).

of the method used, *i.e.* less than 0.31 μM[1]). From the ^{14}C plateaux, the average number of molecules of cholic acid bound per albumin molecule present (r) and the molar concentration of unbound cholic acid (c) were calculated and the results plotted by the method of Scatchard[9] (Fig. 3). With a single class of binding sites this manoeuvre yields a straight line.

Inhomogeneity of the binding sites for cholic acid is well shown by the non-linearity of the Scatchard plot. The line cannot be extrapolated to obtain K_s, the equilibrium constant, or n, the number of binding sites per protein molecule. However, graphical analysis of the line by the method of Rosenthal[10] is compatible with two classes of cholic acid binding sites on albumin. It suggests a primary class of sites of K_e approximately 3.2×10^4 M^{-1}, with n approximately 1.2 per albumin molecule; and a secondary class, operating at much higher cholic acid concentration (over 1 mM) of K_e about 2×10^3 M^{-1} and n approximately 6.5. It was in the latter range of high concentration that Rudman and Kendall performed their experiments, and in this range our results are similar to theirs. But our data reveal in addition the existence of the primary sites, whose affinity is an order of magnitude higher and which operate at serum cholic acid concentrations likely to be seen, for example, in the stagnant loop syndrome. (Note that Rudman and Kendall's K is the reciprocal of K_e; K for cholic acid secondary sites calculated from our data is 5×10^{-4}, very similar to the value of 6.5×10^{-4} found by them).

Fig. 3 also shows the binding of cholic acid to whole fasting human serum. In this case r was calculated as if cholic acid was bound solely to albumin (*i.e.* r = moles of cholic acid bound to serum ÷ moles of albumin present). The resulting Scatchard plot is virtually identical with that for 4% albumin solution. This suggests that cholic acid binding to whole serum can be accounted for by albumin. It becomes unnecessary to postulate a serum binding protein other than albumin for cholic acid. In serum the apparent K_e for primary binding sites for cholic acid is 2.8×10^4 M^{-1}, with n approximately 1.4.

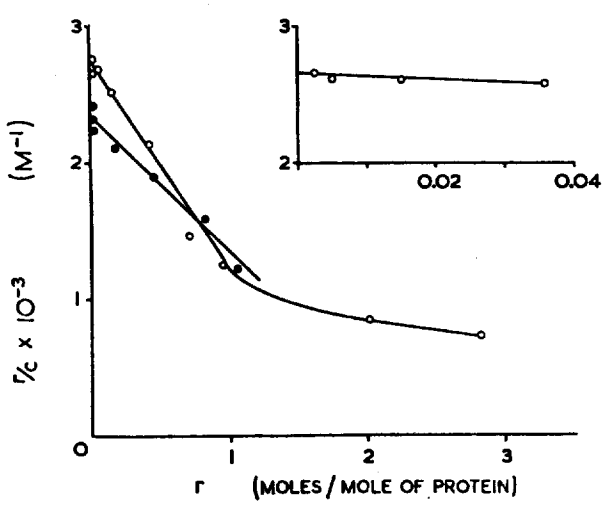

Fig. 4. Binding of taurocholic acid in fasting human serum (closed circles) and in 4% human albumin (open circles) at 37°. Inset—expanded scale to show near-linearity of graph near r/c axis.

Similar data for taurocholic acid are shown in Fig. 4. In this case, the affinity of binding is about 15 times weaker; K_e for the primary sites on albumin is approximately 2.0×10^3 M^{-1} and n about 1.2 per molecule. The binding may be accounted for by one primary site with this K_e, together with secondary sites of K_e about 1.4×10^2 M^{-1} and n approximately 7. Again, at taurocholic acid concentrations above 1 mM, we have found secondary binding sites with characteristics similar to Rudman and Kendall's results at high concentrations, but at lower concentrations we have found stronger binding.

The binding of taurocholic acid to whole human serum is also shown in Fig. 4. As with cholic acid, the binding curve is the same with serum as with 4% albumin solution; it is not necessary to postulate a binding protein other than albumin for either cholic or taurocholic acids. Values for apparent primary taurocholic acid binding sites in serum are K_e approximately 1.6×10^3 M^{-1} and n approximately 1.4 per albumin molecule.

The values obtained for the binding constants are summarised in Table I.

TABLE I

EQUILIBRIUM CONSTANTS AND BINDING SITES ON ALBUMIN FOR CHOLIC AND TAUROCHOLIC ACIDS
Calculated from observed binding data by method of Rosenthal[10].

	Cholic acid		*Taurocholic acid*	
	K_e (M^{-1})	*No. of sites/mole*	K_e (M^{-1})	*No. of sites/mole*
Primary sites	$3.2 \cdot 10^4$	approx. 1.2	$2.0 \cdot 10^3$	approx. 1.2
Secondary sites	$2.0 \cdot 10^3$	approx. 6.5	$1.4 \cdot 10^2$	approx. 7.0
Secondary sites (values given by Rudman and Kendall)	$1.43 \cdot 10^3$	approx. 4		

Conclusions; implications of albumin binding of cholic and taurocholic acids

Two conclusions may be drawn from these experiments.

1. The binding of cholic acid by serum albumin is very much more avid than that of taurocholic acid (K_e 15 times greater). This might be expected by analogy with the binding of neutral steroids; conjugated neutral steroids are generally less strongly bound to albumin than the free parent steroid. In general, the avidity of albumin for steroids is inversely related to their polarity[11,12]. Indeed Rudman and Kendall found this to be true of hydroxylated bile acids. The physiological importance of the stronger protein binding of free cholic acid, as compared with the taurine conjugate, is discussed below.

2. The binding of cholic and taurocholic acids in serum appears to be entirely to albumin in our experiments. We did not find evidence of significant globulin binding as suggested by Rudman and Kendall[1] at high concentrations.

Binding to albumin implies a very high binding capacity, for the concentration of albumin in human serum is of the order of 500 μM, contrasting with the normal range for total serum bile acids of 2.2–4.5 μM. The highest pathological level we have seen (in obstructive jaundice) was 285 μM. As there is at least one binding site per albumin molecule, and there are more numerous secondary sites, the capacity of serum for bile acids is very large. Because of the large capacity, we should expect the percentage binding of bile acids to be little affected by changes in their concentration. This is illustrated in Fig. 5; the percentage binding both to albumin and whole serum

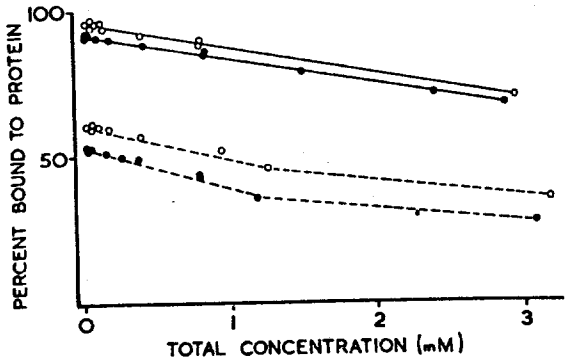

Fig. 5. Percentage binding of cholic acid (——) and taurocholic acid (------) as function of their total concentrations at 37°. Closed circles = fasting human serum; open circles = 4% human albumin in phosphate buffer.

of cholic and taurocholic acids is little affected by the concentrations of the latter, especially at near-physiological concentrations.

Results in patients with high levels of endogenous bile acids

Two patients with stagnant loop syndrome due to diverticulosis of the small intestine were studied. Their serum albumin concentrations were 3.4 and 3.2 g/100 ml. Very high levels of conjugated bile acids were present in their serum (Table II), but the protein binding of cholic and taurocholic acids was entirely normal in both. For example, cholic acid in both was over 95% bound, and taurocholic acid about 50% bound. This normal binding at high concentration would be expected in view of the large binding capacity of serum for both acids.

DISCUSSION

We have confirmed Rudman and Kendall's observation of bile acid binding by plasma proteins. At lower concentrations including physiological ones, however, the binding is much stronger than their data suggest. The primary site for cholic acid in serum is capable of binding about 95% of the total cholic acid concentration present, leaving only 5% available at equilibrium for free diffusion into tissues, *e.g.* the liver and skin.

By contrast, about half of the total taurocholic acid concentration in serum is available for free diffusion at equilibrium—ten times as much as in the case of cholic acid. If this finding of greater binding affinity for cholic acid than for taurocholic acid is applicable to other free and conjugated bile acids, it may be relevant in under-

TABLE II

SERUM BILE ACID BINDING IN TWO PATIENTS WITH THE STAGNANT LOOP SYNDROME

	Cholic acid		*Taurocholic acid*	
	Total concn. (μM)	*% bound*	*Total concn. (μM)*	*% bound*
Patient 1	4.8	93.4	<0.12	43
Patient 2	3.2	92	<0.12	43
Normal	<0.31	90–95	0.12–0.72	46–49.5

standing some disorders affecting bile acid metabolism. For example, in the stagnant loop syndrome, remarkably high concentrations of unconjugated bile acids may be present in serum, far exceeding the levels of conjugated acids[2]. This occurs despite the normally rapid uptake of cholic acid by the liver[3]. The stronger protein binding of unconjugated bile acids may be a factor contributing to their high serum concentrations in this syndrome. The absence of pruritus in the stagnant loop syndrome, despite high plasma concentrations of unconjugated bile acids, may also be related to their plasma protein binding; for this symptom is common in obstructive jaundice where an excess of less strongly protein-bound conjugated bile acids is found.

Strong binding of bile acids by serum albumin may also be of importance in facilitating their transfer from the intestinal mucosal cell into portal blood. Relatively little is known about this phase of bile acid absorption, in contrast with the well-documented mechanisms for transfer from intestinal lumen to mucosal cell, chiefly active transport[13] and passive nonionic diffusion[14].

ACKNOWLEDGMENT

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Production of Tumours in Mice by Deoxycholic Acid

J. W. COOK[1] pointed out that the sterols and bile acids contain in their molecules condensed carbon-ring systems to which are attached a side-chain in such a position that a new 6-membered ring can be formed so as to give the 1 : 2-benzanthracene ring system without molecular rearrangement or group migration. The bile acids of the higher vertebrates are all mono-, di-, or tri-hydroxy derivatives of cholanic acid, which compound can be obtained *in vitro* from sterols by degradation, and the principal bile acids bear the names of lithocholic acid (3-hydroxycholanic acid), deoxycholic acid (3 : 12-dihydroxycholanic acid) and cholic acid (3 : 7 : 12-trihydroxycholanic acid). J. W. Cook and G. A. D. Haslewood in 1934[2] showed that, in the formation of dehydronorcholene from deoxycholic acid by the procedure of Wieland, such a ring-closure to the 1 : 2-benzanthracene ring system had actually occurred. Dehydronorcholene gave on dehydrogenation the benzanthracene hydrocarbon methylcholanthrene, which was found to be strongly carcinogenic. Afterwards, Fieser and Newman[3] showed that methylcholanthrene could be obtained also from cholic acid, which is the chief acid of human bile, and the parent hydrocarbon cholanthrene was synthesized by J. W. Cook, G. A. D. Haslewood and A. M. Robinson[4] and shown to be carcinogenic.

The discovery that a carcinogenic compound could be obtained from deoxycholic acid led us to test for possible action of this kind the acid itself, which was applied in solution in a mixture of alcohol and benzene to the skin of 20 mice in the ordinary way. An epithelioma at the site of application developed in a single mouse in 776 days. The experiment was repeated upon, in all, 80 mice, of which some lived more than 800 days, but no more tumours were obtained. Afterwards, deoxycholic acid was injected in sesame oil into the right flank of 10 mice of mixed stock, of which 5 lived for more than 6 months. Of these five, three developed at the site of injection spindle-celled tumours of the type usually produced by carcinogenic compounds and were killed on the 351st, 355th, and 367th days. The tumours were regarded as malignant on histological grounds, but did not grow when transplanted into other mice of mixed stock. These mice had received 15 injections containing in all 70 mgm. of deoxycholic acid in 300 days. This is, of course, a large amount, and the time required for the development of tumours is long. However, the experiment was repeated using 10 mice of the strain C_3H which, of eight strains examined by Andervont[5], is placed first in the order of susceptibility to subcutaneous tumours induced by carcinogenic hydrocarbons, and of these mice one has now developed a spindle-celled tumour in 155 days (that is, a period less by 200 days than that required by the stock mice) after receiving a total of 28 mgm. in the course of six injections. These results confirm those of Vittorio Ghiron[6], announced at the Third International Cancer Congress at Atlantic City in September 1939, who says "Desoxycholic acid elicited transplantable subcutaneous fibro-sarcomas in a high proportion of the mice and rats injected. This is believed to be the first experimental production of malignant growths with a compound that exists under some conditions in the human body". We have not found any more detailed publication by Ghiron of his results.

Occasionally, tumours are produced in mice by sesame oil alone (W. U. Gardner, personal communication to Dr. Hieger) but we have obtained negative results with many compounds in this solvent, and we do not think that the incidence of tumours recorded above could be attributed to it. We have no evidence that deoxycholic acid can be converted *in vivo* into methylcholanthrene, but at the same time the slow action and large amount required of the acid, and the superiority of injection over external application, are compatible with conversion into some more active compound. Shear[7] obtained negative results by injection of deoxycholic acid, and cholic acid, *sub cutem* in mice, but a single injection only, in glycerol, was given. We are carrying out further experiments with deoxycholic and cholic acids.

Several other investigations in progress here are concerned with the liver in relation to carcinogenesis. Thus Dr. L. D. Parsons[8] has described the increased flow of bile in some tumour-bearing mice, and the use of bile in preparing cell-free filtrates from tumours; Dr. Hieger has confirmed the discovery of Schabad[9] that some extracts of human livers will produce sarcomas in mice, and is preparing these results for publication; and J. W. Cook[10] has described compounds (2 : 2′-azonaphthalene and its transformation product 2 : 2′-diamino-1 : 1′-dinaphthyl) which have a specific action in producing malignant tumours of the liver.

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Effect of Bile Acids on Serum Cholesterol in the Chick.*† (26741)

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Recently Beher *et al.*(1) and Howe *et al.* (2) have reported that several bile acids (hyodeoxycholic acid, chenodeoxycholic acid and lithocholic acid) counteract the hypercholesterolemic effect of cholic acid in mice and prevent cholesterol accumulation in the liver and carcass. Portman and Bruno(3) have reported similar findings with rats. Experimental work on the effect of cholic acid or any other bile acids on serum cholesterol levels in chickens has not been carried out. Since the chicken is known to secrete quite different bile acids than the mouse and rat, it was desirable to investigate the effect of feeding various bile acids on serum cholesterol in chickens.

Experimental. The experiment was conducted with day-old cross-bred cockerel chicks (Cornish male X White Plymouth Rock female) obtained from a local hatchery. They were housed in electrically heated batteries with wire floors and were supplied feed and water *ad libitum*. They received the experimental diets for 3 weeks and were weighed at weekly intervals during this period. At termination of the growth phase of the experiment, blood samples were obtained by heart puncture and the serum separated for total cholesterol determination by the method of Schoenheimer and Sperry(4). The cholic acid, lithocholic acid and hyodeoxycholic acid used in the experiment were obtained from Nutritional Biochemicals Corp. These bile acids were added to the diet at the 0.2% level singularly and combinations of cholic and lithocholic acids, and cholic and hyodeoxycholic acids. The basal diet used was similar to that used in earlier work(4), modified to contain 4.0% corn oil. All substitutions in the diet were made at the expense of fiber.

Three groups of 8 chicks each were started on each of the dietary treatments. The results were analyzed for statistical differences by the Multiple Range Test(5).

Results. The effect of feeding the various bile acids on growth and serum cholesterol is shown in Table I. Addition of lithocholic acid to the basal diet caused a marked depression in growth rate. When cholic acid was added to the diet containing lithocholic acid this retardation in growth rate was reversed. Addition of cholic acid, hyodeoxycholic acid or a combination of these 2 bile acids to the basal diet had no statistically significant effect on growth rate of cockerels. However, it should be noted that rate of growth was faster when both hyodeoxycholic acid and the combination of hyodeoxycholic acid and cholic acid were added to the basal diet.

While lithocholic acid depressed growth, it caused an approximately 3-fold increase in serum cholesterol level of the cockerels. Addition of cholic acid to the diet containing

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TABLE I. Effect of Bile Acids on Growth and Serum Cholesterol of Chicks.

Supplements to basal diet	Avg wt 3 wk, g	Cholesterol/100 ml of serum, mg
None	307 ± 7	178 ± 7
.2% cholic acid	298 ± 7	181 ± 7
" lithocholic acid	176 ± 8	575 ± 26
" hyodeoxycholic acid	319 ± 9	189 ± 9
" cholic acid + 2% lithocholic acid	294 ± 11	298 ± 15
" cholic acid + 2% hyodeoxycholic acid	330 ± 9	169 ± 7

Grouping of treatment results*

Body wt	176	294	298	307	319	330
Serum chol.	169	178	181	189	298	575

* Two values not underscored by the same line are significantly different ($P < .05$).

lithocholic acid appears to have caused a reduction in this hypercholesteremic condition. However, the serum cholesterol level was still approximately twice the normal value. Addition of cholic acid, hyodeoxycholic acid or a combination of the two did not affect serum cholesterol significantly as measured by the statistics applied. A small, non-significant drop in serum cholesterol was observed in chicks receiving the combination of cholic acid and hyodeoxycholic acid.

Discussion. The results indicate in light of other studies that striking species differences may be expected with respect to the effect on serum cholesterol when various bile acids are added to the diet. Howe *et al.*(2) found that lithocholic acid was very effective in lowering serum cholesterol in mice, while the results reported here with chickens are the reverse. It seems possible that this may be caused by differences in the type of bile acids excreted by the 2 species. Haslewood and Wootton(6) reported finding only cholic acid in the mouse (*Mus musculus*), while Japanese workers (reviewed by Anderson, Haslewood and Wootton(7)) have found a large array of bile acids in the domestic chicken. They are chenodeoxycholic acid, isolithocholic acid, tetrahydroxynorsterocholanic acid, 3 α-hydroxy-7-oxocholanic acid and 3-oxochola-4:6-dienic acid. No investigations have been conducted with the chick on production of bile acid artifacts by circulation of the original "native" bile salts through the enterohepatic circulation, or by intestinal micro-organisms. Therefore, it cannot be known exactly which of the above bile acids are "natural" to the domestic chicken. If regulation of bile acid production in the domestic fowl is a homeostatic process as indicated for the rat(8), it may be that lithocholic acid interferes with absorption of chenodeoxycholic acid or some other bile acid that plays a part in the homeostatic control in chicks, or the lithocholic acid itself may be absorbed and play a role in the homeostatic control in the chicken.

Portman and Bruno(9) have reported on the effects of feeding a number of bile salts to rats. They found that all the bile acids tested that had serum cholesterol-elevating activity contained a 12α-hydroxy grouping. Their results are in close agreement with those of Howe *et al.*(2) with respect to activity of various derivatives of cholic acid to raise the serum cholesterol of mice. It is obvious from the results presented in this paper that the theory that bile salts must have a 12α-hydroxy grouping to have serum cholesterol-elevating activity will not apply with the domestic chicken.

Summary. Addition of cholic acid to the diet of chicks does not cause a rise in serum cholesterol as has been reported in mice and rats. Addition of lithocholic acid to the chicken diets caused a marked increase in serum cholesterol. This effect is opposite to that reported to occur in mice and rats.

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OBSERVATIONS ON FEEDING CHOLIC ACID TO BROILERS

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It has long been known that bile has an important role in the digestion and absorption of lipids from the gastro intestinal tract of animals. An excellent review of the role of bile in lipid absorption is given by Deuel (1955). In view of the recent practice of incorporating large quantities of lipids in chicken diets it appeared desirable to investigate the effect of supplementing high fat diets with cholic acid.

Two trials were conducted to determine the effect of cholic acid supplementation of the ration on growth rate, feed efficiency and fat retention of broilers. The composition of the basal ration used in these studies is given in Table 1. The blended fat product that was used as a source of fat was analyzed by gas liquid chromatography and found to have the following fatty acid composition; myristic 2.3%, myristoleic 0.5%, palmitic 26.1%, palmitoleic 5.8%, stearic 15.7%, oleic 43.2% and linoleic 6.4%.

White Rock cockerel chicks were used in these trials. The day-old chicks were individually wing-banded and weighed at weekly intervals during the course of the four-week experimental period. They were housed in electrically heated battery brooders with wire mesh floors. Feed and water were supplied *ad libitum*, and feed consumption was recorded. Feces samples representing excreta for the last 4 days were collected at the temination of Trial 1. Feed and feces samples were analyzed for Cr_2O_3 and fat by the method of Brisson (1956) and A.O.A.C. (1955) respectively.

The results of the two trials are shown in Table 2. Cholic acid was tested alone in Trial 1 and no effect on growth rate was evident. There was a slight decrease in

TABLE 1.—*Composition of the basal ration*

Ingredient	Percent
Corn	46.21
Soybean oil meal (50% protein)	32.00
Fish meal (menhaden)	7.50
Corn gluten meal	2.00
Alfalfa meal (17% protein)	1.00
Defluorinated phosphate	1.50
Salt	0.50
Limestone	0.50
Blended fat	8.00
Choline Cl conc. (25%)	0.20
Mineral mixture (Mn-6%, Fe-20%, Cu-0.2%, I-0.12%, Co-0.02%, Ca-26.5%)	0.20
Methionine hydroxy analog Ca	0.05
Cr_2O_3	0.10
1,2dihydro-6-ethoxy-2,2,4-trimethyl quinoline	0.015
Vitamin mixture*	0.15
Vitamin B_{12} conc. (6 mg. B_{12}/lb.)	0.01
Penicillin conc. (4 gm./lb.)	0.05
Folic acid conc. (4% folic acid)	0.003
ZnO	0.0075
Vitamin A conc. (325,000 USP units/gm.)	0.0022
Vitamin D_3 conc. (950,000 USP units/gm.)	0.0002

* Vitamin mixture contains mg./lb., riboflavin 2,000, pantothenic acid 4,000 niacin 9,000 and choline chloride 90,000.

TABLE 2.—*Effect of cholic acid on growth rate, feed conversion and apparent fat digestion by chicks*

Supplements to the basal ration	Av. wt. at 28 days[1]	Feed/Gain	Apparent fat digestion
	gm.		%
Trial 1			
None	711	1.49	89.3
0.2% Cholic acid	706	1.46	92.4
Trial 2			
None	743	1.47	
0.2% Cholic acid	754	1.45	
0.5% Na_2CO_3	744	1.47	
0.2% Cholic acid +0.5% Na_2CO_3	719	1.48	

[1] Average of four groups of ten White Rock cockerels.

the quantity of feed required to produce a unit of gain when cholic acid was added to the ration. A slight improvement in apparent fat digestion was observed when the basal was supplemented with cholic acid. This increase in fat retention was significantly different from the basal at the 10 percent level of probability. Cholic acid supplementation in the presence and absence of sodium carbonate was evaluated in Trial 2. The data of Trial 2 indicate that there is no consistent effect of cholic acid supplementation on growth rate or efficiency of feed utilization.

SUMMARY

No effect on growth rate or feed efficiency was observed from cholic acid supplementation of a practical ration. However, some improvement in fat absorption as a result of cholic acid supplementation was observed.

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Effect of Bile Acids on Egg Production, Serum Cholesterol and Egg Cholesterol in Hens[1,2]

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Several investigations have been conducted to determine whether bile acids affect serum and liver cholesterol levels when fed to mice and rats (Beher et al., '60; Howe et al., '60; Portman and Bruno, '61). On the other hand, very little experimental work has been carried out to determine the effects of feeding bile acids to chickens. Hegsted et al. ('60) reported that the addition of cholic acid to a diet containing 0.8% of cholesterol caused a small increase in the serum cholesterol of young chickens. Edwards ('61) reported that the addition of cholic acid to a diet without added cholesterol does not cause an increase in serum cholesterol in young chickens as has been reported in mice and rats. He found that the addition of lithocholic acid to the diet of chicks caused a marked increase in serum cholesterol, this effect being opposite to that reported in mice and rats.

The studies reported in this paper were carried out to determine whether adding cholic acid or lithocholic acid to the diet of laying hens would affect serum cholesterol levels, egg production and egg cholesterol levels.

EXPERIMENTAL

Single Comb White Leghorn hens, housed individually in wire cages, were used in the two experiments reported in this paper. Feed and water were supplied ad libitum. Egg production was recorded for 5 days before the hens were fed the experimental diets, and only those hens that were laying were selected for the experiment. Egg production and egg weights were recorded for each individual hen during the experimental period. Hen weights were recorded at the beginning and end of both the experiments. In the second experiment the hens were fed the experimental diets for 20 days before eggs were saved for egg cholesterol determinations. The eggs were homogenized, freeze-dried, extracted overnight with 1,1,1-trichlorethane in a soxhlet extractor and the extract analyzed for cholesterol by the method of Schoenheimer and Sperry ('34). Blood samples were taken from each hen by heart puncture at zero, 5, 10 and 20 days. The serum was separated by centrifugation, and cholesterol was determined by the method of Zlatkis et al. ('53). Cholesterol values and egg production data were subjected to a statistical analysis, and treatment significance was determined by the multiple range test of Duncan ('55) as modified by Kramer ('56).

The composition of the basal diet used is given in table 1. Four diets were each fed to 10 hens in experiment 1. They are as follows: basal; basal plus 0.2% of cholic acid; basal plus 0.2% of lithocholic acid; and basal plus 0.2% of cholic acid plus 0.2% of lithocholic acid. Sixty hens were divided into 4 groups of 15 hens each for experiment 2. The 4 groups were fed the following diets: basal; basal plus 0.025% of lithocholic acid; basal plus 0.05% of lithocholic acid; and basal plus 0.1% of lithocholic acid. Cholic acid and lithocholic acid were added to the diet at the expense of glucose monohydrate in all diets.

RESULTS

Experiment 1. This experiment was conducted to determine the effects of cholic acid and lithocholic acid on serum and

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TABLE 1

Basal diet

	%
Glucose·monohydrate	60.27
Isolated soybean protein	20.00
Corn oil	6.00
Methionine hydroxy analog Ca (90%)	0.74
Glycine	0.33
NaCl	0.75
$MgCO_3$	0.14
Mineral mixture[1]	0.22
Vitamin mixture[2]	0.22
Defluorinated phosphate	3.33
Limestone	4.68
Choline·Cl conc. (70%)	0.29
1,2 Dihydro-6 ethoxy-2,2,4-trimethyl quinoline	0.03
Selka Floc	3.00

1	gm/1000 gm
$FeSO_4 \cdot 7H_2O$	50.0
$CuSO_4 \cdot 5H_2O$	5.0
$CoCl_2 \cdot 6H_2O$	5.0
KI	5.0
$NaMoO_4 \cdot 2H_2O$	0.5
$ZnSO_4 \cdot H_2O$	75.0
$MnSO_4 \cdot 4H_2O$	77.0
Dextrose	782.5

2	gm/1000 gm
Vitamin A (325,000 USP/gm)	12.4
Vitamin E (125,000 IU/lb)	79.1
Vitamin D_3 (950,000 USP/gm)	0.48
Thiamine·HCl	6.0
Riboflavin	10.0
Ca D-pantothenate	10.0
Pyridoxine·HCl	3.0
Niacin	12.0
Folic acid	2.0
Biotin	0.2
Vitamin B_{12}	10.0
p-Aminobenzoic acid	50.0
Inositol	500.0
Menadione Na bisulfite complex	1.6
Dextrose	309.41

egg cholesterol in laying hens. The 40 hens were fed the experimental diets for 30 days.

The data on serum cholesterol is shown is figure 1. Serum cholesterol at 10 and 20 days was found to be significantly influenced by treatment ($P < 0.05$). The serum cholesterol of the cholic acid group was not significantly higher than that of the basal group; but when lithocholic acid was added to the basal diet, serum cholesterol was significantly increased ($P < 0.01$).

Egg production is shown in figure 2. The differences in percentage of egg production between treatments at 10, 15, 20, 25 and 30 days was significant ($P < 0.01$). The hens fed the diets containing lithocholic acid were significantly lower in egg production than either the control or cholic acid fed group ($P < 0.01$). The egg production curve of the hens fed cholic [illegible] is different from those for the other [illegible] ments. At 15 days the egg production [illegible] these hens had dropped markedly, but [illegible] 25 days, these hens had come back to [illegible] duction and thereafter their production [illegible] was approximately the same as that of the control group.

Egg cholesterol was not determined because the hens receiving the diets containing lithocholic acid had stopped laying after 20 days on the experimental diet.

The hens fed the diets containing lithocholic acid lost a small amount of weight during the 30-day experimental period. The birds fed the basal diet and the basal plus cholic acid did not show any marked weight changes during the experiment.

After the hens had been fed the experimental diets for 30 days, they were fed a regular commercial laying mash, and egg production was recorded to determine how long the hens previously fed lithocholic acid would remain in the molt. After 30 days the hens previously fed the diet containing 0.2% of cholic acid plus 0.2% of lithocholic acid had resumed production but it was 15 days before the hens fed 0.2% of lithocholic acid had resumed production.

Experiment 2. This experiment was designed to determine the effects of different levels of lithocholic acid on the serum and egg cholesterol levels of laying hens. By using various levels of lithocholic acid it was anticipated that eggs might be collected from a group with elevated serum cholesterol while still producing sufficient eggs for cholesterol analysis.

The serum cholesterol data is presented in figure 3. Variation due to treatment in the serum cholesterol at 5, 10 and 20 days was statistically significant ($P < 0.05$). At 5 days the hens receiving 0.05% of lithocholic acid had lower serum cholesterol than the other groups; however, this did not continue for the rest of the experiment. The serum cholesterol of the group fed 0.1% of lithocholic acid was found to be significantly higher than that of the hens fed the other diet by 10 days ($P < 0.05$). This was still true at 20 days. While the comparison is not statistically valid, it is interesting to compare the serum cholesterol

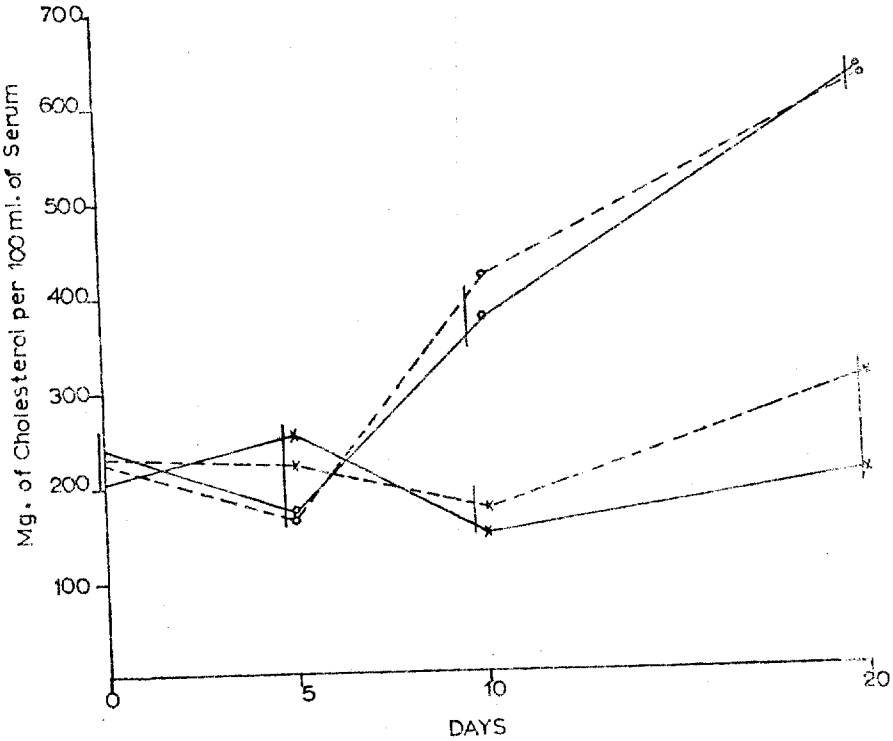

Fig. 1 Effect of feeding cholic acid or lithocholic acid on serum cholesterol. Values bordered by the same adjacent vertical line are not significantly different ($P < 0.05$).

Legend: Control, x———x; Control plus 0.2% cholic acid, x - - - - x; Control plus 0.2% lithocholic acid, o———o; Control plus 0.2% cholic acid, plus 0.2% lithocholic acid, o - - - - o.

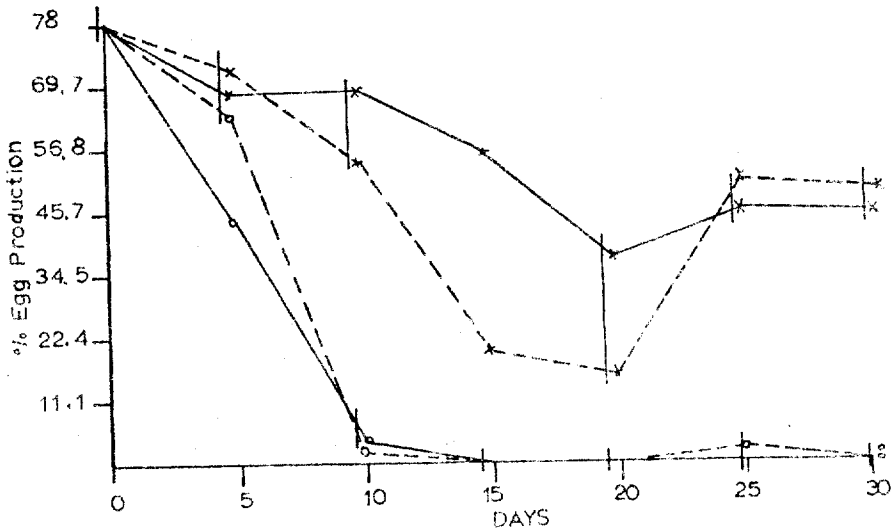

Fig. 2 Effect of feeding cholic acid or lithocholic acid on egg production. Values bordered by the same adjacent vertical line are not significantly different ($P < 0.05$).

Legend: Control, x———x; Control plus 0.2% cholic acid, x - - - - x; Control plus 0.2% lithocholic acid, o———o; Control plus 0.2% cholic acid, plus 0.2% lithocholic acid, o - - - - o.

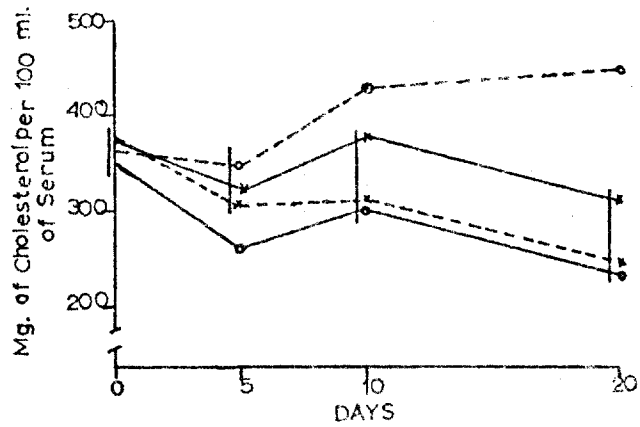

Fig. 3 Effect of feeding different levels of lithocholic acid on serum cholesterol. Values bordered by the same adjacent vertical line are not significantly different ($P < 0.05$).

Legend: Control, x———x; Control plus 0.025% lithocholic acid, x - - - - x; Control plus 0.05% lithocholic acid, o———o; Control plus 0.10% lithocholic acid, o - - - - o.

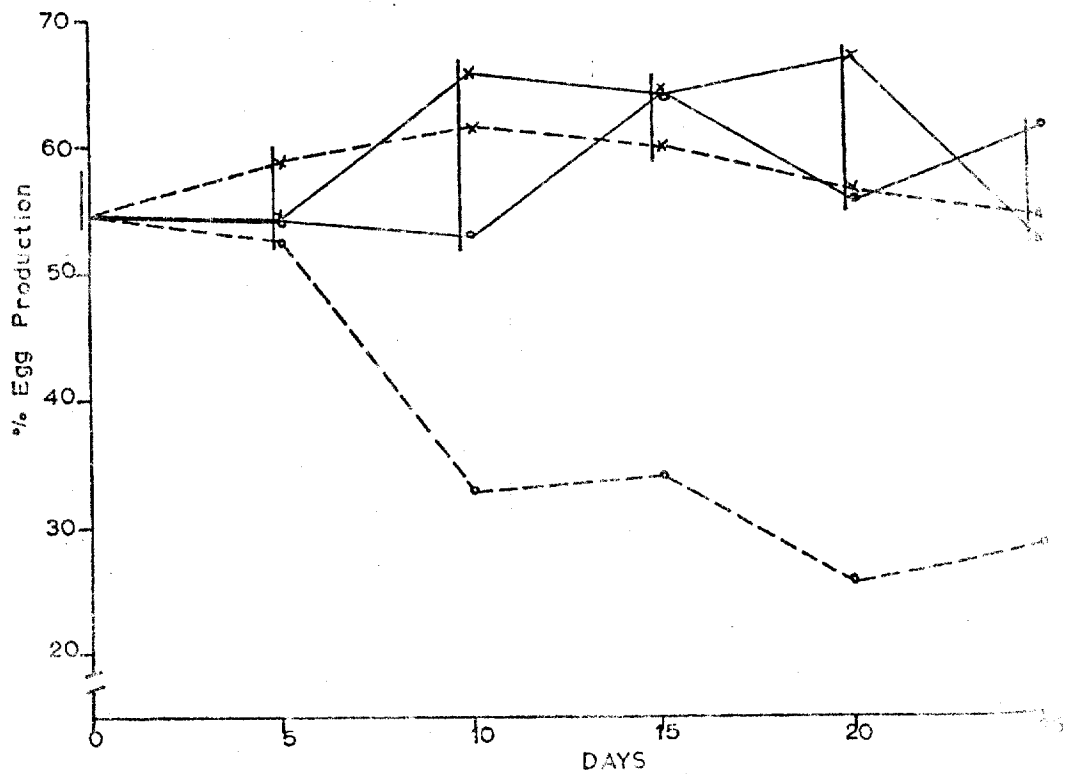

Fig. 4 Effect of feeding different levels of lithocholic acid on egg production. Values bordered by the same adjacent vertical line are not signicantly different ($P < 0.05$).

Legend: Control, x———x; Control plus 0.025% lithocholic acid, x - - - - x; Control plus 0.05% lithocholic acid, o———o, Control plus 0.10% lithocholic acid. o - - - - o.

terol of the hens receiving 0.1% of lithocholic acid in this experiment with those receiving 0.2% of lithocholic acid in the previous experiment. The birds in experiment 1 receiving the higher level of lithocholic acid had serum cholesterol levels approximately 50% greater than those receiving the 0.1% level of lithocholic acid in experiment 2.

No significant changes in body weights occurred between the hens fed the various experimental diets in this experiment.

Egg production data for the second trial are shown in figure 4. The hens that received 0.1% of lithocholic acid produced significantly fewer eggs than those in any of the other groups.

Only one hen molted in the control group, whereas three of the hens in the 0.1% lithocholic acid group, four in the 0.05% lithocholic acid group, and three in the 0.025% lithocholic acid group molted. This is in contrast with all of the hens that molted when fed 0.20% lithocholic acid in the first experiment.

The results of the analysis for cholesterol content of the eggs collected after the hens were fed the experimental diets for 20 days are shown in table 2. The milligrams of cholesterol per gram of egg fat increased significantly ($P < 0.01$) when the hens were fed the diet containing 0.1% of lithocholic acid. No differences were noted between the other treatments as regards egg cholesterol levels. The addition of 0.1% of lithocholic acid to the diet also caused an increase in the total lipid content of the eggs. Eggs from hens fed the lower levels of lithocholic had an intermediate increase in total lipid. The weights of eggs from the hens fed the various diets did not vary significantly. This indicates that this change in cholesterol content of the egg could be expressed in a number of ways and there would still be significantly more cholesterol in eggs from the hens fed the diet containing 0.1% of lithocholic acid.

DISCUSSION

The present study is one of few reports to show that dietary manipulation has significantly influenced egg cholesterol level. Fisher and Leveille ('57) studied the effect of dietary fats on yolk cholesterol. They concluded that the cholesterol content of egg fat was essentially unchanged by alterations in the fatty acid composition of egg fat which was influenced by dietary manipulations. Daghir et al. ('60) reported that feeding hens diets supplemented with 12% of soybean oil resulted in significantly lower serum cholesterol, whereas it had no effect on egg cholesterol level. In contrast with these reports, Combs and Helbacka ('60) reported that the type of fat added to the diet of laying hens influenced the egg yolk cholesterol and egg yolk lipids. Kurnick et al. ('58) observed that feeding 1% of cholesterol in the diet of laying hens caused an increase in serum cholesterol but no change in the cholesterol content of egg yolks. Edwards et al. ('61) showed that eggs from hens of different strains may differ significantly in their fat and cholesterol content, therefore, breed differences could explain some of the differences in results reported.

Popjak and Tietz ('53) reported that the membranes surrounding the growing ovum, consisting of the theca interna and the granulosa, synthesize fatty acids and cholesterol from acetate-C^{14} *in vitro*. These reports point out that it is still not known whether an increase in serum cholesterol will cause an increase in egg cholesterol. Little is known about the site of cholesterol

TABLE 2

Effect of dietary lithocholic acid on egg lipid and cholesterol

Supplement to the basal diet	Whole egg lipid	Egg lipid cholesterol
	%	*mg/gm*
None	44.09 [1]	38.25 [1]
Lithocholic acid, 0.025%	47.00	36.18
Lithocholic acid, 0.050%	47.05	38.51
Lithocholic acid, 0.100%	49.95	55.39

[1] Values bordered by the same adjacent vertical line are not significantly different; whole egg lipid ($P < 0.05$), egg lipid cholesterol ($P < 0.01$).

synthesis nor how it is deposited in the egg yolk. Thus it is difficult even to speculate on the mode of action of lithocholic acid in the hen which results in the changes observed in these experiments.

The results obtained in these experiments with laying hens confirm the earlier results of Edwards ('61) with young cockerels. The only noticeable difference in the results with hens is that the addition of cholic acid did not counteract the effects of lithocholic acid in raising serum cholesterol with hens as it did with young cockerels.

The second experiment with the laying hens indicates that the level of dietary lithocholic acid is very critical. The lower serum cholesterol levels obtained at 10 and 20 days, when 0.05 or 0.025% of lithocholic acid was present in the diet, should not be ignored even though they are not statistically different from the controls.

SUMMARY

It was found that lithocholic acid alone or in combination with cholic acid in the diet when fed to hens caused an increase in serum cholesterol and an abrupt decrease in egg production. Cholic acid supplementation had no effect on serum cholesterol but caused a temporary decrease in egg production which returned to the control level after 25 days. A second experiment was conducted to determine the effect of level of lithocholic acid in the diet on laying hens. Diets containing none, 0.025, 0.05 and 0.10% of lithocholic acid were fed to laying hens. The results show that the feeding of 0.10% of lithocholic acid significantly decreased egg production, while increasing serum cholesterol level, total egg lipids and egg cholesterol. None of the supplements had any significant effects on egg size.

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THE TOXIC CONSTITUENT OF THE BILE*

By William C. Emerson, M.D., Detroit, Michigan

THE fact that bile is toxic has long been observed. Bunting and Brown[1] in experimental work on rabbits showed that by either injecting bile intraperitoneally or by dividing the gall bladder and allowing the bile to escape into the peritoneal cavity that death occurred within twenty-four hours and was attributed to direct action on the myocardium.

There has long been a controversy as to just which constituent of the bile is toxic. Bouchard,[2] Plaesterer[3] and King and Stewart[4] all claim that the toxic constituent of the bile is the pigment. Rohrig,[5] Feltz and Ritter,[6] Leyden,[7] Rywosch,[8] Stadelmann,[9] and Bickel[10] all maintain that the toxic constituent of the bile is the bile salts, sodium glycocholate and sodium taurocholate. Cholesterin and mucin surely are nontoxic.

Biedl and Krause[11] have shown that the bile salts are strongly hemolytic for blood and that in sufficient concentration they cause a coagulation of myosin to such an extent that the muscle loses its contractility. It is undoubtedly this action which causes death due to myocardial failure when whole bile or bile salts are absorbed by the blood over a long period of time. They also showed that when bile salts were injected intravenously, a slowing of the pulse, with a marked fall in the arterial blood pressure occurred.

King and Stewart[4] prepared their pigment from pig's bile. We repeated their work and found that their pigment when dried to constant weight equaled 8.5 per cent. The pigment in dog's bile equals 0.05 per cent. We would not expect pig's bile to contain one hundred sixty times as much pigment as dog's bile. It would be reasonable to assume that their pigment contained other substances. Their dried pigment was analyzed and was found to contain bile salts and large amounts of fat.

This dried pig's pigment, the calcium having been removed by washing with hydrochloric acid, was extracted with hot chloroform, the chloroform evaporated to dryness and the residue dissolved in alcohol. This alcoholic solution of pig's pigment was injected into the femoral vein of a dog. There

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was no evidence of any toxic effect. Twenty-one c.c. of the [illegible] solution, a 9 per cent solution of pigment dissolved in 95 per cent alcohol, was injected, and the blood pressure at the completion of the experiment was higher than at the beginning.

Meltzer and Salant[12] have shown that when a few cubic centimeters of ox bile was given rapidly to a rabbit intravenously the animal died almost immediately in convulsions, while, when given slowly, as much as 50 c.c. of a 50 per cent solution was tolerated. In all our work the material injected was given slowly 2.5 to 3 c.c. a minute.

METHOD

Ox bile was obtained from twenty gall bladders of freshly killed animals. The bile was diluted with an equal volume of normal saline and injected within three hours after collection. Dogs were used in all the experiments.

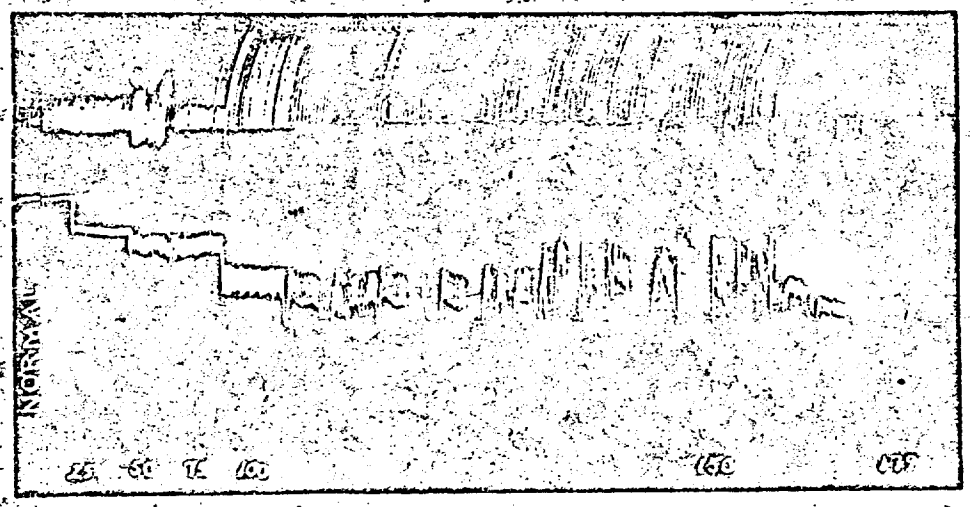

Fig. 1.—Effect on the blood pressure and respiration when bile is injected into the femoral vein.

The animals were anesthetized by the open drop method, later a cannula was placed in the trachea and connected with an ether bottle. A cannula was placed in the carotid artery and connected with a mercury manometer for blood pressure tracing. A tube was placed in the etherization system and connected with a tambour and writing lever for a tracing of the respiratory movements. The femoral vein was exposed and bile diluted with an equal volume of normal saline was injected from a burette. Blood pressure and respiration tracings were made after the injection of every 25 c.c. of the diluted bile.

A 3 per cent solution of sodium glycocholate was injected into the femoral veins of ten normal dogs. Blood pressure and respiration tracings were made as in the control experiments.

A 3 per cent solution of sodium taurocholate was injected into the femoral veins of ten normal dogs. Blood pressure and respiration tracings were made as in the control experiments.

The [illegible] of the sodium taurocholate were prepared as follows: The commercial product of both was obtained. This material was recrystallized three times, the final product being a pure white powder. The melting point of the sodium glycocholate was 156° C. and the melting point of the sodium taurocholate was 95° C. This purified material was dissolved in distilled water and injected as described above.

On ten consecutive days ox bile from twenty freshly killed animals was obtained. The bile was diluted with an equal volume of normal saline and the mixture divided into two equal parts. One part, whole bile diluted with an equal volume of normal saline, was injected into a dog. The other half, also diluted with an equal volume of normal saline, was treated three times with blood charcoal and the final product, a clear light yellow liquid, was injected into a dog. This clear light yellow liquid when compared in the colorimeter against bile diluted with an equal volume of normal saline was found to contain only 5 per cent of pigment, the remaining 95 per cent having been removed by the blood charcoal. This experiment was repeated on ten consecutive days, ten samples of bile being used and twenty dogs, two each day, injected, one with whole bile and one with bile after 95 per cent of the pigment had been removed.

Pigment obtained from human gallstones was dissolved in alcohol and injected into a dog.

RESULTS

Ten specimens of bile were injected into the femoral veins of ten normal dogs. The lethal dose is summarized in Table I. The lethal dose for whole bile when injected into the femoral vein gave an average of 9.6 c.c. per pound. Fig. 1 shows the characteristic gradual fall in blood pressure which occurs when bile is injected into the femoral vein. The fall in blood pressure is later accompanied by a striking arrhythmia and at times there is an apparent cessation of cardiac activity.

TABLE I

DOG NUMBER	WEIGHT OF DOG POUNDS	LETHAL DOSE WHOLE BILE PER POUND C.C.
1	16.5	12.0
2	14.25	8.55
3	18.75	10.5
4	19.75	9.7
5	18.	10.2
6	12.5	8.4
7	15.75	8.77
8	10.25	10.5
9	19.	9.9
10	15.	8.0
Average		9.6

Three per cent sodium glycocholate was injected into the femoral veins of ten normal dogs. The lethal dose is summarized in Table II. The lethal dose of 3 per cent sodium glycocholate when injected into the femoral vein gave an average of 8.5 c.c. per pound. Fig. 2 shows the same gradual fall

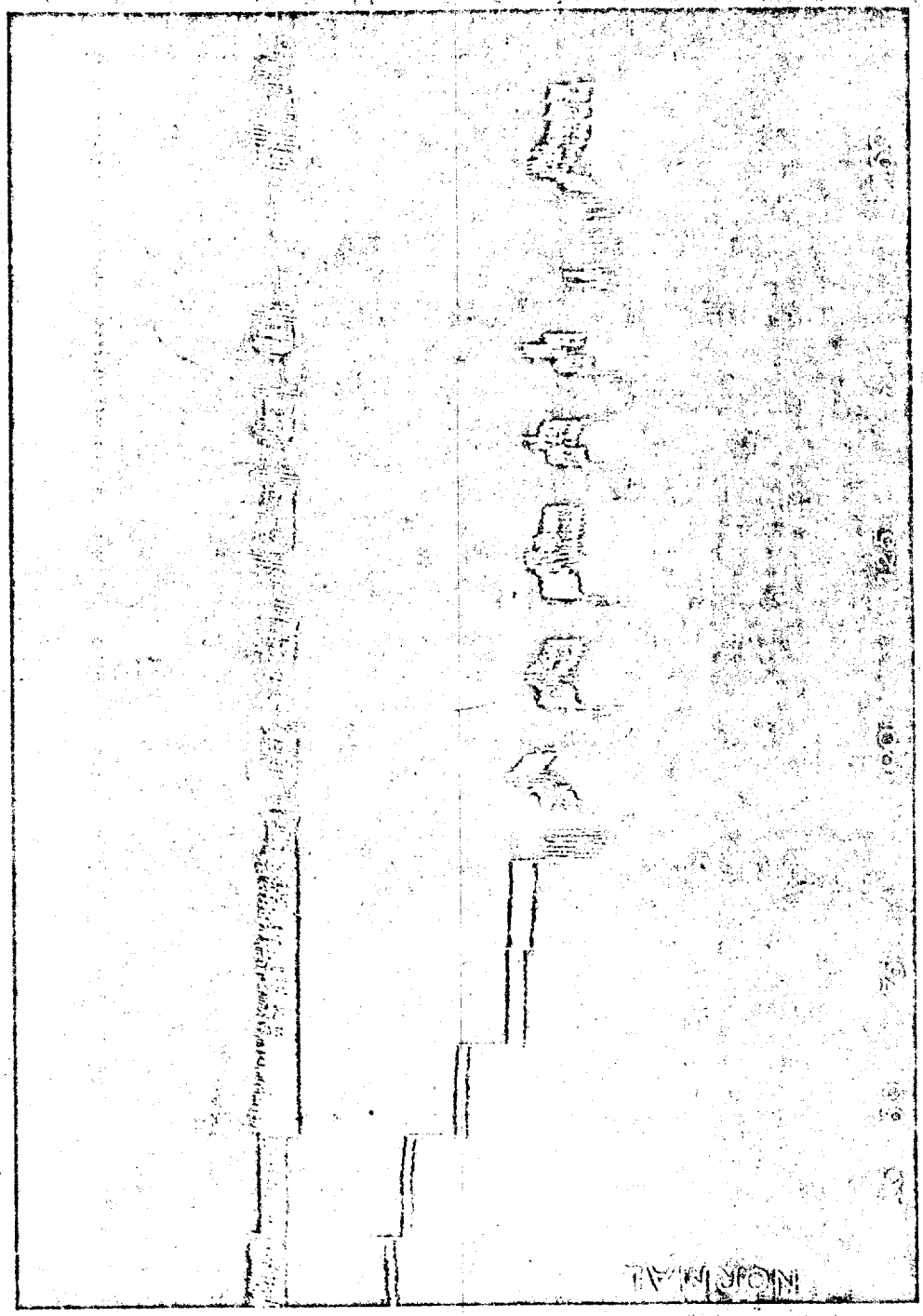

Fig. 2.—Effect on the blood pressure and respiration when 2 per cent sodium glycocholate is injected into the femoral vein.

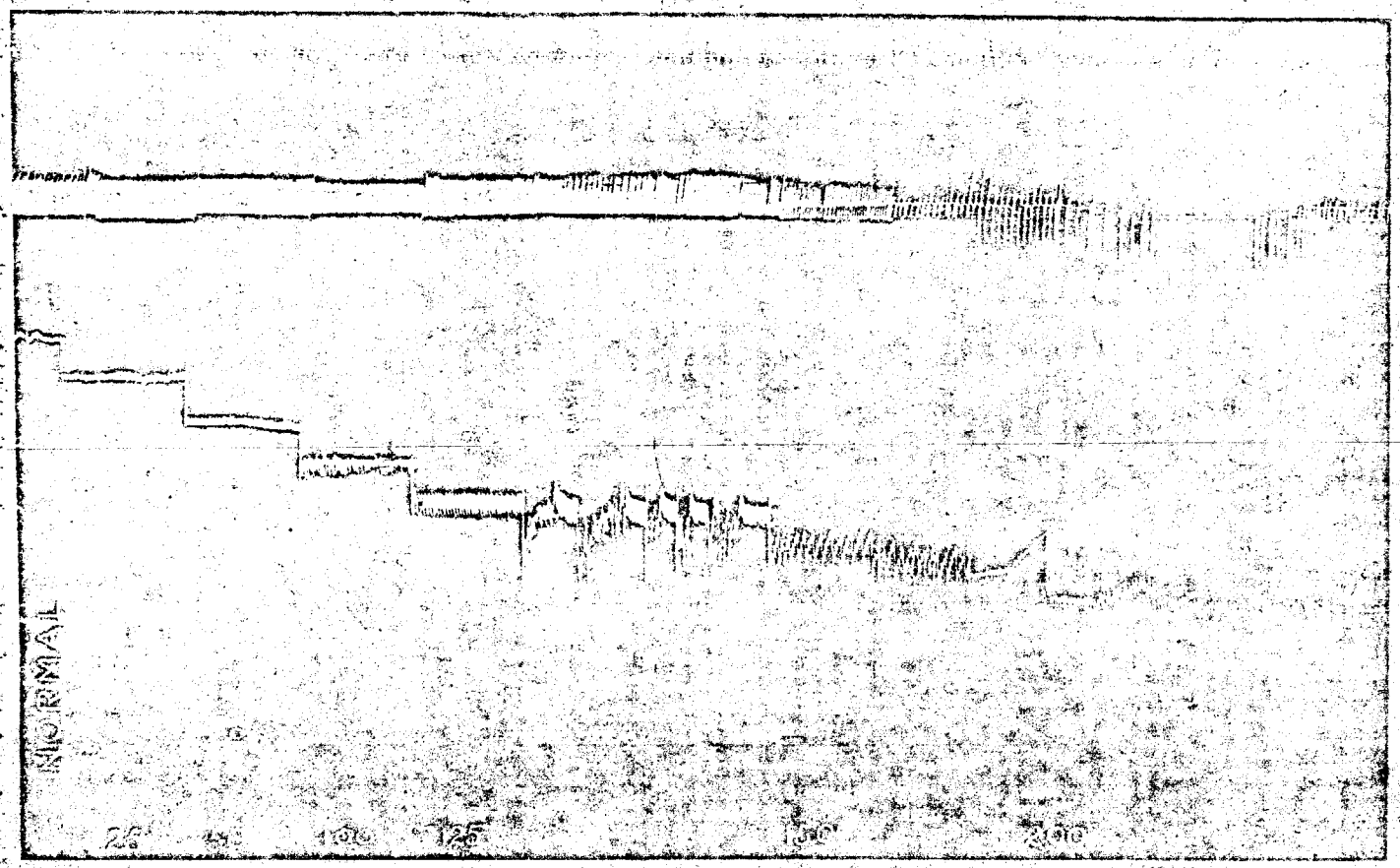

Fig. 3.—Effect on the blood pressure and respiration when 2 per cent sodium taurocholate is injected into the femoral vein.

in blood pressure later accompanied by a striking arrhythmia as occurred in the control tracing when whole bile was injected.

Three per cent sodium taurocholate was injected into the femoral veins of ten normal dogs. The lethal dose is summarized in Table II. The lethal dose of 3 per cent sodium taurocholate when injected into the femoral vein gave an average of 10.1 c.c. per pound. Fig. 3 shows identically the same fall in blood pressure accompanied by a marked arrhythmia as occurred in the control tracing when whole bile was injected.

TABLE II

DOG NUMBER	WEIGHT OF DOG POUNDS	LETHAL DOSE 3% SODIUM GLYCOCHOLATE PER POUND C.C.	DOG NUMBER	WEIGHT OF DOG POUNDS	LETHAL DOSE 3% SODIUM TAUROCHOLATE PER POUND C.C.
11	18.5	8.0	21	19.0	12.0
12	18.0	7.5	22	7.5	10.9
13	11.5	6.0	23	21.25	6.6
14	14.0	7.8	24	17.5	6.2
15	14.25	8.07	25	14.5	10.4
16	17.5	9.9	26	13.0	10.0
17	10.5	9.9	27	9.25	10.8
18	19.0	9.5	28	12.5	11.2
19	21.0	9.10	29	13.75	11.4
20	14.0	10.2	30	9.5	11.5
Average		8.5			10.1

TABLE III

BILE SPECIMEN	WEIGHT OF CONTROL DOG POUNDS	LETHAL DOSE WHOLE BILE PER POUND C.C.	WEIGHT OF SECOND DOG POUNDS	LETHAL DOSE WHOLE BILE MINUS 95 PER CENT PIGMENT PER POUND C.C.
1	10.5	6.2	18.0	4.6
2	10.0	7.3	20.0	10.9
3	33.0	7.8	38.0	8.2
4	7.5	6.6	9.0	10.5
5	12.5	6.7	12.0	7.3
6	26.5	9.6	11.5	9.7
7	16.5	5.7	23.0	9.4
8	14.5	5.5	14.0	10.5
9	18.5	5.7	21.0	5.4
10	17.0	6.8	23.0	7.0
Average		6.8		7.3

Ten samples of ox bile were diluted with an equal volume of normal saline. Half of each sample was treated with blood charcoal. The treated bile after 95 per cent of the pigment had been removed and the untreated bile were injected into the femoral veins of twenty normal dogs, two dogs being used for each sample of bile.

The lethal doses are summarized in Table III. The average lethal dose for the untreated bile is 6.8 c.c. per pound. The average lethal dose for the bile after 95 per cent of the pigment had been removed was 7.3 c.c. per pound.

Fig. 4 shows the same fall in blood pressure accompanied later by the striking arrhythmia as occurred when whole bile was injected into the femoral vein.

Pigment obtained from human gallstones dissolved in alcohol was in-

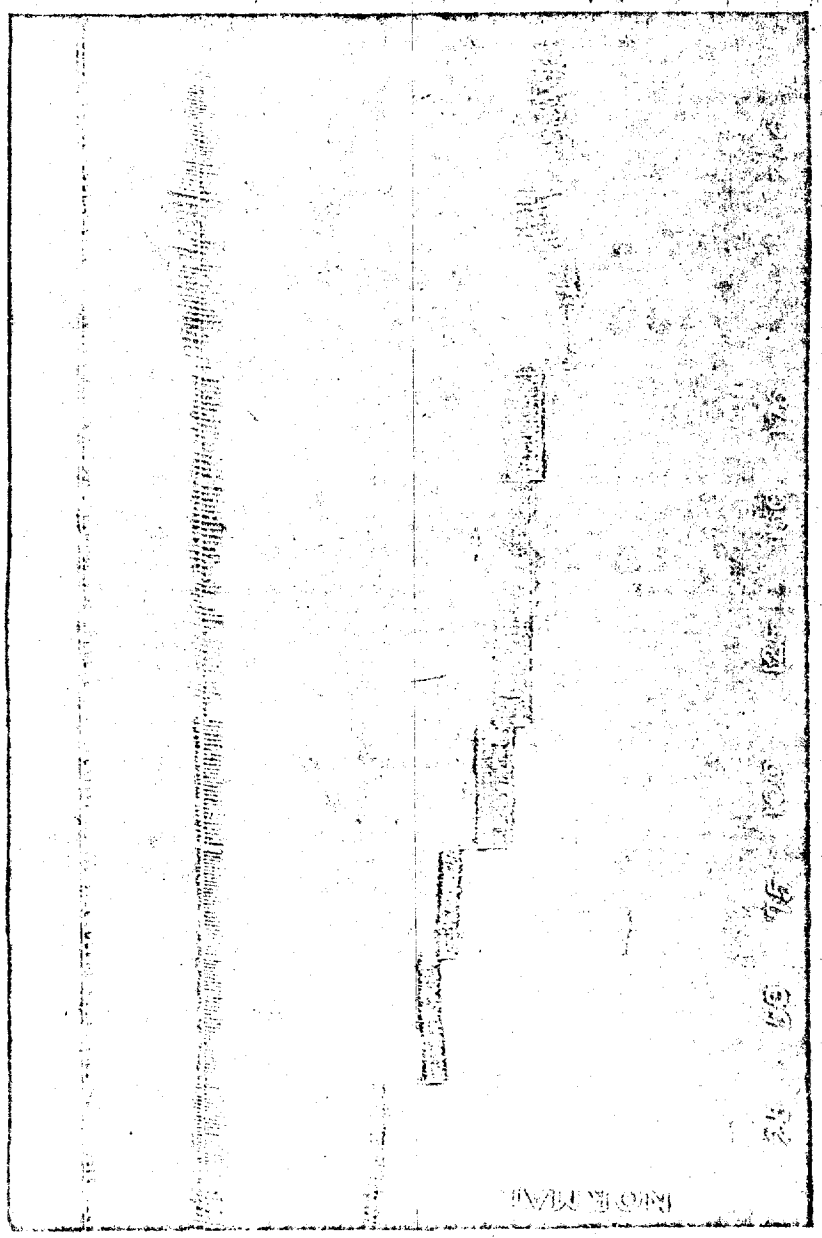

Fig. 4.—Effect on the blood pressure and respiration when bile after 95 per cent of the pigment had been removed was injected into the femoral vein.

jected into the femoral vein of a dog. There was no effect on the blood pressure or respiratory rate. Twenty-five c.c. per pound was injected, and the blood pressure was higher at the completion of the experiment than at the beginning.

DISCUSSION

The fact that the average lethal dose of sodium glycocholate injected at a uniform rate of speed is 1.1 c.c. per pound less than the average lethal dose of bile and that the average lethal dose of sodium taurocholate injected at a uniform rate of speed is 0.5 c.c. per pound greater than the average lethal dose of bile would suggest that the toxic constituent of the bile is the bile salts, sodium glycocholate and sodium taurocholate.

The blood pressure and respiration tracings of the dogs injected with sodium glycocholate and sodium taurocholate being identical with the tracing of the dog injected with whole bile would further indicate that the bile salts are the toxic constituents of the bile.

Sodium glycocholate is more toxic than sodium taurocholate due to the fact that the average lethal dose of sodium glycocholate is 8.5 c.c. per pound whereas it required an average of 10.1 c.c. per pound of sodium taurocholate to kill the animals.

The average lethal dose of bile after 95 per cent of the pigment had been removed being 7.3 c.c. per pound as compared to an average of 8.8 c.c. per pound when whole bile was injected would clearly indicate that the bile pigment is not the toxic constituent of the bile.

That the bile pigment is not the toxic constituent of the bile is further indicated by the fact that the tracing of the blood pressure and respirations in a dog injected with bile after 95 per cent of the pigment had been removed is identical with the tracing when untreated or whole bile was injected, and also by the fact that when pigment was injected there was no fall in blood pressure nor was there any arrhythmia produced.

CONCLUSIONS

1. The toxic constituent of the bile is the bile salts, sodium glycocholate and sodium taurocholate.

2. Sodium glycocholate is more toxic than sodium taurocholate.

3. Bile pigment is not toxic and has no effect on the blood pressure or respiratory rate when injected into the blood stream.

This work was suggested and done under the direction of Dr. Edward C. Dodds.

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A Bronchoconstrictory Action of some Bile Acids.

By

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It is well known that bile acids play a part in choleresis and in digestion and absorption in the gut. Other effects of bile acids in the organism have also been described. In the past the cathartic action of bile has been empirically known and made use of; the experimental finding that bile acids given orally stimulate the peristaltic movements of the gut has offered an explanation of this effect (for references see EMMELIN 1941, DACSO et al. 1945). The bradycardia which sometimes accompanies jaundice has generally been supposed to be caused by bile acids accumulated in the blood. This hypothesis has given rise to a great number of experimental investigations concerning the action of bile acids on the heart; several different effects have been described but the results do not seem concordant (for references see WASTIM et al. 1939, 1940). Bile acids have also been observed to act on the uterus (HOFBAUER 1928). In this paper a bronchoconstrictory action of some bile acids will be described.

Methods.

The experiments were carried out on cats and guinea-pigs. 22 cats were used, 8 decerebrated and 14 under chloralose. Drugs were injected through a cannula in a femoral vein. In some of the experiments the blood pressure in the femoral artery was registered with a membrane manometer. The bronchial tone was registered according to KONZETT and RÖSSLER (1940). As contractions of the respiratory muscles may

interfere with this registration some of the experiments were carried out on cats with widely opened thorax and sectioned phrenic nerves. In some of the experiments curare was given so as to obtain a complete paralysis of the respiratory muscles.

WEST (1938) has found that curare has a bronchoconstrictory effect, which according to ANREP et al. (1939) is caused by histamine liberated by curare from the skeletal muscles. In our experiments curare only elicited a slight bronchoconstriction which did not interfere with the experiment.

11 guinea-pigs were used. 7 were anæsthetized with urethane; drugs were given via the jugular vein. In 4 animals the lungs were perfused with body-warm Tyrode solution through a cannula inserted in the pulmonary artery. Injections were made into the rubber tubing close to the cannula. The perfusate was drained off through a cannula placed in the left auricle. In the guinea-pig experiments the Konzett-Rössler method was also used.

In most of the experiments sodium glycocholate (Merck) was used, but sodium taurocholate (Merck), sodium desoxycholate (B. D. H.) and the sodium salt of acidum dehydrocholicum (Leo) were also investigated.

Results.

Fig. 1 shows the effect of sodium glycocholate on the cat's bronchi. 5 mg of glycocholate injected rapidly intravenously elicits a marked bronchoconstriction. The effect disappears in some minutes. For comparison it may be mentioned that this dose given intravenously has a marked choleretic effect in the cat (ROTHLIN and SCHARCH 1944).

Our experiments show that 5—10 mg of glycocholate per kg of body weight has a marked bronchoconstrictory effect in cats. In most of the experiments even 1 mg/kg causes bronchoconstric-

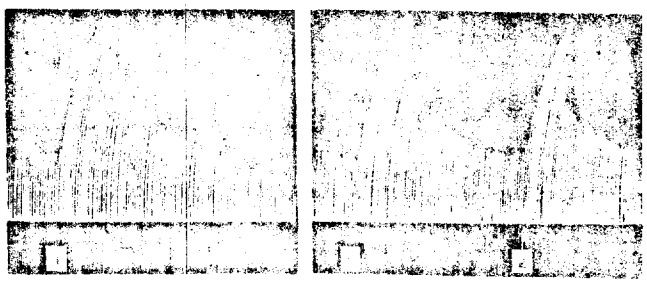

Fig. 1. Bronchoconstrictory action of sodium glycocholate. Curarized cat, 2.1 kg, under chloralose. Between the two sections of the tracing the vagi were cut in the neck. 1: 5 mg sodium glycocholate/kg of body weight. For comparison 5 γ histamine base/kg were injected at 2. Time in minutes.

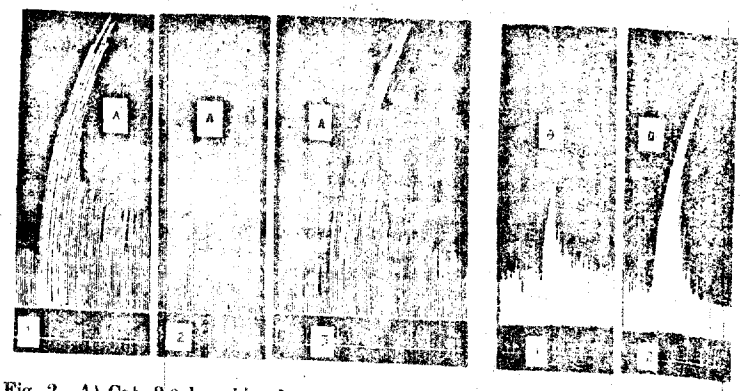

Fig. 2. A) Cat, 2.0 kg, chloralose. 1) 5 mg sodium glycocholate/kg injected in the femoral vein 2) the same dose in the portal vein 3) 50 mg glycocholate/kg in the portal vein. B) Cat, 3.7 kg, chloralose. 1) 5 mg glycocholate/kg in the femoral vein before and 2) after ligature on the hepatic artery and the portal vein. Time in minutes.

tion whereas 0.5 mg/kg is without effect. Taurocholate also elicits bronchoconstriction: 15—20 mg/kg has a marked effect, 1 mg/kg has none. Desoxycholate is active only in a highly toxic dose (about 50 mg/kg). Dehydrocholate even in an amount of 50 mg/kg has no bronchoconstrictory effect.

The conjugated bile acids are thus found to have the highest bronchoconstrictory activity. We have investigated the possible bronchoconstrictory action of glycine or taurine alone. In experiments where 5 mg of taurocholate/kg had a marked effect 10 mg of taurine had none; where 2 mg of glycocholate/kg caused bronchial constriction 10 mg of glycine/kg did not.

In guinea-pigs under urethane glyco- and taurocholate elicit bronchoconstriction. The sensitivity of the bronchi to these bile salts is found to be lower in these experiments than in the experiments on cats: 15—25 mg/kg of glycocholate and somewhat more taurocholate is required to obtain bronchial constriction.

Duration of the effect. The short duration of the bronchoconstriction caused by bile acids is striking. It is well known that bile acids injected intravenously are rapidly removed from the blood (for references see JOSEPHSON 1941). The part played by the liver can be well demonstrated in experiments of this kind. Fig. 2 shows that sodium glycocholate is about ten times less active on the bronchi when injected in the portal instead of the femoral vein. If on the other hand the liver is removed from the circula-

tion, the glycocholate, given in the femoral vein, elicits a much larger bronchoconstriction than it does when the liver circulation is intact. It is note-worthy that the duration of the effect is not markedly augmented in this experiment. This might indicate that other mechanisms play a part in the inactivation of bile acids. We have observed that a dose of glycocholate mixed with blood from the experimental animal a few minutes before the injection gives a smaller bronchoconstriction than the same dose dissolved in Tyrode solution.

Mode of action of bile acids. Several authors have maintained that bile acids stimulate parasympathetic centres in the medulla oblongata thus causing *e. g.* bradycardia in jaundice. The bronchoconstriction described in this paper, however, does not seem to be elicited in a similar way. The effect appears even if the vagus nerves are cut in the neck (fig. 1). HEEB (1940) has shown that in guinea-pigs the lung also receives bronchoconstrictory nerve fibres from the stellate ganglion. We have therefore in experiments on cats with sectioned vagi also removed the stellate ganglia and the 2—3 cranial thoracic ganglia on both sides. In these experiments bile acids cause bronchial constriction. (It might be added that we have observed only bronchodilatation, never constriction, after electrical stimulation of the stellate ganglion in the cat.) From our experiments it may thus be concluded that the bile acids act peripherally on the bronchi. This opinion is further supported by our observation that a certain dose of glycocholate elicits a greater bronchoconstriction when injected in the right ventricle than when given in the carotid artery. RIES and FITIA (1933) claim that bile acids increase the sensitivity of certain effectors to stimulation of parasympathetic nerves. It is also established that bile acids can inactivate the acetylcholine esterase (SOBOTKA and ANTOPOL 1937). These findings, however, do not seem to explain the bronchoconstrictory action of bile acids. In cats with sectioned vagi we have injected glycocholate at a constant slow rate and in a concentration which causes only a slight bronchoconstriction; constriction elicited by electrical stimulation of the vagi is not found to be markedly increased by the glycocholate. Bile acids are also active in atropinized cat.

The bronchoconstrictory affect of bile acids is eliminated by injection of theophylline monoethanolamine (25 mg/kg), this drug is known to render the bronchi insensitive not only to vagal stimulation and acetylcholine but also to histamine (EMMELIN,

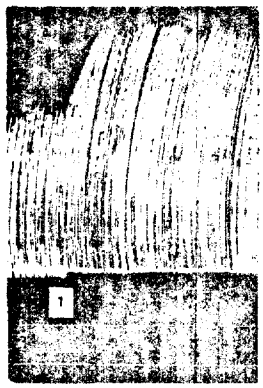

Fig. 3. Isolated guinea-pig's lungs, perfused with Tyrode solution. 1) Injection of 5 mg sodium glycocholate. Time in minutes.

KAHLSON and LINDSTRÖM 1941). Phenobarbitone also (50—100 mg/kg) antagonizes the bronchoconstrictory effect of bile acids.

The observation that bile acids elicit bronchoconstriction in isolated perfused lungs further supports the view that the point of action is situated within the lung (fig. 3).

Comment.

Some of the biological effects of bile acids seem to be related to the surface activity of these substances. ROTHLIN and SCHALCH (1944) comparing some bile acids found a close relation between surface activity, toxicity and hemolytic activity. With regard to these three properties desoxy- and taurocholic acids were found to be most active, glycocholic acid less and dehydrocholic acid least active. Choleresis and activation of lipase, on the other hand, are independent of these qualities. In our experiments the sequence is: glyco-, tauro-, desoxy-, dehydrocholic acid; this is true also when equimolar solutions are compared. Thus the bronchoconstrictory action of bile acids does not seem to depend on the surface activity, nor does there seem to be any relation to choleretic or lipase activating properties.

Summary.

A constrictory action of some bile acids on cat's and guinea-pig's bronchi is described.

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Research Notes

TOXICITY OF LITHOCHOLIC ACID FOR THE CHICK

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(Received for publication February 28, 1963)

Lithocholic acid is considered to be a metabolite derived from chenodeoxycholic acid by intestinal bacteria during enterohepatic circulation (Norman and Sjövall, 1960) and traces of this substance are normally found in the intestine and in the bile of most animals.

Edwards (1961) reported that feeding of 0.2% lithocholic acid to chicks results in a threefold rise in serum cholesterol and in a reduction of growth. When fed to laying hens, the bile acid not only causes hypercholesterolemia but also an abrupt decrease in egg production (Edwards *et al.*, 1962). This effect on serum cholesterol in the chick is opposite to that observed in other animals. In mice and rats, lithocholic acid decreases serum cholesterol levels and counteracts the hypercholesterolemic effect of cholic acid (Howe *et al.*, 1960; Howe and Hutchison, 1962). During the preparation of this manuscript Leveille *et al.* (1962) reported a twofold increase in liver size in chicks fed 0.2% of lithocholic acid and this increase is not explained by differences in fat or moisture content. In rabbits, gastric instillation of lithocholic acid gives rise to cirrhosis of the liver (Holsti, 1960).

Because of the possible importance of this compound to chick growth, the present experiment was performed to investigate the mode of action by which lithocholic acid depresses growth and causes hypercholesterolemia in chickens.

Thirty-two 3-week old broilers were divided into 4 groups of equal average weight and were fed the experimental diets for 2 weeks. The composition of the basal 6% corn oil-casein-sucrose diet was the same as in our previous investigations (Eyssen and De Somer, 1963). One group was fed the basal diet, the other three groups received a supplement of 0.2% lithocholic acid, 0.5% cholesterol, and 0.5% cholesterol plus 0.2% lithocholic acid respectively. Weight gain and feed consumption were recorded daily. Serum and liver cholesterol were determined by the method of Abell *et al.* (1952).

Table 1 shows that feeding 0.2% lithocholic acid reduced the food intake by 25 to 30% and the weight gain by about 50%. Serum cholesterol and total liver cholesterol were significantly increased regardless of the presence or absence of cholesterol in the diet, although the effect of the bile acid was more pronounced on the diet without added cholesterol.

The most conspicuous effect, however, was the increase in liver weight. The livers of birds fed lithocholic acid were greatly enlarged and were light brown to pale yellowish. The bile bladders were filled with a viscous yellow-green fluid contrasting sharply with the clear dark-green bile of the control animals. Histologically the changes consisted of an extensive bile duct proliferation, distorted and disrupted hepatic lobules and marked fibrosis.

Despite of a four- to fivefold rise in liver cholesterol concentration, the liver weight was not increased significantly in the birds of group 3 which received the 0.5% cholesterol ration without lithocholic acid. This clearly proves that the toxic reac-

TABLE 1.—*Effects of feeding chicks during 2 weeks with 0.2% lithocholic acid*

	Experimental diets (*)			
	1 Basal	2 +0.2% lithocholic acid	3 +0.5% cholesterol	4 +0.5% cholesterol +0.2% lithocholic acid
Average food uptake gm./bird/day	27	20	28	30
Average weight gain gm./bird/day	21	11.6	22.3	12.3
Serum cholesterol mg./100 ml.	162	504	397	686
Liver cholesterol mg./100 gm. wet liver	350	414	1,666	690
Average liver weight gm./bird	12.0	30.8	12.5	31.1
Average total liver cholesterol mg./liver	42	127	208	214

(*) eight animals per group.

tions to lithocholic acid in group 2 are not due to the accumulation of cholesterol *per se*.

Although the hepatotoxic effect might explain the growth depressing activity of lithocholic acid for chicks, the present investigation does not warrant any conclusion as to the exact basic mechanisms underlying its toxicity. Preliminary results indicate that other bile acids such as chenodeoxycholic acid, cholic acid, or deoxycholic acid are devoid of liver toxicity. Since lithocholic acid (3α-hydroxycholanic acid) differs by only one hydroxyl group from chenodeoxycholic acid (3α-7α-dihydroxycholanic acid), which is the physiological bile acid of the chick, it could be that the former acts as an antimetabolite in the bile acid metabolism.

The growth-depressing activity of lithocholic acid—a microbial transformation product—also suggests that it would be of interest to investigate whether the intestinal flora of the chick could produce enough toxic bile acids to interfere with the growth of the animal. In this case the growth-promoting effect of dietary antibiotics eventually could be explained by inhibition of these particular microorganisms, a hypothesis recently discussed by De Somer *et al.* (1962).

ACKNOWLEDGMENT

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EFFECT OF VARIOUS DIETARY BILE ACIDS ON NUTRIENT ABSORPTION AND ON LIVER SIZE IN CHICKS

BY

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(Received for publication 23-12-1964).

Besides the "primary" bile acids formed directly from cholesterol, there is another class of bile acids consisting mainly of bacterial metabolites formed through the action of intestinal microorganisms during enterohepatic circulation of the bile (5, 16, 23). Several of these bacterial metabolites have varying effects on cholesterol concentration and synthesis rates. BEHER *et al.* (2) and HOWE *et al.* (20) reported that hyodeoxy- and lithocholic acids counteract the hypercholesterolemic effect of cholic acid and prevent liver-cholesterol accumulation in mice. Similar findings were reported by PORTMAN and BRUNO (30) with rats. These effects are opposite to those reported in chicks by EDWARDS (10), who found that feeding of 0.2 % of lithocholic acid resulted in a threefold rise in serum cholesterol. Hyodeoxycholic acid was without effect in this species (10).

Less information is available on other possible side effects of these compounds. It has been reported that certain unconjugated bile acids could be toxic to the intestinal mucosa of rats (7), and that gastric instillation of lithocholic acid induces cirrhosis of the liver in rabbits (19). The pyrogenic and inflammatory properties of lithocholic acid in man have been described by PALMER *et al.* (27). EDWARDS (10, 12) found that the hypercholesterolemic effect of lithocholic acid in chicks is associated with marked growth depression in the young birds and an abrupt decrease in egg production in laying hens. More recently it has been observed independently by LEVEILLE *et al.* (22) and by EYSSEN and DE SOMER (14) that dietary lithocholic acid increases the liver size of chicks and causes proliferation of bile ductules.

During our preliminary investigations it was also found that lithocholic acid causes malabsorption of nutrients (14). An analogous syndrome of growth-depression and malabsorption has been observed in chicks fed an antibiotic-free casein-sucrose diet (13). This syndrome is caused by intestinal bacteria since it can be cured by antibiotics, and formation of toxic bile acids by the gut flora has been proposed as a possible mechanism to explain these findings (8). Therefore the present investigations were performed to study the effect of several bile acids and their bacterial degradation products on growth of chicks and on absorption of nutrients.

Materials and Methods

All animals were Hyline broiler chicks obtained from a commercial poultry farm, and were housed in electrically heated batteries with rised wire screen floors under continuous illumination, feed and water being supplied *ad libitum*.

The composition of the basal casein-sucrose diet was the same as in our previous investigations (13). Unless otherwise stated 100 parts per million (ppm) of the antibiotic virginiamycin were added to the basal ration. In all experiments an intestinal tracer, chromic oxide (Cr_2O_3), was added in a concentration of 2 g per kg feed. Bile acids were mixed with the diet as a 5 % alcoholic solution.

Feces used for chromic oxide or for fatty acid determinations were collected on glass plates placed underneath the wire floors. The plates were removed daily and the feces dried under reduced pressure over P_2O_5. Fatty acid determinations were made by the method of Van de Kamer *et al.* (32) and chromic oxide was determined by the method of Edwards and Gillis (9). Serum- and liver-cholesterol concentrations were determined by the method of Abell *et al.* (1).

Lithocholic, cholic and deoxycholic acids were purchased from N.B.C., (Cleveland, Ohio). The 3-ketocholanic acid was prepared by dichromate oxidation of lithocholic acid, followed by purification and cristallisation. Taurolithocholic acid was prepared by the method described by Norman (25). The other bile acids were purchased from Steraloids, Inc., (New York).

Results

Effects of various dietary bile acids in young cockerels.

A first series of experiments was performed to investigate whether a malabsorption syndrome occurs during the growth depression caused by feeding lithocholic acid.

In these experiments lithocholic acid was compared with chenodeoxycholic acid, which is the physiological bile acid of the chick, and with cholic- and deoxycholic acids. Cholic acid has been reported to improve fat absorption in the chick (11), and deoxycholic acid has been described as toxic *in vitro* for the intestinal mucosa of the rat (7).

Therefore these bile acids were fed for 2 weeks at the 0.2 % level

to groups of 3-week-old cockerels. Each group consisted of 8 birds of the same initial body weight. As shown in Table I the feeding of chenodeoxycholic, cholic, or deoxycholic acid did not cause growth depression. Both chenodeoxycholic and cholic acid improved intestinal fat absorption, whereas for deoxycholic acid no statistically significant difference was observed.

Lithocholic acid was the only bile acid that caused growth depression, malabsorption of fats, and impaired efficiency of feed utilization. The most conspicuous effect of lithocholic acid, however, was the threefold increase in liver size. The livers of these animals were pale yellowish to light brown and of a very firm consistency. As has been reported in a preliminary research note (14), pathological examination of these livers showed extensive proliferation of cholangiocytes and marked fibrosis. Similar findings were reported by LEVEILLE *et al.* (22).

Since EDWARDS (10) reported that administration of lithocholic acid to chicks causes a threefold rise in serum-cholesterol concentrations, the lipid metabolism of our animals fed bile acids was further investigated.

Table II shows that feeding of lithocholic acid resulted in a threefold rise in serum-cholesterol concentrations and total liver cholesterol, whereas the other bile acids were without effect. To demonstrate that the sterols, which accumulated in the serum of birds fed lithocholic acid were really cholesterol, different systems of gas-liquid chromatography were applied. In each case, the peak containing the majority of the sterols had the retention time of authentic cholesterol.

Table II also shows that lithocholic acid does not cause fatty acid infiltration of the liver, because after saponification and extraction a less than normal amount of fatty acids per gram liver was found. This is in agreement with the results of the histological examination, which indicated that the increase in liver size was mainly due to proliferation of bile-duct cells.

As the specific toxic effect of lithocholic acid eventually could be explained by interference with the metabolism of chenodeoxycholic acid, which is the main constituent of chick bile, ursodeoxycholic acid and hyodeoxycholic acid, were also examined for their possible toxic effects. Ursodeoxycholic acid is found in the bile of certain bear species (17) and small amounts of this acid occur in the rat (24) and in man (31). Hyodeoxycholic acid is a bacterial degradation product of hyocholic acid and is one of the major constituents of pig bile (4). An analogue of lithocholic acid, namely 3-ketocholanic acid, was also included in this study.

TABLE I

Effects of feeding 0.2 % of various bile acids on growth, fat absorption and liver weight in 3-week-old cockerels

	Experimental diets (1)				
	Basal	Lithocholic acid	Chenodeoxycholic acid	Deoxycholic acid	Cholic acid
Average daily weight gain g/bird	23.6	12.9	20.2	22.5	24.6
Feed efficiency g feed/g weight gain	1.5	2.4	1.6	1.7	1.7
Fecal fatty acids (2) mg/g feed intake	8.8	14.9	4.9	6.1	4.7
Liver weight g/bird	12.9	31.5	12.9	14.2	14.1
Liver weight g/100 g body weight	2.1	6.5	2.3	2.4	2.3

(1) 3 animals per group. (2) 6 % corn oil in diet.

TABLE II

Effects of feeding 0.2 % of various bile acids on serum and liver lipids in 3-week-old cockerels

	Experimental diets (1)				
	Basal	Lithocholic acid	Chenodeoxycholic acid	Deoxycholic acid	Cholic acid
Serum cholesterol mg/100 ml	152	463	150	161	155
Liver cholesterol mg/100 g	371	483	393	377	365
Total liver cholesterol mg/liver	48	152	51	53	51
Liver fatty acids meq/100 g	11.4	8.2	11.2	9.9	11.1
Liver fatty acids meq/total liver	1.5	2.5	1.5	1.4	1.6

(1) 3 animals per group.

As shown in Table III, both hyodeoxycholic and ursodeoxycholic acids were devoid of toxic effects at least in our short term experiment of 2 weeks. Ursodeoxycholic acid improved fat absorption and, in this respect, can be compared to cholic acid or to chenodeoxycholic acid. Hyodeoxycholic acid on the other hand, did not improve fat absorption, and this observation has been confirmed in other experiments. Of the three bile acids under investigation, only 3-ketocholanic acid was shown to cause a typical intoxication syndrome with increased liver size, hypercholesterolemia, reduced growth, and malabsorption of nutrients. It cannot be concluded from these experiments, however, whether this is due to the unmodified substance itself, since it could be that rehydroxylation occurs in the liver thereby giving rise to lithocholic acid.

In other experiments several attempts were made to protect animals from lithocholic acid toxicity. Addition to the diet of 0.1 % methionine and 0.1 % taurine was without effect, thus indicating that a deficiency in taurine or methionine is not the prime factor in the pathogenesis of the intoxication syndrome. Since it is generally accepted that the unconjugated bile acids are more toxic than their taurine or glycine derivatives, taurolithocholic acid was also investigated. To prevent deconjugation by intestinal bacteria the concentration of the antibiotic in the diet was increased from 100 to 500 ppm. Although the presence of traces of free lithocholic acid in the gut cannot be excluded, repeated thin-layer chromatography on the intestinal contents showed only conjugated lithocholic acid. Nevertheless, when fed in equimolecular amounts, taurolithocholic acid was as toxic as its unconjugated derivative.

Evolution of the morphological alterations of the liver after feeding lithocholic acid.

In order to follow the evolution of morphological alterations, a group of 40 chicks was fed the basal diet plus 0.2 % lithocholic acid. Two animals were killed at regular intervals and their liver compared with livers of control animals receiving the basal diet only.

Although in most cases, signs of necrosis, regeneration, and bileduct proliferation were seen simultaneously, we divided the sequence of histological events into three chronological periods.

The first period extended from 12 to 36 hours after the first feeding of lithocholic acid. Macroscopically, the liver was slightly enlarged and showed numerous small hemorrhagic spots scattered over the whole parenchyma. This last observation was made after 24 hours only.

Microscopically, numerous necrotic areas were observed as early as

TABLE III

Effects of feeding 0.2 % of various bile acids on growth and on serum and liver lipids in 3-week-old cockerels

	Experimental diets (1)				
	Basal	Dehydrocholic acid	Hyodeoxycholic acid	Ursodeoxycholic acid	3-Ketocholanic acid
Daily weight gain g/bird	22.3	21.8	22.9	21.5	16.2
Liver weight g/bird	15.0	15.1	14.6	15.2	29.3
Liver cholesterol mg/100 g	388	373	372	390	398
Serum cholesterol mg/100 ml	121	142	126	133	491
Fecal fatty acids mg/g feed intake	8.9	5.3	9.0	4.5	19.2

(1) 8 Animals per group.

12 hours after the feeding of lithocholic acid. These necrotic areas were localized either in the outer or in the middle part of the lobule and were observed through the whole liver parenchyma. In these focal lesions the liver cells had lost their cell boundaries and had pycnotic nuclei. No glycogen was found in these necrotic masses, and only exceptionally a few necrotic cells were loaded with fat droplets. In some portal areas a round-cell infiltration was present.

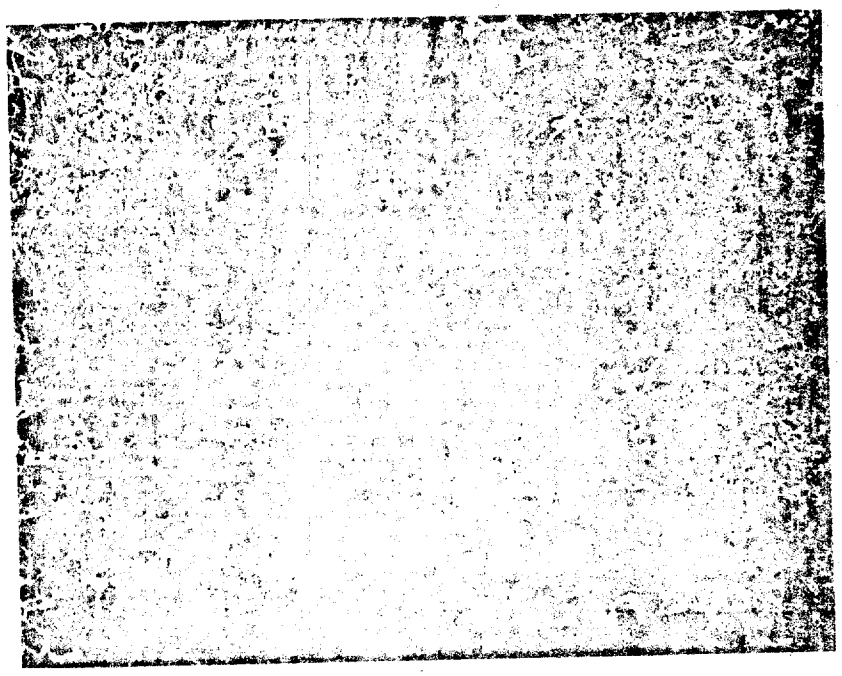

FIG. 1

Chicken liver 36 hours after being fed a diet containing 0.2 % lithocholic acid. Several large necrotic areas some of which are infiltrated by proliferating fibroblast-like cells. In the center and on the left side of the figure is seen the tendency of these proliferating cells to form tubular structures. × 150 — H & E stain.

After 24 and 36 hours, numerous hemorrhagic patches were localized either in and around the necrotic areas or at a certain distance from them. In a few necrotic areas, young fibroblast-like cells were observed, whereas around them were sometimes found small groups of proliferating and invading cells with a rather elongated nucleus and a basophilic cytoplasma (FIG. 1).

During the second period, which lasted for approximately 3 to 4 weeks,

there were simultaneously signs of necrosis, cellular proliferation, and regeneration of parenchymal cells.

Macroscopically, the liver was greatly enlarged and had a firm consistency. The color was pale yellow-brown and spotted. The gall-bladder was frequently enlarged and contained turbid gall.

Microscopically, the necrotic areas were invaded by little-differentiated cells with pale elongated nuclei, in which prominent nucleoli

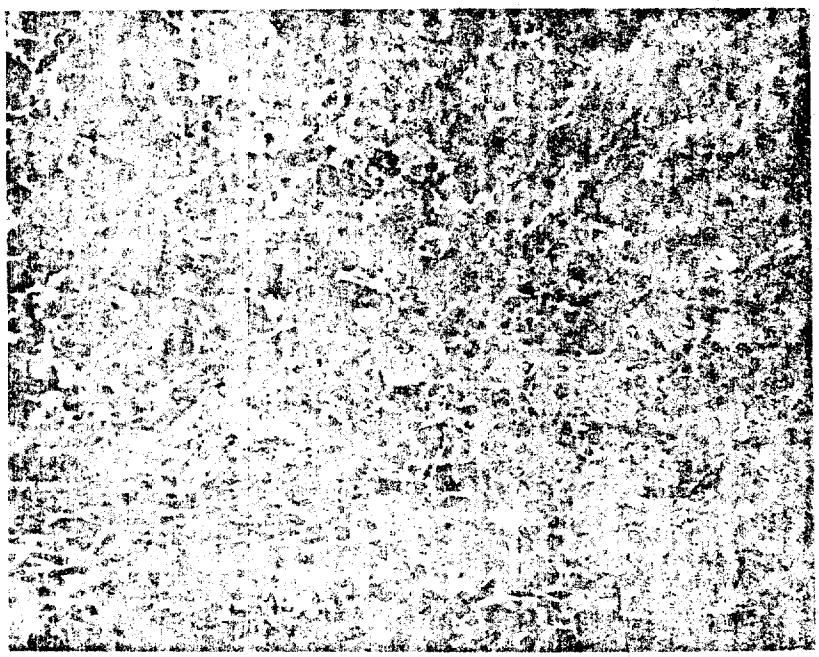

FIG. 2

Chicken liver 54 days after the first feeding of lithocholic acid. Marked proliferation of tubular structures with sequestration of small groups of hepatocytes and infiltration of the surrounding parenchyma by ductular cells. × 375. — Trichrome Masson stain.

were observed, and with rather basophilic cytoplasma. Many mitotic figures were observed among these cells. They appeared to differentiate very early to cholangiocytes and to form small bile-duct structures (FIG. 2). This differentiation was particularly pronounced in the periportal areas and very quickly spread over the whole liver parenchyma causing the bileduct structures (FIG. 3). The cellular proliferation was accompanied by an extensive increase of reticulin fibers. Many of these fibers were swollen mostly in the periportal areas but also infiltrating

between the liver tubules together with the proliferating bile-duct structures. Signs of regeneration of the hepatocytes were visible, and many mitotic figures were found among the parenchymal cells.

The third period, which began approximately one month after the first feeding of lithocholic acid, was characterized essentially by the development of a very pronounced cholangiofibrosis.

Macroscopically, the liver was enormously enlarged and very firm.

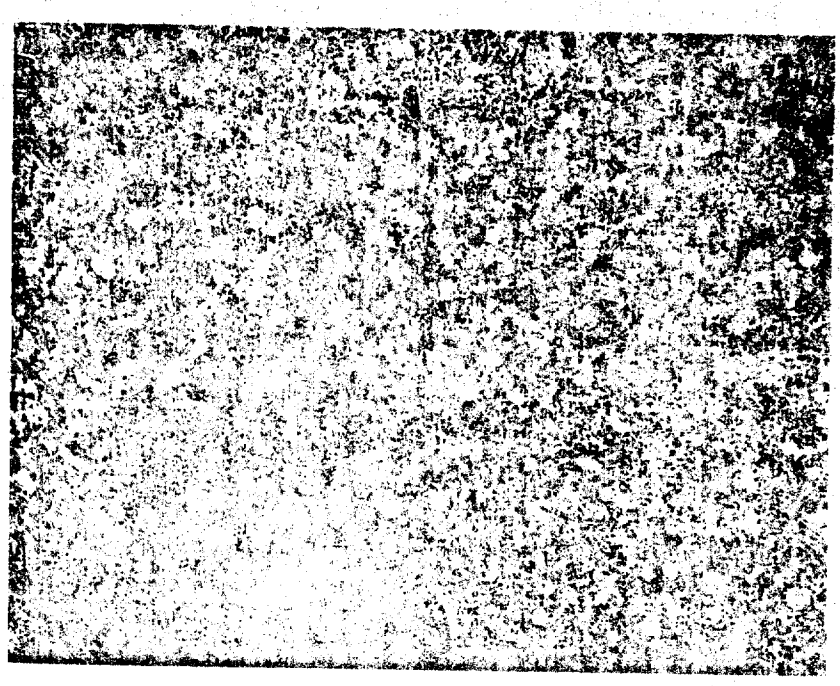

FIG. 3

Marked ductular proliferation mostly located in the periportal area and extending in the surrounding parenchyma. × 50 — H & E stain.

The surface was no longer smooth but gradually became nodular, resembling a cirrhotic liver (FIG. 4). The color was pale yellow-brown. Ascites was always present after 2 months and a fibrinous exudate was found sometimes in the peritoneal cavity. The gall-bladder was greatly enlarged and contained very turbid gall in which a dark stained precipitate was found.

Microscopically, there was gradually increasing proliferation of bile-duct structures invading progressively the whole liver parenchyma. This

proliferation led to complete destruction of the liver structure and gave rise to extensive cholangiofibrosis. The number of healthy hepatocytes decreased steadily, and after 2 to 3 months only small groups diffusely spread over the liver were observed.

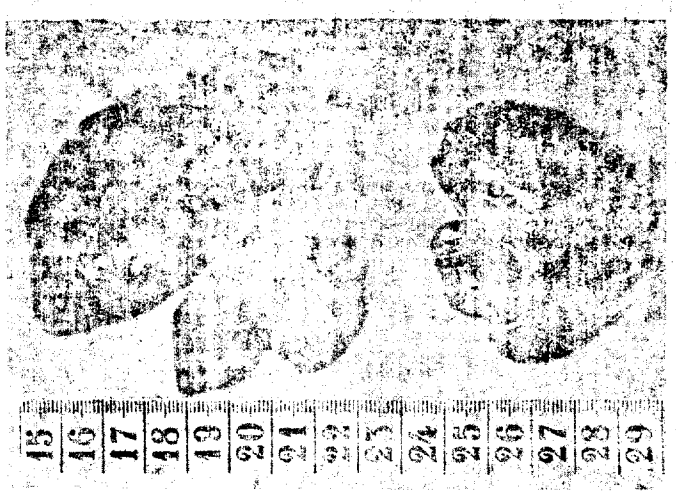

FIG. 4

Enlarged liver with nodular and light colored surface of chicken fed a diet containing 0.2 % lithocholic acid for one month. To the right normal liver of the control group.

DISCUSSION

The present investigations confirm the previous reports by LEVEILLE *et al.* (22) and by EYSSEN and DE SOMER (14), who found that the growth depression caused by feeding chicks with 0.2 % lithocholic acid is associated with hypercholesterolemia and an increase in liver size due to proliferation of bile-duct cells. In addition, our experiments have shown that the first lesions in the liver are characterized by numerous necrotic areas which were observed as early as 12 hours after the feeding of lithocholic acid. Thereafter, a period of regeneration of hepatocytes and proliferation of cholangiocytes begins, and is followed by cholangiofibrosis and a nodular cirrhotic liver with ascites and fibrinous exudation in the peritoneal cavity. Malabsorption of nutrients and increased fecal fat output are common features. These events clearly demonstrate that bile-acid metabolites produced by the intestinal flora can be highly toxic to the host.

Whether the increase in serum cholesterol after feeding of lithocholic acid is due to feed-back inhibition of the degradation of cholesterol to bile acids or merely to damage and functional block of hepatocytes is not entirely clear. But the observation that chenodeoxycholic acid — the physiological bile acid of the chick — does not cause hypercholesterolemia, suggests that feed back inhibition is not sufficient explanation.

Although it seems reasonable to accept the premise that the hepatotoxicity and the intestinal malabsorption can explain the poor weight gain during lithocholic acid feeding, it cannot be concluded from our experiments whether these disturbances are due to the intact lithocholic acid molecule or to a transformation product formed in the intestine or in the liver. In the liver of the rat, for instance, lithocholic acid has been shown to be actively metabolized giving rise to several more polar compounds (3, 26, 28). No definite data are available on the metabolism of lithocholic acid in the liver of the chick, but preliminary experiments indicate that only minor traces of metabolites are found in the bladder bile during a period of 2 days after the administration of lithocholic acid. Yet the first lesions appear in the liver in 12 hours. Metabolites do not appear in appreciable amounts until lithocholic acid has been fed for 3 to 4 days, when a twofold increase in liver size is observed. This delay suggests that the appearance of metabolites is the sequence, not the cause, of a profoundly disturbed hepatic bile-acid metabolism.

Whatever may be the toxic substance involved, the early lesions are characterized by focal necrosis and suggest a direct toxic action on the hepatocytes. The ductular reaction following these early lesions has also been observed in other animals after experimental intoxication with ethionine or carcinogens (15) and might be the result of excretion and concentration of irritating materials in the ductules. Interference with the metabolism of chenodeoxycholic acid and accumulation of toxic intermediates are another possible explanation. Nevertheless, hyodeoxycholic acid and ursodeoxycholic acid, which can also be considered to be analogues of chenodeoxycholic acid, are not toxic.

Our observation that the di- or trihydroxylated bile acids are not toxic rather seems to indicate that the hepatotoxic properties are restricted to certain monohydroxy- or monoketo bile acids. Analogous conclusions were drawn by PALMER *et al.* (27) from their studies on the pyrogenic and inflammatory properties of bile acids in man. Only the 3 α-hydroxy bile acids were strong pyrogenic and inflammatory compounds and polyhydroxylation inhibited these activities. It is also noteworthy that the pyrogenic neutral steroid hormones described previously, belong to the 5 β-H series and have the molecular configuration of the bile acids

(21). Moreover, many of the most active compounds are 3 α-hydroxy derivatives such as etiocholanolone.

Finally, there seems to be a species-dependent difference in susceptibility to the harmful action of lithocholic acid. As shown by HOLSTI (19), cirrhosis of the liver is easily induced in the rabbit following ingestion of lithocholic acid. On the other hand, other work in this laboratory (to be published) has shown that it is difficult to cause ductular cell reaction by feeding lithocholic acid to rats or mice. In rats it was found that lithocholic acid was not toxic when added to a complete diet, but it induced ductular cell reaction when the animals were fed a low-protein diet. Yet the rat is known to metabolize lithocholic acid fairly rapidly into several more polar compounds (3). In view of these results, it seems an attractive hypothesis to postulate that the transformation in the liver results in detoxication by hydroxylation of the bile acid. Perhaps this defense mechanism is acting in normal rats and mice but could be lacking in the protein-deficient rat, in the chick, and in the rabbit.

Since it has been reported very recently that injected lithocholic acid is only slowly metabolized in humans with bile fistulas (6), it is not impossible that lithocholic acid is also involed in pathogenesis of human liver disease. Lithocholic acid is found in the human intestine (18), and ductular cell reaction has been observed in postnecrotic cirrhosis and viral hepatitis (29). Eventually the primary lesions provoked by the virus or the toxic chemical could abolish the defense mechanism by which the normal hepatocyte can detoxify lithocholic acid or another harmful bile acid. Subsequently the latter could cause proliferation of bile-ducts, as in the protein-deficient rat, eventually followed by fibrosis and cirrhosis, as in the chick or rabbit.

SUMMARY

The effects of dietary bile acids were investigated in one-day-old chicks and in three-week-old cockerels.

Feeding 0.2 % of lithocholic acid caused malabsorption of fats, impaired efficiency of feed utilization and a twofold increase in liver weight after 2 weeks of treatment. Body weight gain was reduced by 20 to 40 % and a threefold rise in serum-cholesterol and total liver-cholesterol concentrations was observed. The 3-ketocholanic acid caused an identical syndrome.

Histologically the liver changes consisted of necrosis of hepatocytes

followed by proliferation of cholangiocytes and cholangiofibrosis. One month after the first feeding of lithocholic acid ascites and fibrinous exudation were always present in the peritoneal cavity.

Other bile acids such as cholic, chenodeoxycholic, dehydrocholic, hyodeoxycholic, and ursodeoxycholic acids were devoid of liver toxicity, improved intestinal fat absorption and did not significantly affect serum- or liver-cholesterol concentrations.

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OBSERVATIONS CONCERNING THE PRODUCTION AND EXCRETION OF CHOLESTEROL IN MAMMALS

X. Factors Affecting the Absorption and Fate of Ingested Cholesterol*

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Previous studies (1, 2) from this laboratory have indicated that the supply of endogenous cholesterol to the plasma of the rat is primarily a function of the liver. The rate of this supply may be increased by the administration of thyroid extract (3, 4) or decreased by administration of thiouracil (3, 4), by partial hepatectomy (5), and by carbon tetrachloride intoxication (5). Other studies (6, 7) have demonstrated that the removal of cholesterol from the plasma is also a function of the liver, which first stores the cholesterol (8), then converts most of it into bile acid and excretes it (8). The rate of removal of cholesterol from plasma has been found to be increased by administration of thyroid extract (3) and decreased by administration of thiouracil (3), accumulation of bile acid in plasma (9) and by injection of the surface active substance, triton WR-1339 (10).

Despite the importance of the above endogenous phases of cholesterol metabolism in the regulation of cholesterol in plasma, considerable evidence is available which suggests that the absorption of exogenously derived cholesterol may affect not only these same endogenous phases (11) but also may alter the plasma level of cholesterol of the rabbit (12) and, under certain circumstances, also that of the dog (13) and the rat (14).

Although absorbed cholesterol has been known to enter the intestinal lymph since demonstration of the fact by Mueller (15, 16), quantitative studies concerning the amount of cholesterol absorbed have been done by analyzing the feces of animals given known amounts of cholesterol (17-19). This latter method has been employed (despite the ambiguity possible because of the breakdown of cholesterol in the intestine, as well as its secretion therein) because of earlier technical difficulties associated with chronic cannulation of the lymph duct of experimental animals, and because of uncertainty as to the fraction of absorbed cholesterol which was carried in the lymph.

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However, the difficulties associated with cannulation of the thoracic duct have been overcome, in the rat, by the techniques introduced by both Reinhardt (20) and Bollman and Flock (21). Later, too, it was found in this laboratory (22) that the intestinal lymph serves as the *only* pathway for the absorption of cholesterol (22). In view of these recent discoveries then, the total amount of cholesterol absorbed from the gut can be estimated quantitatively by analysis of the complete collection of intestinal lymph alone.

Therefore, in the studies to be reported herein concerning the factors affecting the absorption and fate of ingested cholesterol, we have analyzed the intestinal lymph, as well as the feces, of several series of rats under various experimental conditions.

Methods

A. Determination of Intestinal Absorption of Cholesterol

1. Collection and Analysis of Feces.—The feces of individually caged male Long-Evans rats were separately collected for 72 hours. At the end of this time, the rats were anesthetized, the small and large intestines were removed and opened, and their contents added to the respective individual fecal collections. Each collection then was analyzed for (*a*) "total sterols;" *i.e.*, total digitonin precipitable substance, (*b*) total cholesterol–Lieberman-Burchard positive material, calculated as cholesterol, and (*c*) "non-cholesterol sterols," obtained by subtracting (*b*) from (*a*). The chemical procedures associated with this analysis have been published elsewhere (23).

All animals were allowed to ingest water and the sterol-free diet, *ad lib.* This latter diet consisted of dextrose 72 per cent, sodium caseinate 14 per cent, gluten 6 per cent, cellulose fiber 5 per cent, U.S.P. salt No. 2, 3 per cent, supplemented with methyl linoleate 60 mg. per 100 gm. of diet and litrison[1] vitamin mixture 200 mg. per 100 gm. of diet. When excess free cholesterol was given it was dissolved in 3 cc. of olive oil and administered by stomach intubation. When excess cholate was given, it was dissolved in the equivalent quantity of 1 N NaOH, diluted with water, and also administered by stomach tube. Cannulation of the bile duct was done according to previously described methods (24), except that the rats were confined in restraining cages so that the catheter could be brought out directly through the abdominal wall. This avoids the necessity of piercing the leg of the animal.

2. Collection and Analysis of Intestinal Lymph.—Intestinal lymph was collected from the abdominal portion of the thoracic duct just above the cysterna chyli according to previously described methods (22). All rats were allowed to ingest 0.45 per cent saline solution and sterol-free diet *ad lib.* In some of the experiments, lymph was collected daily for 72 hours. The total cholesterol, and in some experiments the free cholesterol, of the lymph samples was determined according to methods previously described for blood (25).

B. Determination of Cholesterol in Blood and Tissues

Cholesterol in blood was determined by previously described methods (25), and that in tissues was determined in the same manner as for feces (23).

I. THE INTESTINAL ABSORPTION OF CHOLESTEROL

A. Effect of Cholesterol in Diet

1. Rats Given No Cholesterol.—When rats are placed upon a diet containing no cholesterol, and when, in addition, all bile is diverted from the intestine,

[1] Provided through the courtesy of Hoffman-La Roche, Inc., Rahway, New Jersey.

the flow of lymph will contain a physiologic minimum of cholesterol. This cholesterol may possibly be derived directly from the blood stream (26). Thus (see Table I) the residual content of lymph cholesterol under these circumstances averaged 2.9 mg. per 24 hour collection. If bile is allowed to enter the

TABLE I

The Intestinal Absorption of Cholesterol in Rats Deprived of Bile Acids and in Rats Given Excess Cholate

	No. of rats	Average weight	Intestinal lymph					
			0–24 hrs.			24–48 hrs.		
			Vol.	Total cholesterol		Vol.	Total cholesterol	
		gm.	cc.	mg./100 cc.	mg./24 hrs.	cc.	mg./100 cc.	mg./24 hrs.
*A. Rats Deprived of Bile Acids**								
1. Given No Cholesterol								
Average	6	295	16.7	18	2.9	—	—	—
Range	—	219–324	11.5–27.5	12–22	1.7–3.7			
2. Given 100 Mg. of Cholesterol								
Average	14	299	26.7	23	5.5	51.9	13	5.9
Range	—	249–377	12.0–46.0	3.4–45	1.0–8.6	17.5–79.0	3.3–17	2.3–10
S.E. mean	—	—	±3.0	±8.8	±0.51	±10.4	±2.39	±1.59
B. Rats Given Excess Cholate‡								
1. Given No Cholesterol								
Average	9	276	44.0	38	15.3	—	—	—
Range	—	231–340	27.0–86.0	28–54	7.5–19.8	—	—	—
S.E. mean	—	—	±5.8	±3.82	±1.07	—	—	—
2. Given 100 Mg. of Cholesterol								
Average	7	254	52.0	79	39.9	—	—	—
Range	—	213–282	34.0–69.0	60–99	29.4–51.8	—	—	—
S.E. mean	—	—	—	—	—	—	—	—

* External bile fistula established.

‡ Sodium cholate equivalent to 50 mg. of cholic acid given daily by stomach tube.

lumen of the intestine, endogenous cholesterol, previously secreted into the intestinal contents, is reabsorbed. This reabsorption was sufficient to increase the cholesterol content of a 24 hour collection of intestinal lymph (see Table II) to an average of 11.4 mg. Approximately two-thirds of this was in esterified form. Therefore, the rat, although fed a cholesterol-free diet, nevertheless absorbs about 8.5 mg. of cholesterol per day. Furthermore, it excretes at least an additional 5.4 mg. per day in the feces, since Table III shows that 16.2 mg.

TABLE II

The Intestinal Absorption of Cholesterol in Rats after the Ingestion of Various Amounts of Cholesterol

	No. of rats	Average weight	Intestinal lymph													
			Volume	0–24 hrs						Volume	24–48 hrs.		Volume	48–72 hrs.	
				Cholesterol							Total cholesterol			Total cholesterol	
				Total		Free		Ester							
		gm.	cc.	mg./100 cc.	mg./24 hrs.	mg./100 cc.	mg./24 hrs.	mg./100 cc.	mg./24 hrs.	cc.	mg./100 cc.	mg./24 hrs.	cc.	mg./100 cc.	mg./24 hrs.
A. Rats Given No Cholesterol															
Average	26	290	36.0	31.5	11.4	9.4	3.2	22.1	7.4	—	—	—	—	—	—
Range	—	216–412	15.7–63.0	13–55	7.1–16	3.0–15	1.9–4.1	10–35	4.2–9.7	—	—	—	—	—	—
S.E. mean	—	—	±2.5	±2.1	±0.6	±0.8	±0.24	±2.3	±0.47	—	—	—	—	—	—
B. Rats Given 50 Mg. of Cholesterol															
Average	6	276	41.0	43.0	17.0	—	—	—	—	—	—	—	—	—	—
Range	—	248–335	26–58	31–73	11–25	—	—	—	—	—	—	—	—	—	—
S.E. mean	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
*C. Rats Given 100 Mg. of Cholesterol**															
Average	33	286	44.0	63.0	25.1	20	6.4	64	18.1	68	37	21.8	80	26	14.8
Range	—	215–327	17–107	25–104	15–39	7–34	3.7–10	30–110	11–29	39–128	16–70	14–32	39–134	9–50	8.0–23
S.E. mean	—	—	±4.0	±6.8	±1.23	±2	±0.6	±6.0	±1.7	±8.4	±4.2	±1.6	±14.7	±5.5	±1.51

* Analyses of free and esterified cholesterol done on only 12 of 33 rats.

of cholesterol was found in the 72 hour feces collection. The actual excretion may be greater, as some cholesterol presumably is converted to coprosterol before excretion. These data then demonstrate that the rat probably secretes at least 13.9 mg. of cholesterol (8.5 mg. + 5.4 mg.) into the lumen of his in-

TABLE III

The Intestinal Excretion of Cholesterol in Rats after the Ingestion of Various Amounts of Cholesterol

	No. of rats	Average weight	Intestinal collection—72 hrs.						Average total sterol retained (4)‖
			Total sterol (1)*		Non-cholesterol sterol (2)‡		Cholesterol (3)§		
		gm.	*mg./100 gm.*	*mg./72 hrs.*	*mg./100 gm.*	*mg./72 hrs.*	*mg./100 gm.*	*mg./72 hrs.*	*mg./72 hrs.*
A. Rats Given No Cholesterol									
Average	17	238	1302.0	37.6	677.0	21.5	567	16.2	—
Range	—	180–336	840–2230	18–53	310–1290	10–34	370–940	8.0–24	—
S.E. mean	—	—	±94.0	±2.45	±63.0	±1.8	±41.0	±1.1	—
B. Rats Given 50 Mg. of Cholesterol									
Average	10	204	2225	64.0	975.0	30.0	1250	36.0	23.6
Range	—	187–226	1750–2880	44–100	585–1785	22–48	950–1690	23–52	—
S.E. mean	—	—	±102.0	±4.8	±103.0	±2.8	±64.0	±2.76	—
C. Rats Given 100 Mg. of Cholesterol									
Average	15	265	2730	104.0	961.0	37.0	1769	67.0	33.6
Range	—	200–326	810–4250	83–130	300–1770	12–64	1050–2530	45–83	—
S.E. mean	—	—	±155.0	±3.2	±106.0	±3.9	±97	±2.1	—

* (1) equals total digitoxin-precipitable substance.

‡ (2) equals difference between (1) and (3).

§ (3) equals Lieberman-Burchardt positive substance.

‖ (4) equals (ingested cholesterol + average total sterol excreted of series *A* rats) − (average total sterol excreted by experimental series).

testine per day. No more than about 2.0 mg. of this is derived from bile (5).

2. *Rats Given 50 Mg. of Cholesterol.*—When six rats each were given 50 mg. of cholesterol in 3 cc. of olive oil and then placed upon the sterol-free diet, it was found (see Table II) that the cholesterol content of their lymph increased to an average of 17.0 mg. during the first 24 hours. Analyses of the feces of 10 rats similarly fed (see Table III) revealed that they apparently absorbed 23.6 mg. of exogenously derived cholesterol in 72 hours or about 47 per cent of the total amount given.

3. Rats Given 100 Mg. of Cholesterol.—Rats given 100 mg. of cholesterol in 3 cc. olive oil, showed an average of 25.1 mg. of cholesterol (see Table II) in the lymph collected during the first 24 hours. If this quantity is compared with that found in lymph when no cholesterol was given (11.4 mg., see Table II), it can be seen that an increase of 13.7 mg. (25.1 − 11.4) attributable to the administered cholesterol, was absorbed the 1st day. Again, as Table II indicates, the major portion of the absorbed cholesterol was in the esterified form. Analyses of the lymph on subsequent days (see Table II) indicate that increased absorption also occurred during the 2nd and 3rd days. If the same type of calculation is made as used above to determine the excess amount of cholesterol absorbed, apparently 27.5 mg. of the 100 mg. of cholesterol administered was absorbed over the 72 hour period. This is probably a minimum amount, because it is unlikely that the basic cholesterol content of the intestinal lymph of the rat (on a cholesterol-free diet) will continue to be as much as 11.4 mg. daily, after the first 24 hour collection, as assumed in making this calculation.

The feces of 15 rats fed 100 mg. of cholesterol were analyzed for total sterol and for cholesterol. As Table III indicates, 33.6 mg. of this cholesterol was considered to be absorbed over the 72 hour period. The 18 per cent discrepancy between the value of 27.5 mg. obtained in direct recovery from lymph and that of 33.6 mg. calculated from the deficit in the feces could be due to various factors; the most likely one is our assumption that the irreducible minimum of cholesterol in lymph of the rat fed a cholesterol-free diet will continue to be 11.4 mg. of cholesterol daily even after the first 24 hours, which of course may not be true. The lymph content of cholesterol probably decreases with time on a sterol-free diet.

B. Effect of Absence of Bile

1. Rats Given No Cholesterol.—Six normal rats were deprived of intestinal bile by cannulation of their bile ducts; the thoracic ducts were cannulated and then the rats were placed upon a cholesterol-free diet. As can be seen from comparison of the figures for cholesterol found in the 24 hour lymph collection of a normal rat (Table II) with that found for the bile duct cannulated animal (Table I), a marked reduction occurred in the quantity of cholesterol found in the intestinal lymph. Apparently then, the absence of bile seriously interferes with the reabsorption of cholesterol excreted by the intestinal wall. The residual lymph content of cholesterol even in absence of bile may represent a direct contribution from the blood (26).

2. Rats Given 100 Mg. of Cholesterol.—Intestinal lymph, collected daily for 48 hours from bile duct cannulated rats fed 100 mg. of cholesterol, also showed (see Table I) a drastic reduction in cholesterol content as compared (see Table II) with that of lymph collected from intact rats receiving the same quantity of cholesterol.

C. *Effect of Excess Cholate*

1. Rats Given No Cholesterol.—The oral administration of 50 mg. of cholate to rats fed a sterol-free diet increased the lymphatic absorption of cholesterol. The average cholesterol found in the lymph of the first 24 hours was 15.3 mg. (see Table I) which, when compared to the 11.4 mg. found in the untreated rat (see Table II) represents an increase of 24 per cent.

TABLE IV

The Intestinal Excretion of Cholesterol in Rats Given Excess Cholate (50 Mg. per Day)

	No. of rats	Average weight	Intestinal collection—72 hrs						Average total sterol retained
			Total sterol		Non-cholesterol sterol		Cholesterol		
		gm.	mg./100 gm.	mg./72 hrs.	mg./100 gm.	mg./72 hrs.	mg./100 gm.	mg./72 hrs.	mg./72 hrs.
A. Rats Given No Cholesterol									
Average	10	286	1176	37.0	614	19.2	562	17.9	—
Range	—	245–336	905–1460	27–44	431–924	13–29	453–728	10–29	—
S.E. mean	—	—	±55	±2.1	±47	±1.5	±27	±1.38	—
B. Rats Given 100 Mg. Cholesterol									
Average	10	258	3392	88.7	1209	32.0	2183	56.7	48.9
Range	—	210–299	2390–3960	69–134	505–1760	13–61	1530–2500	44–72	—
S.E. mean	—	—	±49	±5.7	±120	±4.2	±122	±2.56	—

2. Rats Given 100 Mg. of Cholesterol.—The feeding of excess cholate to rats also getting 100 mg. of cholesterol markedly increased the intestinal absorption of cholesterol. The average cholesterol in the lymph of 7 rats so treated (see Table I) was 39.9 mg.,—an increase of 59 per cent over the control value of 25.1 mg. shown in Table II. Analyses of the feces of 10 rats similarly fed excess cholate showed (see Table IV) less fecal sterol than was found in rats not receiving cholate supplementation (Table III), and therefore an increase of 45 per cent in the apparent absorption of cholesterol.

II. THE FATE OF ABSORBED CHOLESTEROL

A. *The Plasma Cholesterol during and after the Absorption of Cholesterol*

The plasma concentration of cholesterol was not found to change significantly following the administration of 100 mg. of cholesterol, even if cholate also was given. The average plasma cholesterol of 10 rats given 100 mg. of

cholesterol plus the sterol-free diet was 61, 60, 62, and 64 mg. per 100 cc., before, and 24, 48, and 72 hours, respectively, after the ingestion of the cholesterol. The average plasma cholesterol of 8 rats given 100 mg. of cholate in addition to the 100 mg. of cholesterol was 57 and 61 mg. per 100 cc., before and 72 hours after, respectively, the ingestion of the two substances.

B. The Deposition of Cholesterol in the Liver and Viscera

A previous study (9) had shown that when excess cholesterol, in the form of hypercholesteremic serum (27), was injected intravenously into rats, its disappearance from the blood proceeded *pari passu* with its deposition in the liver. The other tissues of the body, however, showed no significant increase in cholesterol following this type of cholesterol administration. Furthermore, although the ingestion of a high cholesterol diet (containing 2 per cent cholesterol) by the rabbit led to an immediate hypercholesteremia within 72 hours, no excess cholesterol was found in any tissue except the liver (28).

TABLE V

The Deposition of Intestinally Absorbed Cholesterol

No. of rats	Weight	Plasma cholesterol		Liver			Viscera*		
		Before experiment	72 hrs. after exp.	Dry weight	Cholesterol		Dry weight	Cholesterol	
						Total organ			Total organ
	gm.			*gm.*	*mg./100 gm.*	*mg.*	*gm.*	*mg./100 gm.*	*mg.*
A. Normal Control Rats on Stock Diet									
5	181	51	—	2.31	893	20.7	4.87	1190	57.3
Range.....	166–200	50–53	—	1.96–3.16	715–1100	17–28	4.23–5.80	1060–1380	54–62
B. Rats Given High Cholesterol Diet for 72 Hrs.									
9	180	51	61	1.88	2150	39.1	4.80	1276	61.1
Range.....	146–220	49–73	49–87	1.47–2.53	1450–3230	28–54	4.65–5.07	1180–1350	58–66

* Viscera include the entire contents of both peritoneal and thoracic cavities with the exception of the liver.

In view of these findings, the livers of young rats, and those of young rats fed for 72 hours on a diet containing 2 per cent cholesterol, were analyzed for cholesterol. Similar analyses were made of the combined extrahepatic organs (intestines, kidney, adrenal glands, testes, heart, and lungs) after similar feeding of the high cholesterol diet.

The liver content of cholesterol in the 9 rats given a high cholesterol diet for 72 hours increased markedly (see Table V) as compared to the values found in

the control rats. This average increase of approximately 19 mg. of cholesterol, representing an increase of almost 100 per cent, of course accounts for only a fraction of the amount of cholesterol absorbed but it must be remembered that undoubtedly a considerable amount of cholesterol also was converted into and excreted as cholic acid in these young rats, as previously demonstrated (29).

Despite the marked increase in the cholesterol content of the livers of rats given a cholesterol-rich diet, there was only a slight increase in the cholesterol content of the extraheptaic organs. Thus the viscera of 9 rats (see Table V) contained an average of 61.1 mg. of cholesterol as compared to the control value (see Table V) of 57.3 mg.,—an increase of only 7 per cent. Apparently then, only the liver plays a prominent part in removing dietary cholesterol from the blood.

The fundamental importance of the liver in removing the newly absorbed cholesterol from the blood was shown more strikingly by the following experiments. 5 of 10 rats were subjected to partial hepatectomy (approximately 48 per cent of the total liver was removed) and then all the rats were given 100 mg. of cholesterol in 3 cc. olive oil daily by stomach tube. The average plasma cholesterol of the operated rats rose from 56 mg. per 100 cc. (range: 49 to 62 mg./100 cc.) before, to 115 mg. per 100 cc. (range: 97 to 130 mg./100 cc.) 48 hours after, the partial hepatectomy. The plasma cholesterol of the control rats on the other hand did not increase, being 66 before and 51 mg. per 100 cc., 48 hours after the beginning of the cholesterol feeding.

DISCUSSION

The preceding studies indicate that the intestine of the rat is capable of excreting at least 13.9 mg. of cholesterol per day and that a portion of this secretion is reabsorbed to enter the blood by way of the intestinal lymph.

Following the ingestion of measured amounts of cholesterol, the rat was found to absorb a fraction inversely proportional to the dose. Thus, it appeared capable of absorbing about 47 per cent of a 50 mg. dose, but only about 34 per cent of a 100 mg. dose. The preponderance of the cholesterol was in the esterified form, as previously noted by Mueller (15), and by Bollman and Flock (21).

At least 82 per cent of the amount of cholesterol, calculated from the results of fecal analysis to have been absorbed over a period of 72 hours, was actually recovered in the lymph collected over the same period. Considering the assumptions upon which these calculations were based, the discrepancy of 18 per cent s understandable. Since, however, it was found in an earlier study (22) that all cholesterol absorbed from the intestines enters the general circulation *via* the intestinal lymph, the calculations based upon the lymph studies appear to be the most reliable.

The importance of bile in the absorption of cholesterol, first observed by

Mueller (15), and later also by various investigators (30, 31), was confirmed in these studies. The diversion of bile from the intestine led to a marked decrease in the intestinal lymph cholesterol both in animals fed a diet free of cholesterol and in those given 100 mg. of cholesterol. However, contrary to the findings of Bloom *et al.* (32), we found a slight absorption of cholesterol even in the absence of bile. The feeding of bile salt in the form of cholate increased the absorption of cholesterol previously excreted by the intestine itself, and it markedly increased the total amount of absorption of exogenously derived cholesterol.

From these findings concerning the intestinal activity of cholate, it becomes apparent that cholate acts in at least two ways in respect to cholesterol metabolism. First, it promotes the intestinal absorption of cholesterol. Secondly, when in excess in the plasma, it produces hypercholesteremia (33) and it seems to do so by preventing the egress of cholesterol from the blood (9). Whether both of these effects of cholate are due to its surface-active properties has not been determined.

Following the ingestion of cholesterol, neither the blood nor the various extrahepatic tissues exhibited a significant acute rise in cholesterol. However, qualitative changes occur in the blood cholesterol, since as has been shown by Biggs *et al.* (12), ingested labelled cholesterol quickly interchanges with blood cholesterol. More recently also, Page *et al.* (26), have demonstrated that the lipoproteins of intestinal lymph change following ingestion of cholesterol and with the drainage of such lipoproteins into the blood stream it seems most likely that for a while at least, the plasma cholesterol is altered qualitatively.

Following the intravenous administration of excess cholesterol, the blood is rid of it primarily by the ability of the liver to first store the cholesterol, then convert it and excrete it into bile as cholic acid (6, 8). The present studies indicate that the liver also functions in the same manner to rid the blood of cholesterol derived from exogenous sources. The liver then appears to occupy a pivotal position in the control of plasma cholesterol regardless of whether such plasma cholesterol arises *de novo* from the liver (1, 2), or is absorbed from the diet. Nevertheless, certain changes of the plasma itself have been shown (9) to be capable of influencing at least the rate of hepatic removal of plasma cholesterol.

SUMMARY

The absorption of oral cholesterol by rats was followed directly by analyses of the thoracic lymph and indirectly by calculating the deficit of fecal cholesterol under the amount fed. The two methods checked within about 20 per cent, with fecal analyses indicating the greater absorption. The absorbed cholesterol was found to be deposited in the liver, only very minor quantities being found in other organs or plasma.

Bile is necessary for normal absorption of cholesterol, although very small amounts are absorbed even in its absence. Excess oral cholic acid increases the absorption of cholesterol by otherwise normal animals. The rat absorbs about 47 per cent of a single 50 mg. dose of cholesterol and about 34 per cent of a 100 mg. dose.

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Effects of Cholesterol and Other Substances on Essential Fatty Acid Deficiencies[1]

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Cholesterol, cholic acid and bile salts, with or without certain vitamins, have recently been added to a number of experimental diets, especially in the study of atherosclerosis. That these compounds affect lipid metabolism is readily apparent from the work of several laboratories. Popjak ('46) reported that cholesterol addition to a diet increased mobilization of fat. Ridout et al. ('52) reported that it resulted in increased glyceride and cholesteryl esters of the liver. Alfin-Slater and co-workers ('54) reported similar results using low-fat diets. Cholate and bile salts were reported (Swell et al., '53) to increase blood cholesterol and perhaps alter fat metabolism indirectly through an effect on cholesterol. Vitamins reported to affect either cholesterol or liver lipids included choline (Meyer et al., '56), pantothenic acid, pyridoxine, riboflavin (Guehring et al., '52) and biotin (Okey et al., '51).

The purpose of this investigation was to study the effects of cholesterol, cholic acid, sodium glycocholate, and B vitamins and combinations thereof on the total carcass and liver lipids and on essential fatty acid- (EFA) deficiency symptoms of rats maintained on low-fat diets free from essential fatty acids.

EXPERIMENTAL

Male weanling rats of the Wistar strain, weighing from 40 to 50 gm were used in groups of 7 for dietary treatments of 5 weeks each. Additional animals were held in the negative control group for longer-term observations. The animals were housed in individual cages and fed the respective diets ad libitum for the 5 weeks. The percentage composition of the basal diet was as follows: glucose,[3] 74; fat-free casein,[4] 20; salts,[5] 4; celluflour,[6]. The vitamins were added in the following amounts in milligrams per kilogram of feed: thiamine·HCl, 20; riboflavin, 10; pyridoxine, 5; Ca pantothenate, 60; nicotinic acid, 25; *p*-aminobenzoic acid, 10; biotin, 2; inositol, 1200; folic acid, 10; vitamin B_{12} (0.1%) 1.8; choline, 1500; menadione, 50; α-tocopherol, 100. Vitamin A powder[7] was added at 2 I.U./gm of feed and vitamin D_2 at 0.2 I.U./gm of feed. The B vitamins were added as a premix with casein carrier, prepared in amounts to last one month. Hydrogenated coconut oil (iodine value, 2.5) was added to the completed diet at a 3% level except as otherwise stated. The diet and premix were stored at 0°C. In the positive control diet soybean oil replaced the hydrogenated coconut oil.

In the first series of experiments (table 1) 13 groups of rats received dietary supplements (1%) as follows: (1) positive control (3% soybean oil), (2) none (basal only), (3) cholesterol, (4) cholic acid, (5) cholesterol and cholic acid, (6) sodium glycocholate, (7) cholesterol and sodium glycocholate; (3a, 4a and 5a) in which 2% of a rice bran concentrate was added to diets 3, 4 and 5, respectively, and (3b, 4b and 5b) in which an additional 50% of the B-vitamin premix was added to diets 3a, 4 and 5, respectively. All of these diets except diet 1 contained 3% of hydrogenated coconut oil and no source of essential fatty acids.

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[1] Journal Paper no. 1257 of the Purdue University Agricultural Experiment Station.

[2] Present address: McCollum Pratt Institute, Johns Hopkins University, Baltimore.

[3] Cerelose, Corn Products Refining Company.

[4] Casein was extracted for three days with ether in a Lloyd's continuous extractor.

[5] McCollum Mix no. 185.

[6] Cellu Flour, Chicago Dietetic Supply House.

[7] Nutritional Biochemicals Corporation.

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TABLE 1

Effects of supplements fed to weanling rats during a 5-week period

Diet no.	Supplement to basal diet[1]	Number of rats			Average gain in weight	Average total lipids in	
		Started	Developed dermatitis	Died		Carcass	Liver
					% of control	*% S.E.*[4]	*% S.E.*
1	Soybean oil (positive control)	5	0	0	100	4.9 ± 0.71	1.8 ± 0.10
2	None	7	0	0	75	4.4 ± 0.46	2.4 ± 0.78
3	Cholesterol	7	5	0	58	3.8 ± 0.57	3.4 ± 0.52
3a	Cholesterol and R.B.[3]	7	7	0	70	3.1 ± 0.28	3.8 ± 0.98[5]
3b	Cholesterol and R.B.[3] + B[2]	7	0	0	75	3.5 ± 0.41	5.0 ± 0.36
4	Cholic acid	7	0	2	36	1.6 ± 0.17	1.9 ± 0.35
4a	Cholic acid and R.B.[3]	7	0	0	45	1.5 ± 0.09	2.5 ± 0.19
4b	Cholic acid + B[2]	7	0	0	30	2.1 ± 0.22	3.9 ± 0.41
5	Cholesterol and cholic acid	7	0	0	59	1.7 ± 0.19	7.4 ± 1.01[5]
5a	Cholesterol, cholic acid, R.B.[3]	7	0	1	46	1.9 ± 0.12	12.9 ± 1.59[5]
5b	Cholesterol and cholic acid + B[2]	7	0	0	41	2.1 ± 0.23	9.4 ± 1.14[5]
6	Sodium glycocholate	7	3	0	66	2.7 ± 0.30	4.4 ± 0.52
7	Cholesterol and glycocholate	7	4	0	58	3.6 ± 0.24	13.7 ± 0.58

[1] All supplements at 1% of diet except as otherwise listed.

[2] B vitamins increased 50%.

[3] R.B. refers to 8% of water-soluble rice bran concentrate (Borden Co., Research Division).

[4] Standard error of the mean.

[5] Includes one atypical figure.

TABLE 2

Influence of saturated fat in EFA-deficient diet (5-week period; 7 rats/group)

Supplement to basal	Average gain in weight		Number showing dermatitis	
	No fat	3% fat[1]	No fat	3% fat[1]
	% of control	*% of control*		
None (control)	100	100	0	0
Cholic acid	41.2	48.0	0	0
Cholesterol	84.5	74.0	0	4
Cholesterol and cholic acid	48.6	79.0	0	1

[1] Hydrogenated coconut oil.

In the second series (table 2), diets 2, 3, 4 and 5 were repeated, with and without the hydrogenated coconut oil, to observe whether saturated fat is necessary for these compounds to exert their effects.

The experiments were terminated at the end of 5 weeks. The animals were weighed, examined for EFA-deficiency symptoms and killed with ether. All of the internal organs and skin were removed. The remaining carcass was weighed, cut into pieces and digested with 40% of NaOH (0.7 ml/gm of tissue) for two hours on the steam bath. The mixture was allowed to cool, 1/5th volume of ethanol was added and the heating continued for an additional hour. Capryl alcohol was added when needed to prevent foaming. When cool, the digest was acidified with hydrochloric acid (2:1), 20 ml of petroleum ether was added and the mixture filtered through Celite.[8] The residue was washed thoroughly with petroleum ether and the filtrate was extracted with 4 25 ml portions of petroleum ether. The ether layer was washed with water and dried over anhydrous sodium sulfate. The ether was evaporated under nitrogen and the residual lipid dried *in vacuo* at 50°C for one hour and weighed. The percentage of total lipid was based on the wet-carcass weight. The liver lipids were determined in a similar manner.

RESULTS

After 5 weeks, growth impairment as indicated by body weight was apparent with all diets fed except the positive control (soybean oil) diet. The weight range of the latter group at the end of the 5 weeks was 246 to 288 gm. The addition of cholesterol, cholic acid and sodium glycocholate retarded gains in weight, the maximum weights being only 58, 26 and 66% of the controls, respectively (table 1).

The addition of cholesterol and/or sodium glycocholate reduced the time required for the appearance of EFA-deficiency symptoms (5 weeks as compared with 8 to 10 weeks for animals fed the basal diet). The deficiency symptoms observed at 5 weeks were dermatitis, dandruff, dull hair coat and some loss of hair.

The most marked effects on liver lipids were obtained by the addition of cholic acid or sodium glycocholate to the cholesterol supplement. These substances increased the liver lipids three- to 4-fold over cholesterol alone. In all cholic acid-containing diets the carcass lipids were greatly reduced. Increasing the B vitamin content of the cholic acid diets tended to increase the liver lipids with a concomitant decrease in body weight.

The animals receiving cholic acid had a severe gastrointestinal disturbance, indicated by diarrhea, and two of them did not survive the 5-week period. In contrast with cholic acid the corresponding bile salt, sodium glycocholate, did not have such severe effects on the animals. The gastroenteritis and the retarding effect of cholic acid on growth were somewhat alleviated by the addition of cholesterol to the diet (diets 3 and 5). However, growth retardation by cholic acid seemed to be greater when the B vitamins were increased 50% or rice bran concentrate was added along with the cholesterol. In the absence of cholesterol the addition of the B vitamins with cholic acid had very little effect on weight, whereas the addition of rice bran concentrate appeared to relieve the in-

[8] Registered trade name for Johns Manville's diatomaceous silica filter aid.

testinal disturbance of the animals and resulted in increased food intake with subsequent weight gains.

The growth-retarding effect of cholesterol and cholic acid was also observed in EFA-deficient diets without fat (table 2). In this series control groups, the average weights of which were taken as 100%, both lacked EFA. The presence of hydrogenated coconut oil in the diet seemed to minimize the retarding effect of cholesterol-cholic acid combination on growth.

DISCUSSION

The manner in which the steroid substances retarded body weight is not clearly understood. However, the beneficial effects of additional B vitamins (50%) or of rice bran concentrate when given with cholesterol suggests the possibility that cholesterol may increase the requirements of certain B vitamins in EFA-free diets. On the other hand, cholic acid seemed able to nullify this effect of the B vitamins; indeed, it appeared as an antagonist. Growth retardation by cholic acid could not be attributed to decreased feed consumption as a result of gastrointestinal disturbance, since rice bran concentrate alleviated the latter but growth was still retarded.

In general, poor growth was accompanied by low carcass fat, as found in earlier studies (Quackenbush et al., '42). Cholesterol alone did not alter the carcass or liver fat substantially but increased the liver fat when fed with cholic acid and especially when fed with sodium glycoholate. Alfin-Slater et al. ('44) found that livers of EFA-deficient rats on a "fat-free" diet contained twice as much total lipid (6 to 7%) and twice as much total cholesterol as those of normal animals at the end of a 20-week period. Livers of our EFA-deficient animals which were fed cholesterol and glycocholate contained twice as much total lipid (13.7%) at the end of 5 weeks. Undoubtedly this effect of exogenous cholesterol was enhanced by the favorable conditions for cholesterol absorption which bile salt and 3% of dietary fat provided (Swell et al., '53). The cholesterol, once it is absorbed, may participate in esterification reactions (Daskalakis and Chaikoff, '55). Since the dietary choline level (0.15%) appears to be adequate for normal lipotropic activity (Ridout et al., '52), the high liver lipid appeared to be the result of interactions between EFA deficiency and absorbed cholesterol.

Whereas cholesterol and sodium glycocholate accelerated the appearance of EFA-deficiency symptoms on the EFA-free fat-containing diet, this effect was not observed on a completely fat-free diet. The time required for the skin symptoms to appear using the latter diet was 8 to 10 weeks for males and 12 to 15 weeks for females. This appears contrary to the findings of Peifer and Holman ('55) who reported accelerated depletion by cholesterol on fat-free diets and attributed the effect to an increased rate of utilization of EFA stores. Perhaps differences in diets and initial storage of EFA by the weanling rats are responsible for our different results. Essential fatty acid depletion in overall body stores is presumed when animals develop skin symptoms, since the lipids from a rat with EFA deficiencies were shown unable to cure the dermatitis (Quackenbush and Steenbock, '42). Total carcass lipids observed in our results, were not altered in most cases. The largest decrease occurred in cholic acid-fed rats but these showed no acceleration of EFA-deficiency symptoms. Therefore, cholic acid seems to have inhibited the formation or accelerated the utilization of body fat without affecting the EFA stores. If cholic acid inhibited the formation of fatty acids, perhaps it formed coenzyme A esters of cholic acid and thereby effectively reduced the synthesis of fatty acids, since coenzyme A esters of cholic acid have been reported by Siperstein and Murray ('56). Such specific action of cholic acid seems an attractive concept; however, it must be borne in mind that numerous factors in poor nutrition can limit total fat levels in the animal body (Quackenbush et al., '42).

SUMMARY

1. Cholesterol and sodium glycocholate accelerated EFA-deficiency symptoms using diets containing 3% of saturated fat; this effect was not observed on fat-free diets. Both substances retarded growth.

2. Cholic acid did not accelerate EFA deficiency symptoms, but retarded growth and body-fat deposition and produced dia

rhea. The diarrhea was alleviated by increased supplements of a rice bran concentrate but growth was not improved. Growth was improved by dietary cholesterol and fat.

3. When cholesterol was fed with either sodium glycocholate or cholic acid, the lipid content of the liver increased several fold. However, sodium glycocholate was the more effective of the two supplements, with cholesterol, to increase liver lipids and accelerate EFA-deficiency symptoms.

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CHOLERESIS AND CHOLERETICA, A CONTRIBUTION TO THE PHYSIOLOGY OF BILE

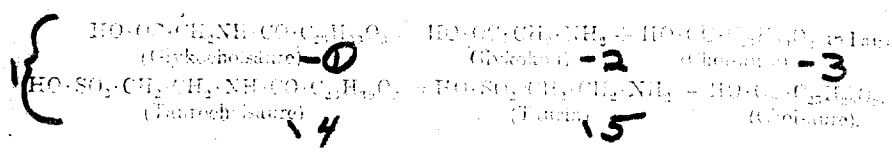
5th Report. Gillert, E. (Medical Clinic II, Charite, Berlin).

Toxicity of Bile Acids

In clinical pathology, the cholemic complex is considered as a form of autointoxication and is essentially related to bile acid poisoning. Pruritus in fresh cases, the tendency to bleeding in extended cases of icterus and the slowing of the pulse are attributed to bile acid intoxication.

Bile acids are highly important both in normal and pathological metabolism; their importance is best characterized with respect to the digestive process in the intestine tract by the phenomena described by the cholein principle of Wieland (1). Since, in addition, bile acids have choleretic effects, the problem of bile acid therapy appears to be of importance for the pathology of the liver and for digestive disturbances. One aspect, however, is missing from the literature: accurate data concerning the toxicity of bile acids. As long as the toxicity of bile acids is unknown, any therapeutic application of the acids appears to be unjustified. The author, therefore, posed the question: what is the lethal dose in anaimals, and, is there a relationship between the chemical structure of bile acids and their pharmacological effects and toxicity? Further, it is necessary to determine the pathological and anatomical intoxication mechanism and to examine the tolerance of bile acids in humans in comparison with their effectiveness.

In recent years, the chemistry of bile acids was developed essentially by Wieland (1), Boderke (2), Schlenk (3), Windaus (4), Borshe (5) and others. A distinction is made between "conjugated bile acids and nonhydrolyzable bile acids". The first, the glycocholic and taurocholic acids, can be decomposed by hydrolyzing agents and cleave nitrogen-containing (glycochol, $HO.OC.CH_2NH_2$) or nitrogen and sulfur-containing (taurine, $HO.SO_2CH_2NH_2$) conjugates; the latter originate in the conjugate bile acids and are composed of C, H and O only. Thus:



Key: 1. Glycocholic acid; 2. Glycochol; 3. Cholic acid; 4. Taurocholic acid; 5. Taurine.

Cholic acid is a trioxymonocarboxylic acid ($C_{24}H_{40}O_5$); from this, by reduction, dioxymonocarboxylic acid, $C_{24}H_{40}O_4$ = desoxycholic acid and, further, Monoxymonocarboxylic acid, $C_{24}H_{40}O_3$ = lithocholic acid, discovered by H. Fischer in 1911 (6), were formed. The latter occurs in very minute amounts only, 100 kg yields 1-2 g, but as the lowest member of the saturated oxymonocarboxylic acids

characteristic of bile, it is of particular theoretical interest (7). Due to lack of material, its investigation had to be omitted.

Oxidation of trioxymonocarboxylic acid (cholic acid, $C_{24}H_{40}O_5$ yields dehydrocholic acid, $C_{24}H_{34}O_5$, a ketonic acid which is commercially available under the name of Decholin (8).

Through the cleavage of alcoholic hydroxides of oxymonocarboxylic acids in the form of water, Wieland (9) found an acid providing a common base for cholic, desoxycholic and lithocholic acids: cholanic acid, $C_{23}H_{39}CO_2H$, the starting material of menthadioxycholanic acid, Degalol (8), which will be discussed later. By partial water cleavage, Bodecker (10) prepared apocholic acid from cholic acid $C_{24}H_{38}O_4 = C_{23}H_{35}(OH)_2CO_2H$, which closely resembles desoxycholic acid and which will also be discussed later.

Of the large number of physiologically and experimentally determined bile acids, only the above are of physiological and pharmacological importance. In humans, practically only cholic acid occurs physiologically, while desoxycholic and lithocholic acids can hardly be found. A greater role, however, is played by the conjugated bile acids bonded by the choleine principle; their quantitative representation has recently been attempted by Rosenthal and V. Falkenhausen (11) et al. (12), without being able to provide definitive quantitative information concerning their occurrence.

Experimental Section

Lethal doses were determined on rabbits. The experiments were performed first without and then partically with superposition of amounts given. The doses determined (see below), however, represent amounts having lethal effect after a single administration. Pathological and anatomical results were obtained through the courtesy of Mr. Plenge, Assistant, Pathological Institute, Charite.

The material for these and in part for other experiments were provided in an extremely generous manner by the J. D. Riedel Co.; it is the pleasant duty of the author to express his appreciation at this time.

1. Na Glycocholate

The substance was injected i.v. as a 2% solution into rabbits.

With a dose of 0.07 g/kg there was no appreciable effect.

With 0.08 g, distress, agitation were observed, otherwise without effect.

With 0.09 g exitus. Dissection yielded parenthymatous hemorrhage in the liver, systole of the left, diastole of the right ventricle, increased postmortal peristaltic.

2. Na Taurocholate

It was again given in a 2% solution by i. v. injection.

With a dose of 0.08 g/kg tonic-clonic cramps, a slowdown of respiration and bowel movement occurred. Mydriasis. Recovery after 6 minutes.

Similar toxic symptoms appeared after a dose of 0.09 g/kg, but recovery was slower.

In the case of a dose of 0.1 g/kg, no cramps, but slowly developing violent opisthotonus was observed. Extinction of the corneal reflex, exophtalmus. Cessation of respiration is interrupted only be deep breathing separated by pauses of several seconds. Respiration is resumed abruptly, beginning with tachypnea.

At 0.11 g/kg immediate exitus with tonic-clonic cramps, exophtalmus and cessation of respiration.

3. Sodium Cholate

Given by i. v. injection of a 1% solution.

0.03 g/kg was tolerated without visible reaction.

With 0.04 g/kg, violent screaming.

With 0.05 g/kg cramps, but with rapid recovery.

With 0.06 g/kg death immediately upon rejection.

4. Sodium Desoxycholate

Given as 1% solution by i. v. injection.

With 0.01 g/kg violent screaming.

With 0.015 g/kg, death immediately after injection.

Dissection: systolic stasis of the left, diastolic stasis of the right ventricle. Scintillation. Strong postmortal peristaltic. Slight pulmonary emphysema. Venous hyperemia of all organs. Slight hemorrhage of the mucous membrane of the stomach.

5. Sodium Apocholate

Given by i. v. injection as a 1% solution.

With 0.03 g/kg no appreciable effect.

With 0.04 g/kg to 0.07 g/kg agitation and violent screaming, otherwise no effect.

With 0.09 g/kg exitus within a few minutes after injection.

6. Dehydrocholic Acid

Used i.v. in the form of 20% Decholin, marketed by the J. D. Riedel Co.

Animal 1: The injection of 2 g/kg lead to immediate exitus with tonic-clonic cramps. Under strong opisthotomus, a few minutes post exit body rigidity occurred which probably should be interpreted as acid rigidity and which disappeared after about 30 minutes to be replaced by death rigidity proper.

Dissection: strong, venous hyperemia, particularly of the liver, diastolic cardiac stasis. Brain without symptoms.

Microscopy: massed lipoids in urine and liver cells.

Liver: parenchymatous turbidity and turbid swelling of liver protoplasma. Beginning dissolution of cell nuclei. Congested blood supply.

Spleen: congested blood supply. Large nucleus center in lively activity. Numerous hemosiderin in reticulum cells.

Kidneys: congested blood supply.

Animal 2: Administration of 1.5 g/kg Decholin, i.v. Exitus during injection at about 1.3 g/kg.

Dissection: cramp-like systole of the left ventricle, beating right auricle, strong excessive blood supply in liver and kidneys and spleen, no hemorrhaging erosion of stomach; hydrops vesicae fell.

Microscopy: strong lipoid deposition in Kupfer's star cells of the liver. Parenchymatous turbidity of liver protoplasma, degeneration of nuclei.

Spleen: very numerous and large nucleus centers in vivid activity. In the nuclei, fatty degeneration of reticulum cells. Moderate amount of hemosiderin in reticulum cells.

Kidneys: blood congestion. Lipoid deposition in the epithelia of straight urine channels.

Heart: slight, fine droplet lipoid deposition in muscle fibers.

Animal 3: Received 1.24 g/kg Decholin, i.v. Exitus occurred with dyspnea and violent tonic-clonic cramps 8-10 minutes after injection.

Dissection: numerous hemorrhages under the thymus capsule. Diastolic heart. Blood congestion of liver, spleen and kidneys. Single coccidia of liver. Swelling of Peyer's piles in the lowest ileum.

Microscopy: no examination

Animal 4: 1.0 g/kg Decholin, i. v. Exitus immediately after injection with respiratory paralysis.

Dissection: ascites, strongly filled stomach, pregnancy, very violent peristaltic movements of the uterus. Very strong swelling of the lymph follicles of the appendix.

Spleen: enlarged, swollen, hyperemic.

Kidneys: no symptoms.

Pulmones: numerous bronchopneumonic centers in both lungs with fresh subpleural hemorrhaging.

Microscopy: heart -- slight, fine droplet lipoid deposition in muscle fibers.

Liver: fatty degradation of star cells. Lymphocytic round cell accumulation in lobular connective tissue. Moderate amount of fine droplet lipoid in liver cells.

Kidneys: blood congestion, otherwise no symptoms.

Spleen: blood congestion. Large lymph follicles with large nuclei, much hemosiderin in pulpa and reticulum cells.

Animal 5: 1.0 g/kg Decholin, i. v. Screaming during injection, recovery shortly afterwards.

The experiment was repeated several times with different animals, same results.

The maximum permissible amount of Decholin in a single i. v. injection thus should be 1 g/kg.

Conditions are different in superposition experiments. The following experiments were performed to investigate these conditions:

Animal 6: 0.4 g/kg Decholin was given i. v. Bowel movement and convulsive running followed. The animal was entirely well 30 minutes later and was again given 0.4 g/kg. Convulsive respiration was observed, slowing and turning into tachypnea after 10 minutes. On 3 subsequent days, 2 x 0.4 g were given i. v. Exitus on the evening of the 3rd day.

Dissection: diastolic cardiac stasis. Hemorrhaging erosion of stomach, blood congestion of liver, spleen and kidneys. Swelling of Peyer's piles in the lowest ileum.

Animal 7: Received 0.7 g Decholin i. v. Screaming during injection. Shortly afterwards, irregular breathing and bowel movement. Symptomless after 10 minutes. Repeated administration of 0.7 g on following day. Rabbit rears with

violent screaming and bowel movement. After about 1 hour, administration of 0.7 g repeated; followed by violent gasping for air; exitus after 20 minutes.

Dissection: systolic heart. Strong hemorrhaging under the thymus capsule. Swelling of Peyer's piles in the lowest ileum. Blood congestion in liver, spleen and kidneys. Coccidia of liver.

It is concluded that either the relatively low toxicity dehydrocholic acid is not eliminated or decomposed within 24 hours, or organic changes take place which reduce the tolerance.

7. Menthadioxycholanic Acid

While the previous experiments referred to a series of intoxications by injection, the following series, as a form of appendix, deals with intoxication by feeding. It is in the nature of bile acids as physiologically vital ingredients of the intestine tract that toxic doses are much greater here. The bile acid that proved to be most toxic in i.v. applications, desoxycholic acid, served as the basis of the investigation, in the form of menthadioxycholanic acid (Degalol; J.D. Riedel Co.).

Animal 1: received 1.0 g/kg Degalol, per os. No symptoms.

Animal 2: received 2.0 g/kg Degalol, p.o., followed by mixed feed. Exitus after 7 days.

Dissection: diastolic cardiac stasis. Abortus. Stomach well filled. Gall bladder tightly filled, other organs without symptoms.

Microscopy: liver coccidia, much lipoid, fine droplets in liver and star cells. No hemosiderin.

Animal 3: received 4.0 g/kg Degalol, p.o., then mixed feed. Death after 3 days.

Dissection: diastolic cardiac stasis. Kidneys, liver and spleen congested with blood. Nodular and perinodular swelling of lymph nodes in the appendix. Local blood congestion in parts of the colon serosa. Hemorrhaging erosion of the stomach, fresh, fibrinous peritonitis.

Microscopy: blood congestion in spleen, little hemosiderin in reticulum cells.

Kidney: lipoid deposition in the epithelia of the boundary layer. Lipoid deposits in the vascular endothelia of the medullary substance.

Heart: strong, nucleus-shaped lipoid depositions. Small round cell infiltrations.

Intestine: strong development of the lymphatic apparatus with fatty degradation of endothelia.

Animal 4: received 6.0 g/kg Degalol, p.o., followed by mixed feed. Death after 2 days.

Dissection: diastolic heart, coccidia of the liver, blood congestion. Liver remarkably brown. Spleen very small. Blood congestion. Stomach very full. Hemorrhaging erosion of stomach to size of a one cent piece, strong lymph node swelling in the appendix. Kidneys congested with blood, but not excessively.

Microscopy: heart -- extensive nucleus-shaped lipoid deposition in fine droplets; liver -- coccidia, lipoid deposition in the liver, occasionally only in Kupfer's star cells and very seldom and individually in liver cells. Blood congestion. No hemosiderin.

Kidneys: nucleus-shaped lipoid in fine droplets.

In summary, the following overview is presented of lethal and just tolerable doses:

TABLE

	1	2	3	4
von Na Glykocholat	0.60 g	0.8 g		
von Na Taurocholat	0.11 "	0.[illegible] g		
von Na Cholat	0.05 "	0.05 g		
von Na Desoxycholat	0.15 "	[illegible] "		
von Na Apocholat	0.09 "	[illegible] "		
von Na Dehydrocholat (Decholin)	1.1 "	1.0 "		
von Monoglycolsäure (Decolin)	—	1.0 "	von 2.0 g an	

Key: 1. Per kg of body weight in rabbits; 2. Lethal; 3. Just tolerable; 4. Lethal in . . . days; 5. Of; 6. Acid; 7. From 2.0 g on.

It is thus seen that for rabbits the sodium salts of taurocholic acid and glycocholic acid and of apocholic acid and cholalic acid have the greatest tolerance. If the tolerance for cholalic acid is calculated for humans, for i.v. application to a person weighing 70 kg, it is seen that an amount of 3.5 g by weight of this cholic acid would still be possible. For sodium dehydrocholate, however, tolerance is still far greater, 20 times this amount. Conversely, tolerance for desoxycholic acid as the sodium salt is only 1/5 of that for the other cholic acids. It should be mentioned from the literature that Weiss (14) and Stadelmann (15) have fed bile acids to dogs with biliary fistula in doses of 5-9 g glycocholic acid per day and that thus the tolerance of dogs should resemble that of rabbits. It must be emphasized, however, that of the physiological bile acids, conjugated acids are the least toxic in animals; this fact is probably related to the cholein principle considering the relationship with taurin and glycocoll, which closely resembles the deposition of fatty acids.

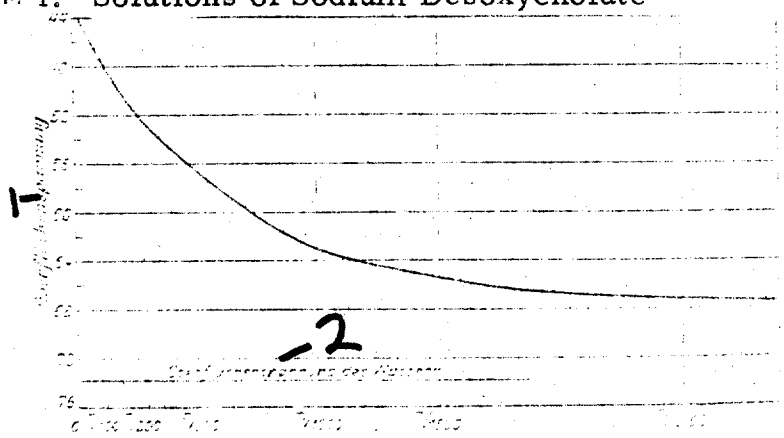
With respect to potential bile acid therapy on humans, evidently the problem of the action mechanism of the individual bile acids and their toxicity is of decisive importance. By definition, the cholein principle, the solubility of bodies insoluble in water in bile acids can be taken as a measure of their biological importance

this has been determined by H. Wieland and H. Sorge (16) for desoxycholic acid and its salts, but not for cholic acid, etc. Since the binding force is substantially greater in the salt form than it is in the state of free acids, salts were generally used in the experiments, with the sole exception of menthadioxycholanic acid. Because, as is known, bile is capable of dissolving fatty acids insoluble in water, which is explained by the cholein principle (Brugsch), the importance of bile salts for the organism may be considered from the viewpoint of bile secretion. In the literature, experiments concerning the effect of bile acids in enhancing bile secretion have been known for a long time, particularly with respect to desoxycholic, the dehydrocholic acid of acid of Neubauer, Brugsch, et al. (17), (18). The extent to which a parallel exists between toxicity and bile secretion enhancement, is a subject for future investigations.

However, a special relationship exists between the surface activity and toxicity of bile acids. Six curves from the authors work (17) are reproduced below; the curves represent surface tension with respect to water: curves are given for sodium desoxycholate, sodium cholate, sodium apocholate and sodium dehydrocholate, also the Na salts of conjugated bile acids, which with respect to their toxicity and surface activity form a sharply defined group.

Sodium desoxycholate has the strongest surface activity and sodium dehydrocholate the lowest, while sodium apocholate and sodium cholate are intermediate.

FIGURE 1. Solutions of Sodium Desoxycholate



Key: 1. Surface tension; 2. Surface tension of water.

FIGURE 2. Solutions of Sodium Cholate

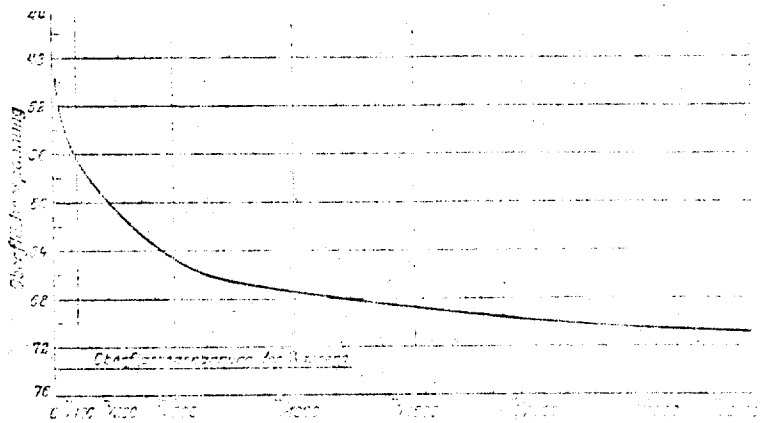


FIGURE 3. Solutions of Sodium Apocholate

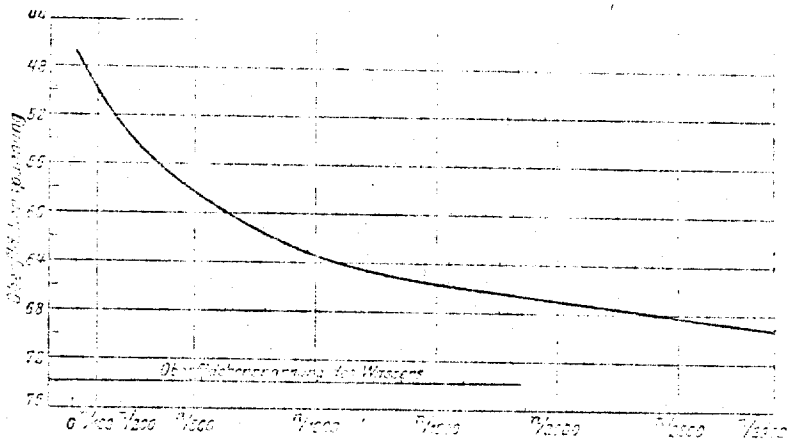
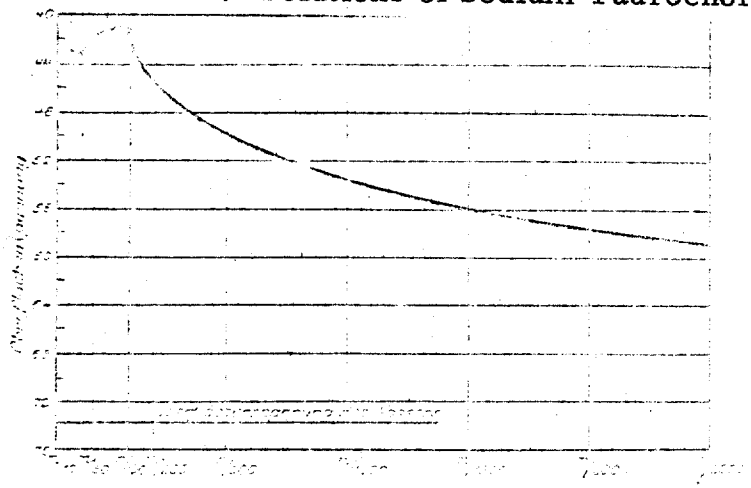


FIGURE 4. Solutions of Sodium Dehydroxycholate

FIGURE 5. Solutions of Sodium Taurocholate



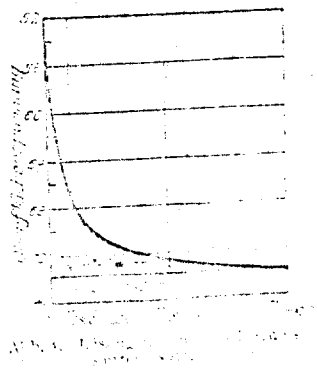
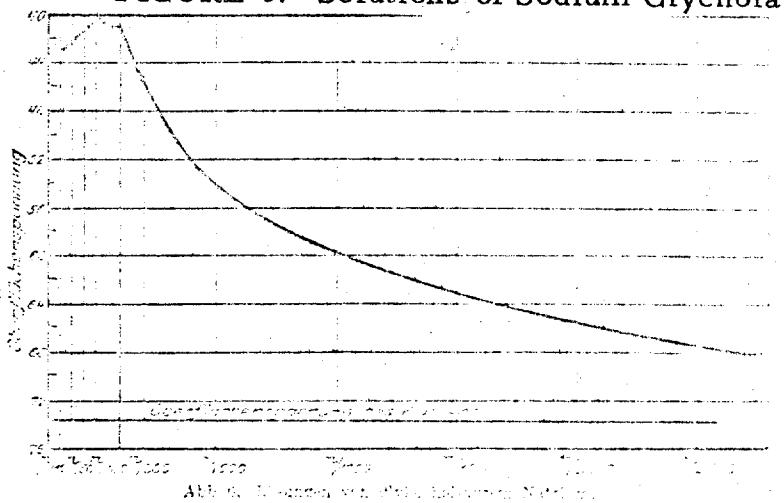
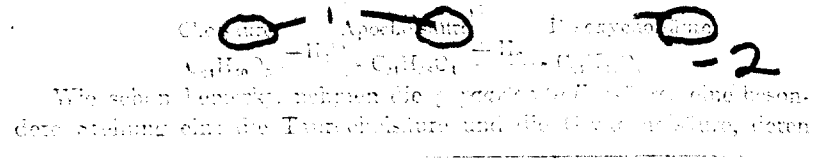


FIGURE 6. Solutions of Sodium Glycholate



Keys as for Figure 1. The following can still be detected as free acids: 1. Desoxycholic acid in a dilution of n/40000, lethal 0.015 g/kg; 2. Cholalic acid in a dilution of n/30000; lethal 0.09 g/kg; 3. Apocholic acid in a dilution of n/30000; lethal 0.06 g/kg; 4. Dehydrocholic acid in a dilution of n/1200; lethal 1.1 g/kg.

The behavior of apocholic acid is remarkable; chemically it is extraordinarily similar to desoxycholic acid, but it is six times less toxic. It belongs, as pointed out in the introduction to the unsaturated oxymonocarboxylic acids (dioxycholic acids), which are formed by the partial cleavage of water and it possesses the property to combine with hydrocarbons, alcohols, ketones, etc. to addition products (18). Its relationship to desoxycholic acid is shown by the following (taken from Meyer-Jacobson Text, IV, Part 2):



(2)

Key: 1. -ic acid

As noted above, conjugated bile acids occupy a special position: the sodium salts of taurocholic acid and glycocholic acid were investigated. Their toxicity is very low, while their surface activity is relatively high.

The following are still detectable as free acids:

1. Taurocholic acid in a dilution of n/40000; lethal 0.11 g/kg;

2. Glycocholic acid in a dilution of n/37000; lethal 0.09 g/kg.

While the toxicity of conjugated bile acids is substantially lower than that of unpaired ones; it is seen that in the case of unpaired bile acids, toxicity is in a reverse relationship with surface activity.

The symptomatology of toxic phenomena with nonlethal doses includes diarrhea, interference with circulatory activity and with respiration. Mydriasis was observed in numerous cases. Compounds of cholic acid with menthol (Degalol) in lethal doses cause clonic-tonic convulsions, clon. opisthotonus, increased peristaltic and dyspnea. Rabbits scream. Further, uterus convulsions were observed (with abortion by pregnant rabbits). The early occurrence of rigidity in the case of superlethal doses is remarkable.

Pathological and anatomical findings show parenchyme damage of the liver protoplasma with damage to the nuclei of liver cells, fatty degeneration of star cells and accumulation of round cells in intralobular connective tissue. In the spleen, multiplication of nucleus centers and hemosiderin deposition in reticular cells are found. Congestive hyperemia of the kidneys with lipoid deposition in the straight urine channels. Lipoid deposits in fine droplets in muscle cells of the heart, swelling of lymph follicles. Bronchopneumatic centers, pleural and peritoneal hemorrhaging and hemorrhaging in the thymus capsule. Hemorrhaging erosion in the stomach and intestine tract. Swelling of nucleus centers and of lymph nodes of Peyer's piles. Death occurs under systolic or diastolic cardiac stasis.

Toxic Mechanism

It is an achievement of Bodecker (19) to have shown that i. v. injected apocholic acid is eliminated quantitatively in a few minutes through the liver, from which it follows, as shown by Brugsch (20), Inger (21) and Gillert (22) that the liver represents a secreting organ of the first importance. Wieland (23) emphasizes the importance of whether blood contains salts of bile acids, a question which in the meantime has been answered positively by Rosenthal and von Falkenhausen (24) and Gillert (25). Although proof is still lacking that liver cells have the function of maintaining the level of bile acids at a certain height, it is highly probable that this is so, similarly to the manner in which liver cells keep the bilirubin level of blood as low as possible (Kraus). In the case of an oversupply of bile acids in an organism, damage to the liver cells would therefore be most likely. Further, results of dissections show that bile acids represent blood and vascular toxins. In the first case, because due to the cholein principle they possess special and characteristic activities, in the second, because, as shown by anatomy, their application may cause hemorrhaging in capillary regions. Finally, bile acids are centrally attacking agents, as exhibited by the clinical image of the respiratory paralysis of poisoned animals. Their death may be interpreted as central.

Summary

1. Bile acid intoxication results in anatomical alterations of different tissues; these are described.

2. The toxic image of bile acids is central, it is characterized by respiratory paralysis, vascular damage and convulsions.

3. The toxicity of unpaired bile acids is approximately a reverse function of their surface activity. Exact values of lethal doses are given.

It is a pleasant duty of the author to thank Professor Dr. Brugsch for his assistance.

THE EFFECT OF DIETARY FAT ON BILE ACID METABOLISM IN MAN[1]

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Abstract

The influence of dietary fat on the biliary bile acid metabolism was studied in a 22-year-old male volunteer by using cholic acid-24-C^{14}. The subject was maintained for 3 successive 15-day periods on homogenized formula diets which provided 45% of calories from butterfat or corn oil, or which were fat-free. The cholic acid turnover was assessed during the final 9 days of each period by analyzing bile samples obtained from the duodenum. On butterfat the daily cholic acid production was 0.13 g, while on corn oil and the fat-free diet it was 0.28 and 0.25 g, respectively. On changing from a free choice to a formula type diet, the glycine/taurine conjugation ratio decreased abruptly from about 2 to nearly 1, and remained at this level as long as the homogenized diet was fed. There was an increase in the proportion of cholic and a corresponding decrease in the deoxycholic acid in the bile with both high fat diets. The reverse changes in these bile acid proportions were associated with the fat-free diet. The fluctuations in the relative concentrations of the other bile acids were less pronounced.

Introduction

The assumption that the well documented hypo- and hyper-cholesterolemic effects of unsaturated and saturated fats upon plasma cholesterol might reflect corresponding alterations in the total body cholesterol pool has prompted attempts to associate these changes with modifications in bile acid and sterol excretion (1–9). Although it has been established that bile acids represent the major products of cholesterol breakdown (10, 11), the degree to which bile acid and sterol metabolism is amenable to dietary influence has remained uncertain (12). This uncertainty has been compounded by the inadequacy of the imposed dietary control and the lack of suitable analytical techniques for a quantitative examination of the complex bile acid mixtures excreted in the feces.

In the present study attempts have been made to correct both of these shortcomings. The effect of dietary fat upon bile acid metabolism has been determined in a patient subsisting on formula rations of known composition under metabolic ward conditions. The utilization of cholic acid-24-C^{14} as a marker (13) coupled with periodic sampling of the gallbladder bile has obviated the need for analyzing the more complex mixture of fecal bile acids. For the identification and quantitative estimation of the bile acids gas-chromatographic techniques developed in our laboratory have been used (14, 15).

[1]This investigation was performed with the aid of grants from the Medical Research Council of Canada and the Ontario Heart Foundation.
[2]The work reported here was done in partial fulfillment for the requirements of the Ph.D. degree in Biochemistry and was performed during the tenure of a Medical Research Council of Canada Fellowship.

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Materials and Methods

Subject

A 22-year-old male had been admitted to hospital with multiple fractures 11 weeks prior to the initiation of the study. By the time the experiments were started fracture healing was progressing well, the patient was ambulant, and was being retained in hospital for continued physical therapy. The patient was housed in a metabolic ward throughout the period of study.

Labelled Material

Cholic acid-24-C^{14} in the form of sodium salt was obtained from the New England Nuclear Corporation, Boston, Massachusetts. It had a specific activity of 16 microcuries per milligram. The material was used without further purification.

Diets

The basal fat-free diet was prepared, stored, and dispensed as previously described (41). The other diets were prepared by substituting 45% of calories from butterfat or corn oil for an equicaloric amount of carbohydrate (Dextrimaltose) in the basal diet. The daily intake was regulated to maintain the subject's weight constant. In addition to the diet the patient was allowed to consume only water, and tea and coffee without milk, cream, or sugar.

Design of Experiment

The study was conducted over three successive 15-day dietary periods during which the formula diets were administered in the following order: first period, 45% calories from butterfat; second period, 45% calories from corn oil; third period, fat-free. There was a 5-day interruption between the second and the third periods, during which time the subject returned to the free-choice diet. (On the second day in this interval, the subject had a general anaesthetic while his cast was changed.) On the seventh day of each dietary period the patient received by mouth prior to his morning meal 5.0 microcuries of cholic acid-24-C^{14} (0.31 mg) in 50 ml of water. The flask and mouth of the subject were rinsed with several portions of water and these were swallowed. Samples of the gallbladder bile were removed from the duodenum on day 0 and after 3, 7, 9, 12, and 15 days on the diet. Blood was drawn at regular intervals after an overnight fast for the determination of plasma cholesterol.

Method of Bile Sampling

Samples of the gallbladder bile were removed from the duodenum by the method of Lindstedt (13). At 7:00 a.m. on the day of sampling the subject was allowed to swallow an intestinal sampling device of the type described by Crosby *et al.* (16) (obtained from College Park Instruments, College Park, Indiana, USA). The tube passed through the pylorus into the duodenum within 2½ to 3 hours. Fifteen Ivy units of a cholecystokinin preparation (Cecekin, Vitrum Pharmaceutical Company, Stockholm, Sweden) was then administered intravenously and within a few minutes a good flow of concen-

trated bile was usually obtained. Ten milliliters of this bile was aspirated under gentle suction into a calibrated test tube. The bile samples were stored at −20° C until used.

Analytical Methods

The plasma cholesterol was determined by the method of Abell *et al.* (17). The glycine- and taurine-conjugated bile acids were isolated by the ion exchange chromatographic procedure described earlier (18). The fractions containing the individual conjugates were pooled separately, neutralized, and evaporated to dryness *in vacuo*. The residue was dissolved in 1.5 *N* NaOH and hydrolyzed in sealed tubes in an autoclave at 120° C for 4 hours. The free bile acids were recovered from the hydrolyzate by diethyl ether extraction following acidification. The acids were dissolved in ether:methanol (5:1) and methylated with freshly distilled diazomethane (14). After evaporation to dryness in tared flasks, the methyl esters were weighed, transferred to a volumetric flask with methanol, diluted to volume, and an aliquot of approximately 2 mg was removed for trifluoroacetylation. Duplicate samples from both conjugate groups from each bile specimen and duplicate samples of the standard mixture of bile acid methyl esters were trifluoroacetylated and gas-chromatographed as previously described (14, 15).

Isolation of Cholic Acid

Cholic acid was isolated only from the glycine-conjugated fractions. An aliquot of the bile acid methyl ester mixture was applied to a 20×2 cm silicic acid column and the cholic acid ester recovered as described by Wootton (19). The column effluent was monitored by thin layer chromatography using the solvent system suggested by Hofmann (20). The heart fractions of the methyl cholate peak were combined, transferred to a volumetric flask, and, after dilution to volume, aliquots used for specific activity measurements. The concentration of total cholic acid in this fraction was determined by the spectrophotometric method of Levin *et al.* (21).

Radioactivity Measurements

The methyl cholate isolated above was plated in an infinitely thin layer on aluminum planchets. The radioactivity was measured in a Nuclear Chicago gas flow counter with an ultra-thin window. Corrections were made for background and coincidence.

Results and Discussion

The effect of the three formula diets on the plasma cholesterol levels is indicated in Fig. 1. The response in the single subject was similar to the average changes observed in large groups of university students subsisting on similar formula diets (22). About 6 days after the commencement of a new diet the plasma cholesterol reached a plateau. In the brief respite from controlled diet, when the patient temporarily returned to a free-choice ration, the plasma cholesterol rose, but failed to reach the pre-diet level in the 5 days. Figure 1

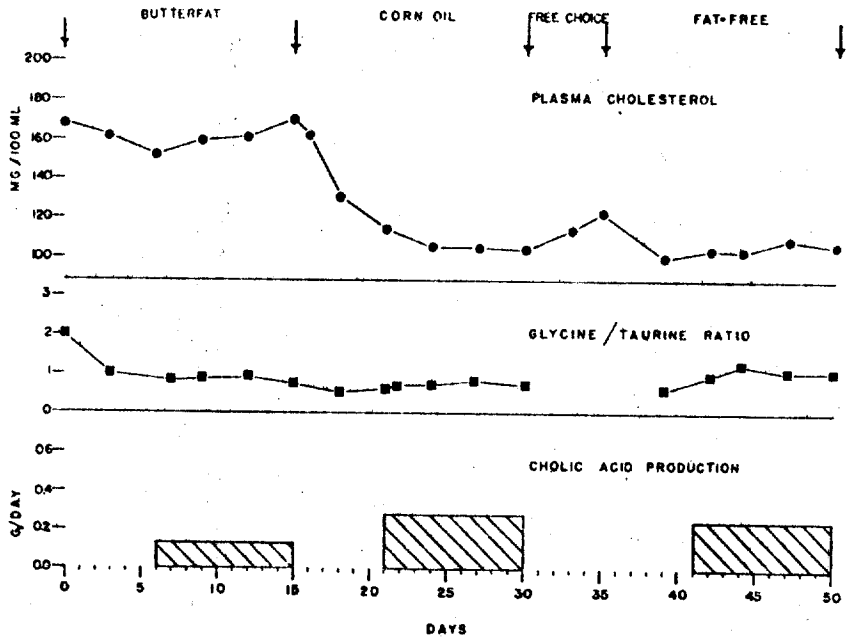

FIG. 1. The effect of butterfat, corn oil, and fat-free diets on plasma cholesterol, glycine/taurine conjugation ratio, and daily cholic acid production.

also indicates the changes in the glycine/taurine conjugation ratio and the estimated daily cholic acid production during the three dietary regimens. The change from a free-choice to the formula diet was accompanied by an abrupt reduction in the glycine/taurine ratio (from an initial value of 2 to about 1). Although further fluctuations in this ratio were noted on transferring to other dietary regimens, these changes were minor and poorly defined, and could not be attributed to any one fat or the absence of it.

The glycine/taurine ratio of 1 observed on these diets is remarkably lower than the mean value of 3.2 noted by Sjovall (23) for subjects on free-choice diets comprised of the usual variety of foodstuffs. It has been demonstrated, however, that dietary taurine in doses as low as 1.5 g per day is capable of increasing taurine conjugation (24). The formula diets supplied to the subject provided an average methionine and cystine intake of 2.4 and 0.4 g per day, respectively. These amino acids might have been converted to taurine in such quantities as to affect the conjugation ratio. It would appear unlikely that the drop in the glycine/taurine conjugation ratio would have resulted just from feeding the test diets in the homogenized state.

The cholic acid production indicated in the lower part of Fig. 1 shows an inverse relationship with the plasma cholesterol levels. The production was lowest on the butterfat diet which produced the highest cholesterol levels. On corn oil or the fat-free diets, which gave low plasma cholesterol levels, the cholic acid production increased significantly. Figure 2 gives the semilogarithmic

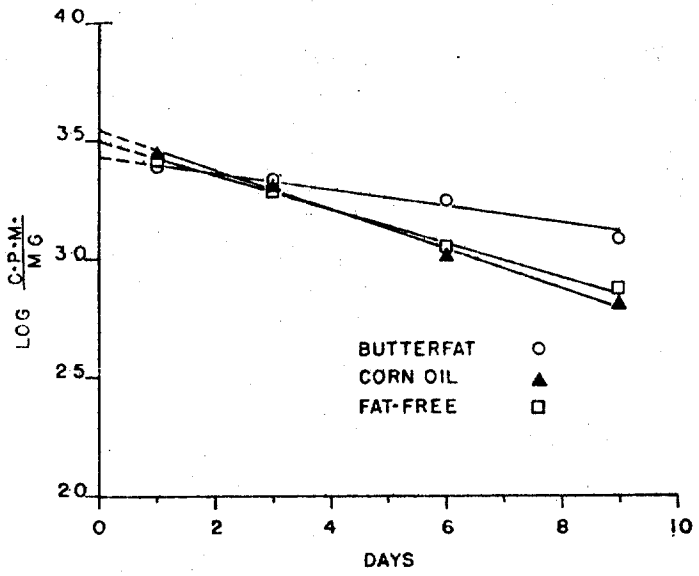

FIG. 2. A semilogarithmic plot of the specific activity of cholic acid in the pool during different dietary regimens.

plots of the specific activities of cholic acid in the bile acid pool on which the calculations of the cholic acid production rates were based. The cholic acid pool size and its half-life on the various dietary regimens is indicated in Table I. Similar changes in cholic acid turnover have been reported by Lindstedt and Steinberg (25) in groups of subjects subsisting on formula diets containing hypo- and hyper-cholesterolemic fats. From earlier studies (1, 2, 5–9) it has been inferred that the unsaturated vegetable seed oils lower plasma cholesterol levels in man at least in part by increasing the bile acid output, whereas a reduction in output has been associated with the hypercholesterolemia brought about by saturated fats. Although by current standards the techniques employed by the above workers in assessing the excretion of bile acids should be considered inadequate, the inferences drawn are compatible with the results of the present study.

TABLE I

Cholic acid turnover during different dietary regimens

Diet	Cholic acid pool size (g)	Cholic acid half-life (days)	Daily production (g/day)
Butterfat	1.67 (100%)*	8.8 (100%)*	0.13 (100%)*
Corn oil	1.49 (89%)	3.7 (42%)	0.28 (216%)
Fat-free	1.59 (95%)	4.2 (48%)	0.25 (192%)

*Calculated on the basis of the results for the butterfat diet, which were arbitrarily assigned a value of 100%.

A suppression of bile acid synthesis during the butterfat diet in comparison to that observed during corn oil and fat-free diets, however, would suggest that the observed differences might have resulted from the presence of cholesterol in butter. On the basis of in vitro assays, it has been demonstrated recently (26) that in man, as well as in other species, exogenous cholesterol is capable of suppressing effectively both cholesterol and, by implication, bile acid synthesis. This interpretation would bring the present data in line with the report of Spritz *et al.* (27) that the synthesis of cholesterol and bile acids was not significantly affected by the type of the sterol-free dietary fat. Profound distortions in the bile acid proportions following dietary alterations, however, would appear to suggest a more complex interaction.

The changes in the individual bile acid concentrations during the three dietary regimens are shown in Fig. 3. These data were obtained from quantitative gas-chromatographic recordings of the type illustrated in Fig. 4 for the glycine- and taurine-conjugated acids after 7 days on the fat-free diet. Although considerable variations were noted in the individual bile acid proportions during a single dietary regimen, the effect of the change in the diet is definitely noticeable, and it is about the same for the acids of both conjugate groups. When the high fat diets were fed, cholic acid concentration increased while that of deoxycholic acid decreased. On transfer from high butterfat to high corn oil diet, there was an immediate but transitory fall in the cholic acid and a corre-

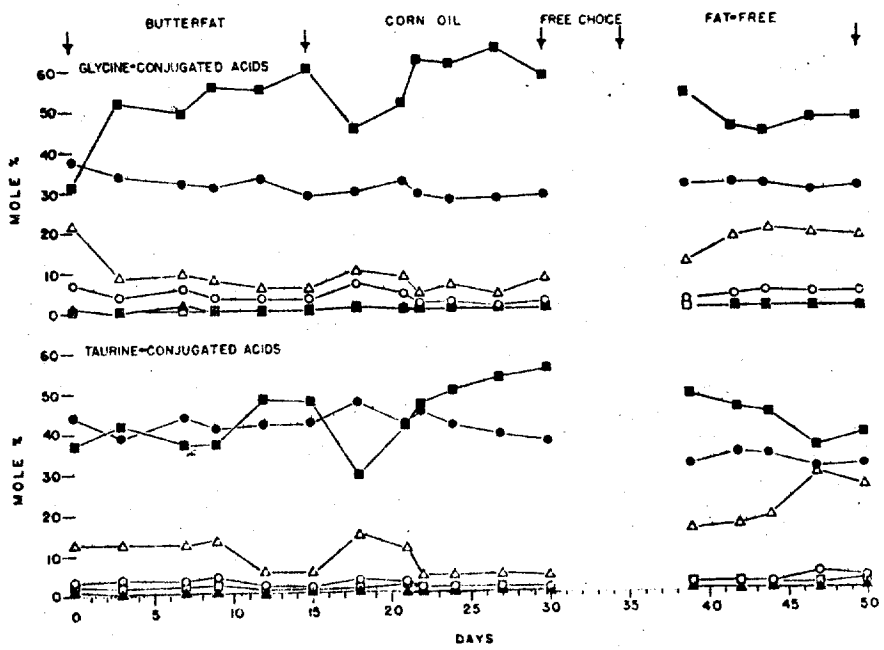

FIG. 3. The effect of dietary fat on the bile acid composition of the gallbladder bile of man. Legend for bile acids: □, lithocholic; ■, cholic; ○, ursodeoxycholic; ●, chenodeoxycholic; △, deoxycholic; ▲, 3-beta-12-alpha-dihydroxycholanic.

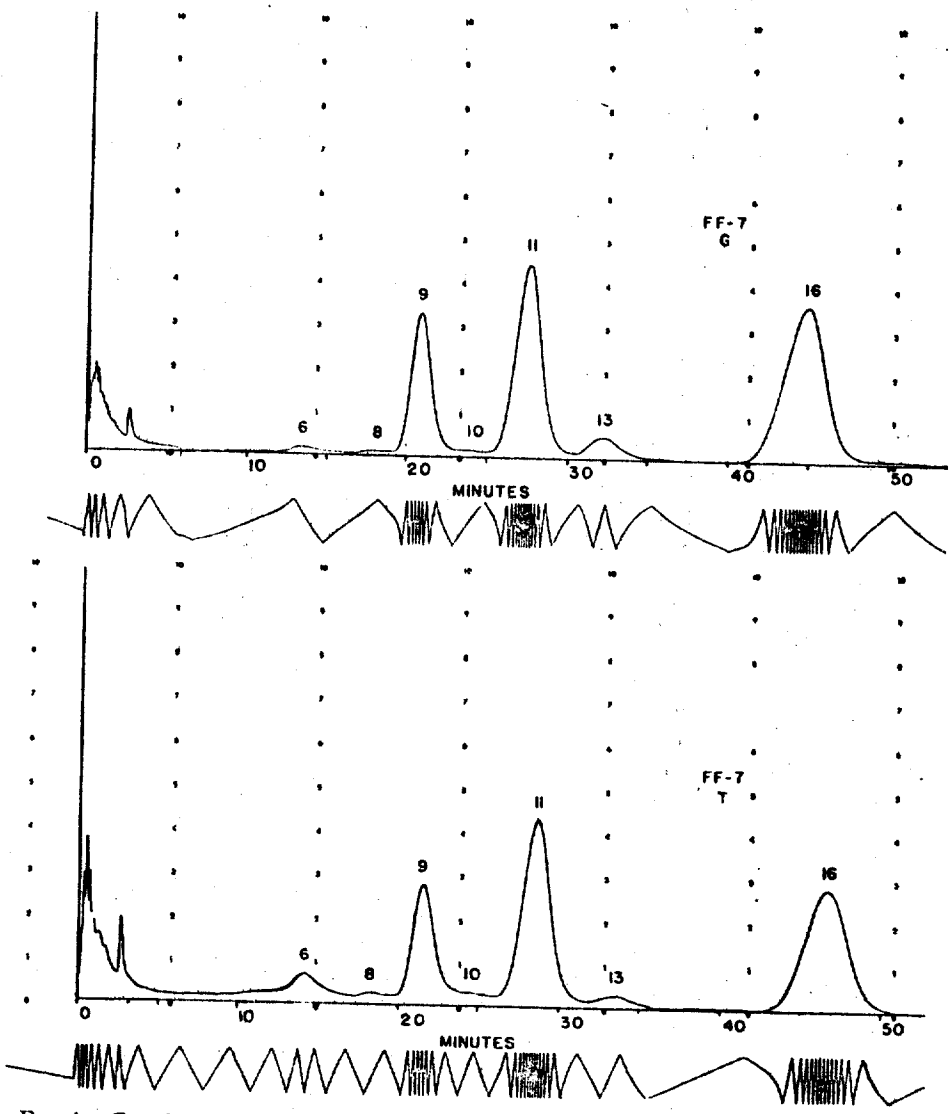

FIG. 4. Gas-chromatographic separation of the trifluoroacetylated methyl esters of bile acids isolated from gallbladder bile after 7 days on the fat-free diet.

Upper half, acids from glycine-conjugated fraction; lower half, acids from taurine-conjugated fraction.

Conditions of chromatography as previously described (14, 15). 6, lithocholic; 8, 3-beta-12-alpha-dihydroxycholanic; 9, deoxycholic; 10, 3-alpha-12-beta-dihydroxycholanic; 11, chenodeoxycholic; 13, ursodeoxycholic; 16, cholic.

sponding (but not identical) increase in deoxycholic acid. On transfer to the fat-free diet there was a similar immediate drop in cholate and an increase in deoxycholate concentration, but these changes, in contrast to those observed on transfer to the other regimens, were maintained for the duration of the experiment. The variations in the relative concentrations of chenodeoxycholic and

lithocholic acids were much less pronounced than those of the other major bile acids. Ursodeoxycholic acid tended to mirror the changes observed with deoxycholic acid in each of the three dietary periods, but the fluctuations were never as marked. The acid tentatively identified as 3-beta-12-alpha-dihydroxycholanic acid, which has thus far been isolated only from human feces (15, 28), was consistently present, although its concentration was low (0.1 to 2.0%). The bile sample drawn after 3 days on the corn oil regimen contained a component (3.8 and 1.7% in the glycine- and taurine-conjugated acids, respectively) which had a relative retention time similar to that reported by Sjovall (29) for the 3-alpha-hydroxy-7-keto cholanic acid, gas-chromatographed under similar conditions.

The formation of deoxycholic acid is dependent upon an intact enterohepatic circulation and the action of the intestinal flora (30, 31). The reduction of deoxycholic acid levels observed on the high butterfat and corn oil diets and the increase noted during the fat-free diet might therefore be related to suppression and stimulation, respectively, of the intestinal flora caused by these diets. The disappearance of deoxycholic acid from the bile of normal subjects subsisting on high fat diets has been noted also by others (32). Furthermore, in animal experiments it has been established that suppression or elimination of the intestinal flora is associated with an increased cholic acid half-life, a reduced bile acid turnover (33, 34), a reduced conversion of cholic to deoxycholic acid (28), and a hypercholesterolemia (35). This pattern was reversed following restoration of the normal intestinal flora. Such a scheme would not explain, however, the increased cholic acid production observed during the ingestion of corn oil. It is possible that this fat might have a more direct effect upon bile acid synthesis in the liver. A specific effect of unsaturated fats is suggested by the work of Lewis (3), who observed an increased cholic acid output in a patient with a complete bile fistula following intravenous infusion of cottonseed oil. It has been proposed that cholesterol is metabolized to bile acids at an accelerated rate when esterified to unsaturated fatty acids (36, 37).

The results of the present investigation would appear to be at variance with those of Hellman and Rosenfeld (4) and Avigan and Steinberg (38), who found no change in bile acid excretion with different dietary fats. In view of extensive bile acid degradation and transformation by the microbial flora (39), it is questionable whether or not fecal bile acid analyses can be relied upon as a valid index of bile acid production, even though an equilibration between the steroid acids of the feces and the circulating cholesterol is eventually obtained (40). A repetition of the type of study described here but involving a larger number of individuals would appear to be desirable.

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New solvent systems for thin-layer chromatography of bile acids

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SUMMARY A modified thin-layer chromatographic technique separates small amounts of glycochenodeoxycholic from glycodeoxycholic acid and the major taurine conjugates and unconjugated bile acids from one another.

KEY WORDS thin-layer chromatography · wedge technique · conjugated · unconjugated · bile acids

OF THE SEVERAL recent methods for thin-layer chromatography of bile acids (1–12) I have found a modification of the Hamilton method as reported by Kritchevsky, Martak, and Rothblat (3) to be more effective than others since it permits separation of up to 12 bile acids in one chromatogram. This modification is reported here.

Methods. Silica Gel G with calcium sulfate binder (Research Specialties Co., Richmond, Calif.) was used although Silica Gel H, binder free (E. Merck, A. G., Darmstadt, Germany), which was investigated later, gave comparable results. It adhered to the chromatoplate more strongly and because of its greater transparency allowed better staining of the bile acids. A slurry of 30 g of Silica Gel G in 58 ml of distilled water was spread 0.2 mm thick with the aid of a commercial apparatus (Microchemical Specialty Co., Berkeley, Calif.) on glass plates measuring 20 by 20 cm.

Commercial cholic acid and deoxycholic acid (Mann Fine Chemicals Inc., New York, N.Y.) and lithocholic, ursodeoxycholic, and chenodeoxycholic acids (Steraloids, Inc., Pawling, N.Y.) were purchased. The conjugated

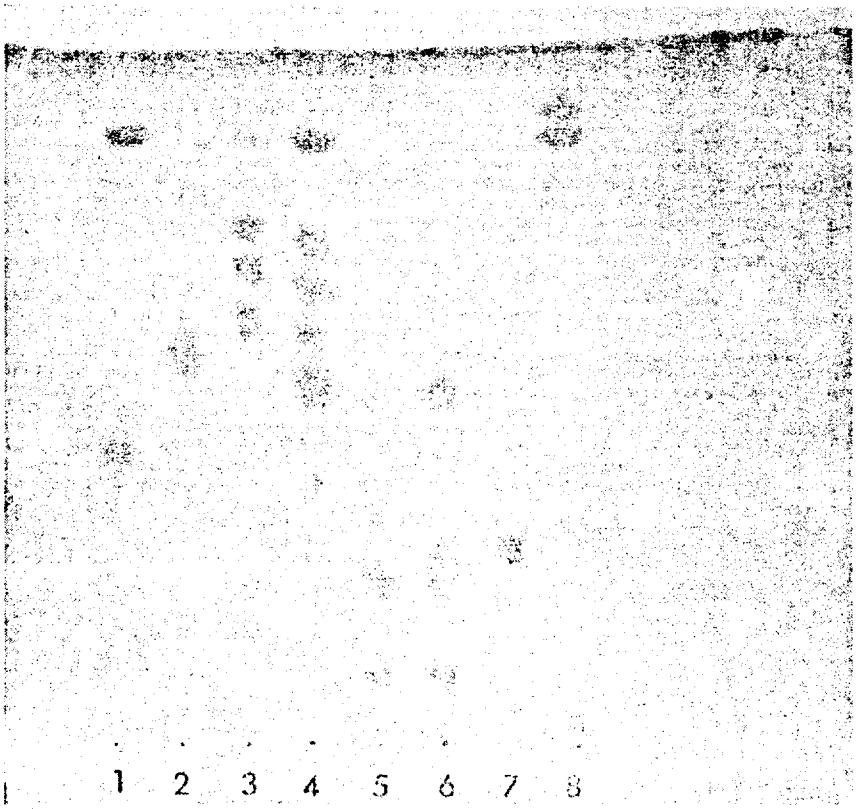

FIG. 1. Thin-layer separation of bile acids in solvent system described under Table 1. *1*, C, LiC; *2*, GLiC; *3*, UDC, CDC, DC; *4*, C, GLiC, UDC, CDC, DC, LiC; *5*, GC, GCD (and impurity); *6*, GC, GCD, GDC, GLiC; *7*, GDC; and *8*, TC, TLiC, LiC (impurity in TLiC).

Abbreviations. Unconjugated bile acids: *C*, cholic; *UDC*, ursodeoxycholic; *CD*, chenodeoxycholic; *DC*, deoxycholic; and *LiC*, lithocholic. Conjugated bile acids: *GC*, glycocholic; *GCD*, glycochenodeoxycholic; *GDC*, glycodeoxycholic; *GLiC*, glycolithocholic; *TC*, taurocholic; *TCD*, taurochenodeoxycholic; and *TLiC*, taurolithocholic.

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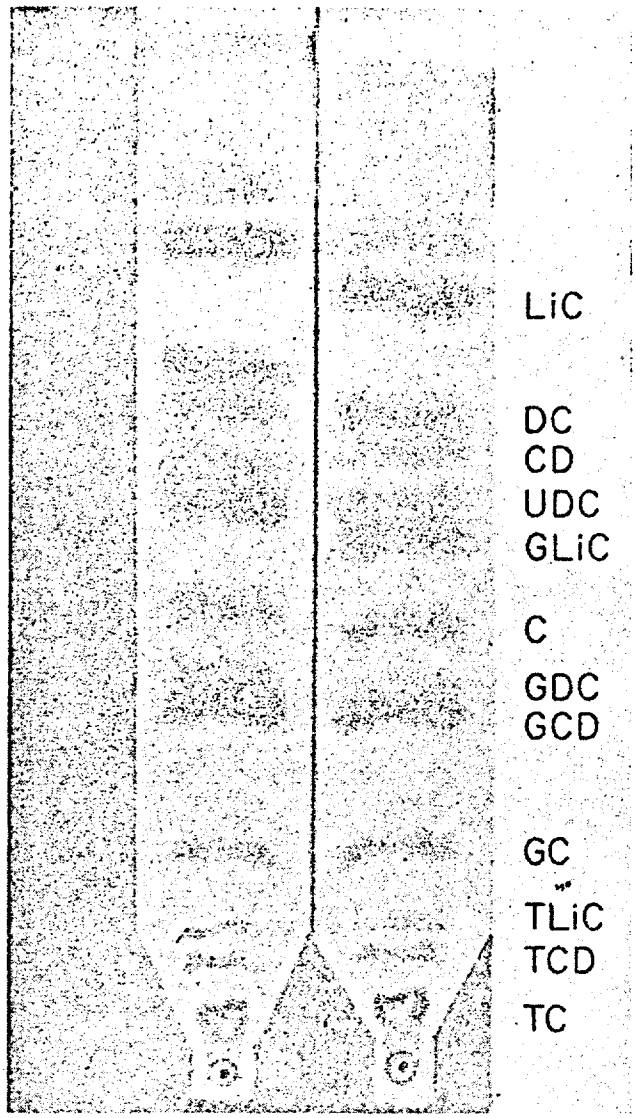

FIG. 2. Thin-layer separation of bile acids by wedge method. Solvent system and abbreviations as in Fig. 1.

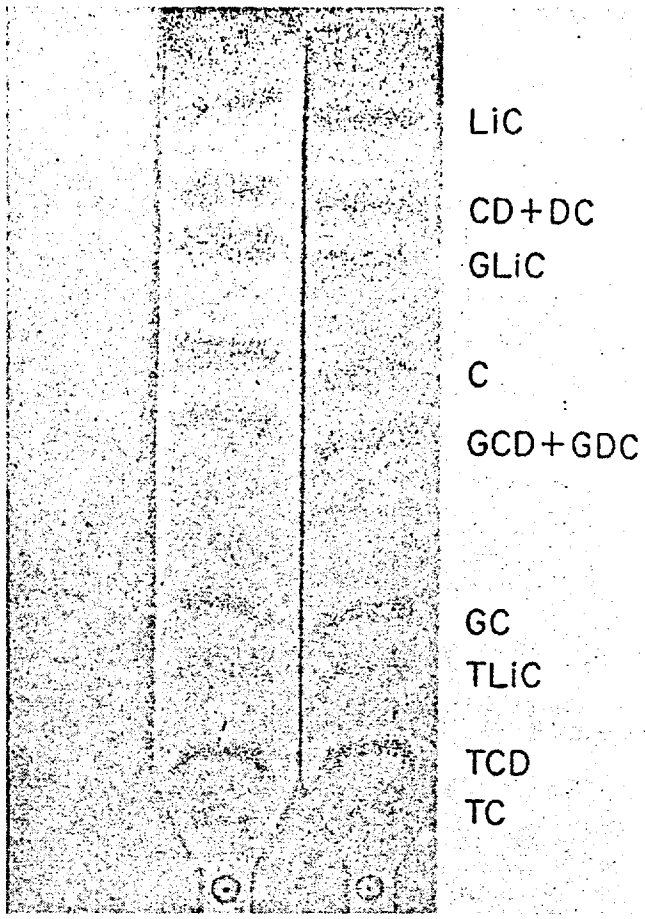

FIG. 3. Thin-layer separation of bile acids in ethylene dichloride-acetic acid-water 1:1:0.1. Abbreviations as in Fig. 1.

bile acids, with the exception of taurolithocholic acid (Steraloids, Inc.), were synthesized by the method of Norman (13) and the bile acids were purified by reverse phase chromatography (14), thin-layer chromatography, or crystallization. The taurolithocholic acid was not purified. The bile acids were applied either as the acid or in the salt form.

Isooctane and isopropyl ether (Fisher Scientific Co.), certified acetic acid (E. I. du Pont de Nemours & Co., Inc.), and isopropyl alcohol (Mallinckrodt Chemical Works, Pharmaceutical Div.) were distilled before use. The bile acids were dissolved in ethanol (1 $\mu g/\mu l$) and 10–20 μg was applied with a sharpened micropipette. The chromatoplates were activated by heating at 100°C for 40–60 min, cooled, and chromatographed. Whether the plates were activated before or after application of the bile acids made no difference to their mobility, but heating after application could conceivably result in some degradation of the bile acids. Later work has shown activation to be unnecessary.

All chromatography was done at 23–25°C in glass tanks of volume 5600 ml. The solvent (see below) was poured into the tank, which was lined with Whatman 3 MM filter paper, and the chromatoplate was inserted without further equilibration. The tank was then covered with a glass plate and sealed with masking tape. The plates were removed when the moving front was 1–2 cm from the top (1–2 hr). After chromatography the plates were dried and sprayed with a 10% solution of phosphomolybdic acid in alcohol and heated for 15 min in an oven at 110°C until the characteristic blue spots appeared. (Detection with a saturated solution of antimony trichloride in chloroform, 50% aqueous sulfuric acid, and iodine was also found to be satisfactory.)

Results. Representative chromatograms after use of the solvent system (modified Hamilton method) isooctane-isopropyl ether-glacial acetic acid-isopropyl alcohol 2:1:1:1 in the standard and wedge methods (15) of chromatography are shown in Figs. 1 and 2 and

TABLE 1 R_f Values of Free and Conjugated Bile Acids

	Method	
	Standard	Wedge
Free bile acids		
Cholic	0.42	0.45
Ursodeoxycholic	0.61	0.58
Chenodeoxycholic	0.62	0.63
Deoxycholic	0.74	0.68
Lithocholic	0.90	0.80
Conjugated bile acids		
Taurocholic	0.0	0.06
Taurochenodeoxycholic	0.0	0.12
Taurolithocholic	0.03	0.16
Glycocholic	0.10	0.22
Glycochenodeoxycholic	0.26	0.36
Glycodeoxycholic	0.30	0.39
Glycolithocholic	0.53	0.55

Adsorbent: Silica Gel G. Solvent mixture: isooctane isopropyl ether glacial acetic acid isopropyl alcohol 2:1:1:1 (see Figs. 1 and 2).

the average R_f values from these two methods are listed in Table 1.

The addition of isopropyl alcohol to the Hamilton system increases the R_f values of all compounds and preserves the excellent separation of the unconjugated dihydroxycholanic acids. The use of straight-chain alcohols in this system was less satisfactory. Although the taurine conjugates remain at or near the base line in the standard method, they move off the point of application in the wedge method and there is complete separation of taurocholic, taurochenodeoxycholic, and taurolithocholic acids. In addition, there is complete separation of glycochenodeoxycholic from glycodeoxycholic acid when their sum is 20 μg or less. Separation of these compounds is incomplete when 30 μg is chromatographed as in Fig. 2.

Another useful solvent system for the chromatography of bile acids by groups is ethylene dichloride acetic acid water 1:1:0.1. Development is more rapid than with Gänshirt, Koss, and Morianz's developer and the mixture contains fewer solvents than some other methods. A chromatogram employing this system and the wedge method is shown in Fig. 3.

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Influence of the diet on the turnover of bile acids in germ-free and conventional rats

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1. [24-^{14}C]cholic acid was given to conventional and germ-free rats and the faecal isotope excretion studied for 8 days. The turnover, pool size and daily excretion of cholic acid and its metabolites were calculated for rats on three different diets, i.e. a commercial type diet (pellets), a semi-synthetic diet without (D7) and with 20% cellulose (D7+cellulose). The transit time of the intestinal contents was evaluated by following the excretion of an orally administered dose of carmine.

2. The amount of bile acids excreted was two to three times higher in both conventional and germ-free rats receiving pellets than in those receiving diet D7. This difference in bile acid excretion between animals receiving different diets cannot, therefore, be caused by an influence of the diet on the gastro-intestinal microflora. This effect of the commercial diet could not be due to a high fibre content, since an increase in the fibre content of the semi-synthetic diet D7 by the addition of 20% cellulose did not reproduce the effect obtained with the commercial diet.

3. The difference observed in bile acid excretion between germ-free and conventional rats on diet D7 was mainly ascribed to the much longer transit time of intestinal contents in germ-free as compared with conventional rats.

The turnover of cholic acid can be calculated by determining the rate of faecal isotope excretion after administration of [24-^{14}C]cholic acid. Studies using this technique in the rat have demonstrated that both the intestinal flora and the diet influence the elimination of cholic acid. The slowest faecal excretion rate of labelled cholic acid has been observed in germ-free rats (Gustafsson, Bergström, Lindstedt & Norman, 1957), the estimated biological half-life of cholic acid being at least three times that in conventional rats on the same semi-synthetic diet (D7). Determination of faecal excretion products of cholic acid gave a mean daily value of 1·9 mg in germ-free rats and 5·1 mg in conventional rats (Gustafsson, Normal & Sjövall, 1960). Portman & Murphy (1958) have demonstrated that the type of diet has a pronounced influence on the turnover of cholic acid in conventional rats. In rats fed on Purina chow, the biological half-life of cholic acid was 2·0 days, whereas with a standard, low-residue, semi-purified diet with sucrose as the source of carbohydrate, and with the same diet supplemented with 20% of cellulose, the half-lives were 4·2 and 1·4 days, respectively. They also found that the mean faecal excretion of cholate by animals given Purina chow was nearly five times that of animals given the sucrose synthetic diet.

Factors associated both with the microflora in the gastro-intestinal tract and with the diet ingested play an important part in the metabolism of bile acids, and, in the conventional animal, these interdependent factors can affect various conditions per-

taining to the formation and passage of bile acids through the body. For example, the rate of elimination of bile acids may be influenced by an effect on their synthesis in the liver; the reabsorption of bile acids from the intestine may be influenced by variations in the formation of their different metabolites, present in different physical states in the contents of the gastro-intestinal tract, and also by alterations in the transit time of these contents due to changes in the muscular activity of the intestinal wall, combined, in rodents, with an effect on the practice of coprophagy.

In the present investigation germ-free and conventional rats receiving the same three diets have been compared, in order to study the influence of the diet *per se* on the turnover of bile acids. Two of these diets were semi-synthetic, one being low and the other high in fibre; a third, commercial diet, with a comparatively high fibre content, was also used. The transit time of the gastro-intestinal contents was studied by administering carmine; coprophagy was prevented in some of the rats.

EXPERIMENTAL AND RESULTS

The following systematic names are given to compounds referred to in this report by trivial names: cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholanoic acid.

Animals

Germ-free rats of the Swedish strain were reared according to the technique described earlier (Gustafsson, 1948, 1959). All the rats were male and their initial ages ranged from 107 to 235 days.

Diet and treatment of animals

All the germ-free animals were housed in metabolism cages in one jacket isolator made of plastic and stainless-steel. After the experiments had been completed with the germ-free animals, the conventional rats were transferred to this isolator and housed in the same manner. Three weeks before the experiments began, the rats were put into metabolism cages, divided into three groups and fed the experimental diets. The first group received the basal semi-synthetic diet, D 7 (Table 1), the second group a diet made up of 4 parts of diet D 7 to 1 part of cellulose (Cellulose Powder for chromatography; Kebo AB, Stockholm, Sweden), and the third group, a pelleted commercial diet. According to the analysis supplied by the manufacturers, the composition of the pellets was (% by weight): protein, 27·5; fat, 10·5; nitrogen-free extract, 41·0; ash, 9·0; fibre, 4·7; water, 7·3. All diets were sterilized by autoclaving for 20 min at 121°. Diets and water were given *ad lib.* From the beginning of the experiments coprophagy was prevented in half of the rats in each dietary group, by restraining cages similar to those described by Bollman, Cain & Grindlay (1948). On the 1st day of the experiment, 0·1 mg sodium [24-^{14}C]cholate (5 μc), autoclaved in 1 ml water, was given orally to each rat by means of a stomach tube. Through the same stomach tube, a suspension of 100 mg carmine in 1 ml water was given. Faeces and urine were collected every 24 h. After 8 days the rats were killed. The liver, stomach, small intestine (divided

into three equal segments), caecum and large intestine were removed separately. The stainless-steel funnels of the metabolism cages were renewed every day and removed from the isolator. After the faeces had been collected, the surfaces of the funnels were washed with 80% ethanol. At the end of the experiment the whole metabolism cage was washed in the same manner.

Table 1. *Composition of the basal diet D7 (per kg diet)*

Ingredient	Amount
Casein	220 g
Wheat starch	635 g
Arachis oil	100 g
Salt mixture*	40 g
Ascorbic acid	1 g
Inositol	1 g
Choline	2 g
Thiamine	50 mg
Riboflavine	20 mg
Pyridoxine	20 mg
Nicotinamide	200 mg
Calcium pantothenate	100 mg
p-Aminobenzoic acid	300 mg
Biotin	1 mg
Folic acid	20 mg
Cyanocobalamin	0·02 mg
Vitamin A (palmitate)	21000 i.e.
Vitamin D (ergocalciferol)	4500 i.u.
Vitamin K (phylloquinone)	10 mg
Vitamin E (tocopheryl acetate)	500 mg

* Hubbell, Mendel & Wakeman (1937).

Extraction of labelled bile acids

Faeces, liver, stomach and intestinal segments were each homogenized in 4 parts of water with a Virtis Homogenizer with propeller for 1 min at 5000 rev/min. Ethanol was added to the homogenate to give a final concentration of 80% (v/v) ethanol. After refluxing twice for 3 h, the residue was further extracted for 48 h with boiling chloroform–methanol 1:1 (v/v). Radioactivity was determined in a Packard Tri-Carb Scintillation counter. Internal standards were always used to correct for quenching.

The amount of faecal labelled bile acids that remained after extraction with 80% ethanol was calculated by determining the amount of isotope recovered in the subsequent chloroform–methanol extracts. As shown in Table 2, 1·7 to 8·2% of the labelled faecal bile acids were not recovered in the 80% ethanol extract from both germ-free and conventional rats, irrespective of the diet. Furthermore, it is interesting to note that there were only small differences between germ-free and conventional rats and between the different dietary groups.

After determining the isotope remaining in the liver and the gastro-intestinal tract, and the isotope in the faeces excreted during the experimental period, the recovery of the isotope as a percentage of the amount given was calculated (Table 3). The mean values for the recovered isotope were consistently lower in the germ-free (86–89%) than in the conventional rats (90–95%). The excretion of labelled compounds in

urine was also determined. Labelled compounds could be demonstrated in only three rats, but the total urinary excretion of isotope was never above 0·1% of the isotope given. The 80% ethanol washings of funnels and cages were refluxed and filtered. In no instance did the extracts contain labelled compounds.

Table 2. *Percentage of administered isotope recovered by extraction from the liver and gastro-intestinal tract of rats 8 days after oral administration of [24-^{14}C]cholic acid, and from the faeces accumulated during this period*

Rearing conditions	Diet	Isotope recovered	
		Mean*	Range
Germ-free	D7	89	88–92
Conventional	D7	94	91–98
Germ-free	D7 + cellulose	88	83–90
Conventional	D7 + cellulose	95	92–99
Germ-free	Pellets	86	83–92
Conventional	Pellets	90	87–94

* Mean value for three rats with and three without prevention of coprophagy.

Table 3. *Amount of labelled bile acids extracted with chloroform–methanol 1:1 from the residue obtained after initial extraction with 80% ethanol from faeces of rats*

Rearing conditions	Diet	No. of extractions	Percentage of recovered isotope present in chloroform–methanol extracts	
			Mean	Range
Germ-free	D7	32	3·6	1·7–4·8
Conventional	D7	48	4·7	3·5–7·0
Germ-free	D7 + cellulose	40	6·7	5·2–7·7
Conventional	D7 + cellulose	40	4·8	2·9–8·2
Germ-free	Pellets	40	4·7	3·4–6·2
Conventional	Pellets	48	2·6	1·8–3·1

Distribution of remaining labelled bile acids in the different parts of the gastro-intestinal tract and liver

Fig. 1 gives the percentage of labelled compounds in the liver, stomach, upper, middle and lower third of the small intestine, caecum and colon 8 days after administration of [24-^{14}C]cholic acid. More than 75% of the isotope recovered from the various organs investigated, from both conventional and germ-free rats, was present in the small intestine, irrespective of the diet. The upper third of the small intestine, including the duodenum, only contained 20% of the isotope present, whereas the remaining 80% was equally distributed between the middle and lower thirds. The amount of labelled bile acids in the liver and bile ducts varied between 0·7 and 5·9%. The mean values for the six groups varied between 1·3 and 3·9%. Labelled bile acids were present in the stomach of all conventional rats and of all germ-free rats, except for four animals. The highest value, 8·8%, was found in a conventional rat kept in a restraining cage. The amounts present in the stomachs of rats in which coprophagy

was prevented were about the same as those in the groups of animals not kept in restraining cages. The caecum contained between 2·1 and 20·5% of the labelled bile acids. No significant difference in the amount present in the caecum was observed between different groups of animals kept on different diets, or between germ-free and conventional animals. Only trace amounts of labelled compounds were found in the colon. Mean values for the different groups varied between 0·8 and 2·6%.

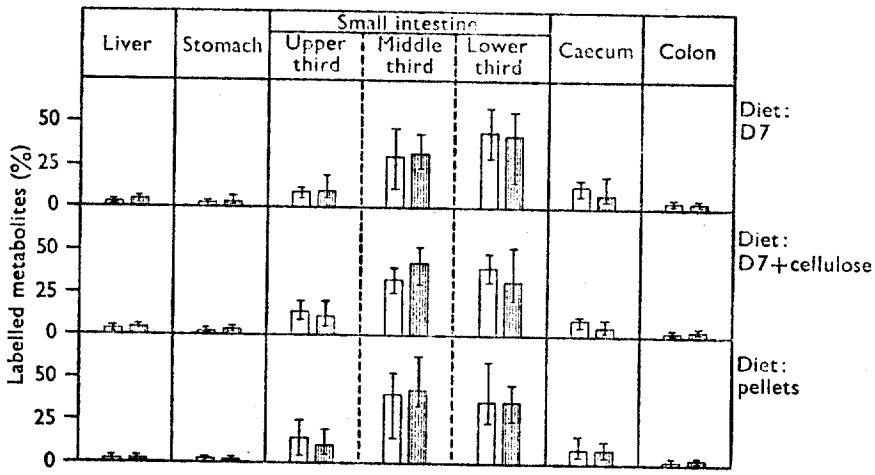

Fig. 1. Percentage distribution of labelled bile acids remaining in the liver and different parts of the gastro-intestinal tract of rats 8 days after administration of [24-^{14}C]cholic acid. Each bar gives the percentage of labelled metabolites and the vertical lines indicate range. □, germ-free rats; ▥, conventional rats.

Elimination rate and turnover of cholic acid

The daily excretion of isotope was determined during 8 days following administration of [24-^{14}C]cholic acid. Fig. 2 gives semi-logarithmic plots of $-\log(1-[u_t/a_0])$ *v.* time, where u_t = cumulative excretion of isotope, to time t; and a_0 = administered amount of isotope. Graphing resulted in straight-line plots which intersected the zero ordinate at about 0·5 day. The half-life times obtained from these graphs (Fig. 2) are given in Fig. 3.

As the elimination of cholic acid from the pool probably follows first order kinetics, this equation will hold:

$$a_f = a_0\, e^{-kt},$$

where a_0 = given amount of isotope, a_f = isotope remaining in the pool at the time t, and k = elimination constant. As the bile acids in the rat are present only in the liver and the gastro-intestinal tract, and only in trace amounts in the rest of the animal (Sjövall & Åkesson, 1955), it is assumed that the pool is equal to the isotope recovered when the rats were killed. From the values of k the biological half-life was calculated according to

$$t_{\frac{1}{2}} = \log 2/k.$$

However, the isotope given was not completely recovered. It is not known whether this was due to losses during sampling or to incomplete extraction and so it is impossible to evaluate which of the two calculated values for half-life is correct. However, the differences between the two estimations are small and do not interfere with the validity of the following conclusion.

Figs. 2 and 3 show that, in germ-free rats, the biological half-life of cholic acid, in animals reared on pellets, was only a third of that observed in animals on diet D7. Addition of cellulose to diet D7 shortened the half-life time of cholic acid, and in animals receiving pellets it was even shorter than in those on the semi-synthetic diets. In

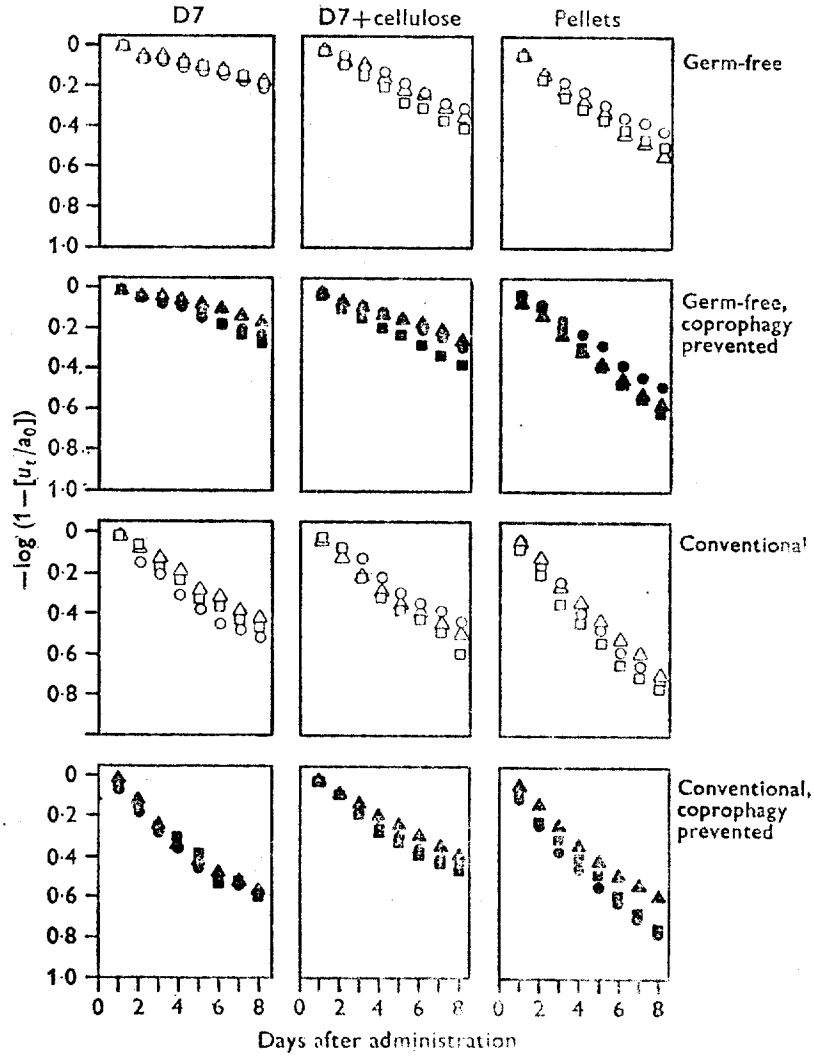

Fig. 2. Semi-logarithmic plot of elimination of [24-^{14}C]cholic acid in rats given diet D7, D7+cellulose, or pellets. u_t = cumulative excretion of isotope to time t; a_0 = administered amount of isotope; ○, △, □, represent individual rats reared in ordinary metabolism cages; ●, ▲, ■, represent individual rats in which coprophagy was prevented by restraining cages.

conventional rats the half-life of cholic acid in animals receiving pellets was slightly shorter than in those receiving diet D7, but the diet did not exert the same influence as that observed in germ-free rats, since the cellulose supplement to diet D7 did not shorten the half-life of cholic acid. The elimination rates of cholic acid in germ-free rats were always slower than in conventional rats on all the diets tested. The greatest

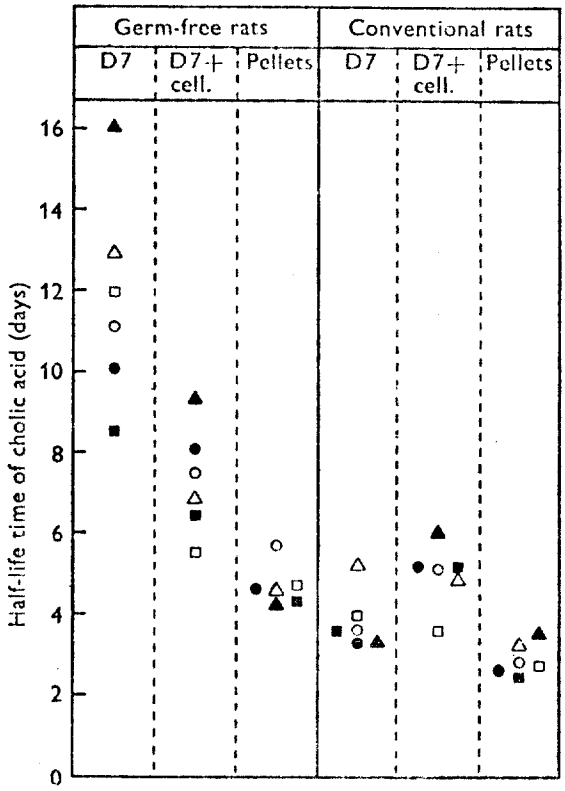

Fig. 3. Half-life times of [24-^{14}C]cholic acid in rats reared on diet D7, D7+cellulose (D7+cell.), or pellets. For symbols see Fig. 2.

differences between germ-free and conventional rats were observed in rats reared on diet D7. Prevention of coprophagy had no obvious influence on the half-life of cholic acid in any of the groups.

Determination of cholic acid and its metabolites present in the liver and intestinal tract

The extracts of labelled bile acids from the small intestine were combined and hydrolysed in N-NaOH for 6 h at 120° in a sealed tube. The unconjugated bile acids were extracted with diethyl ether from an acidified solution and separated by column chromatography by the method described earlier (Gustafsson *et al.* 1960). The chromatographic fractions containing cholic acid were combined and the amount quantitated enzymically by means of a NAD-linked β-steroid dehydrogenase prepared from

Pseudomonas testosteroni, as described by Iwata & Yamasaki (1964). The specific radioactivity of cholic acid was determined.

Assuming that the specific radioactivity of cholic acid is equal to that of its metabolites (Gustafsson *et al.* 1960), and that the specific radioactivity was the same in different parts of the intestinal tract at the time the rats were killed, the total amount of cholic acid plus its metabolites can be calculated by dividing total radioactivity in the rat by the specific radioactivity of cholic acid in the small intestine.

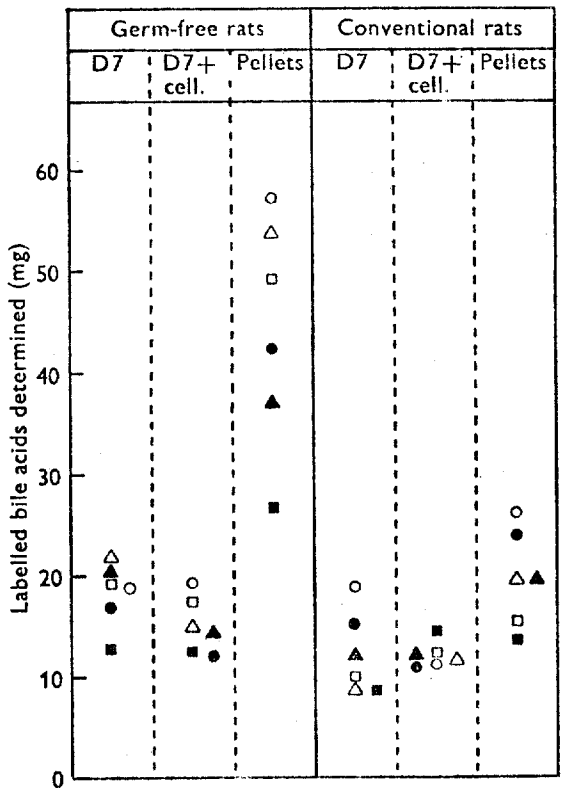

Fig. 4. Estimated amount of cholic acid and its metabolites present in liver and intestinal tract of rats receiving diet D7, D7+cellulose (D7+cell.), or pellets. For symbols see Fig. 2.

The estimated amounts of cholic acid and its metabolites are summarized in Fig. 4. Mean values of 18·5, 15·0 and 44·5 mg labelled bile acids were found in the intestinal contents of germ-free rats reared on diets D7, D7 with cellulose, and pellets, respectively; the corresponding values for conventional rats were 10·7, 12·1 and 19·7 mg. Germ-free and conventional rats reared on diet D7 and D7 with cellulose were found to contain about the same amount of labelled bile acids. However, when the animals were receiving pellets germ-free rats contained approximately double the amount of bile acids found in conventional rats.

Daily excretion of cholic acid and its metabolites

The turnover time, $\overline{T}$, of cholic acid was calculated from the formula

$$\overline{T} = t_{\frac{1}{2}}/\ln 2.$$

$t_{\frac{1}{2}}$ was calculated from determinations of faecal isotope excretion. The daily excretion of cholic acid and its metabolites was calculated by dividing the total amount of these compounds present in the rat by the turnover time. The values for daily excretion so determined are summarized in Fig. 5.

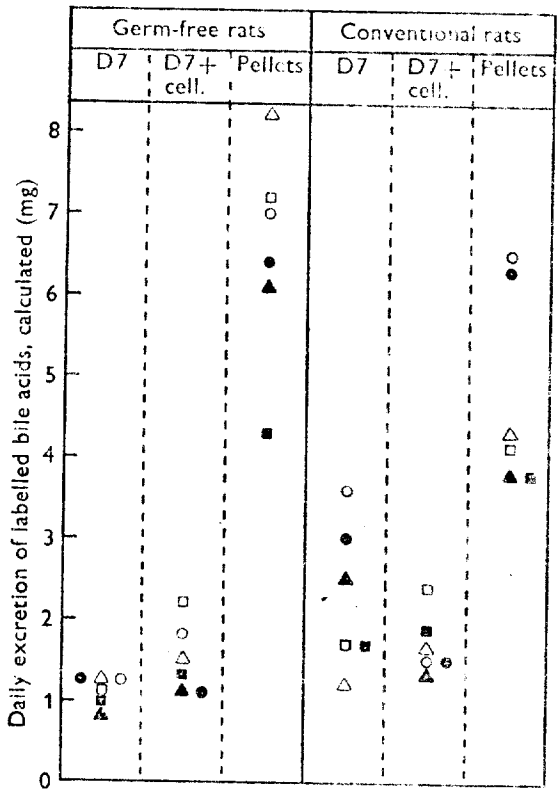

Fig. 5. Calculated values for the excretion of cholic acid and its metabolites in rats receiving diet D7, D7+cellulose (D7+cell.), or pellets. For symbols see Fig. 2.

The average daily excretion of cholic acid and its metabolites was found to be 1·1, 1·5 and 6·6 mg in germ-free rats reared on D7, D7 with cellulose, and pellets, respectively. The corresponding values for conventional rats were 2·3, 1·7 and 4·2 mg. The addition of cellulose to D7 did not increase the bile acid excretion in either germ-free or conventional rats. Feeding with pellets resulted in a significantly higher bile acid excretion than either of the semi-synthetic diets tested.

In rats fed D7 with cellulose, or pellets, the daily excretion was found to be the same in conventional as in germ-free rats. In animals receiving D7 the germ-free rats had a significantly lower bile acid excretion than the conventional rats.

Faecal excretion of carmine

Carmine is widely used as a faecal marker since it is easy to detect in faecal samples. The faecal excretion of carmine was determined during the present investigation in order to study the duration of faecal excretion of an amount of food administered orally.

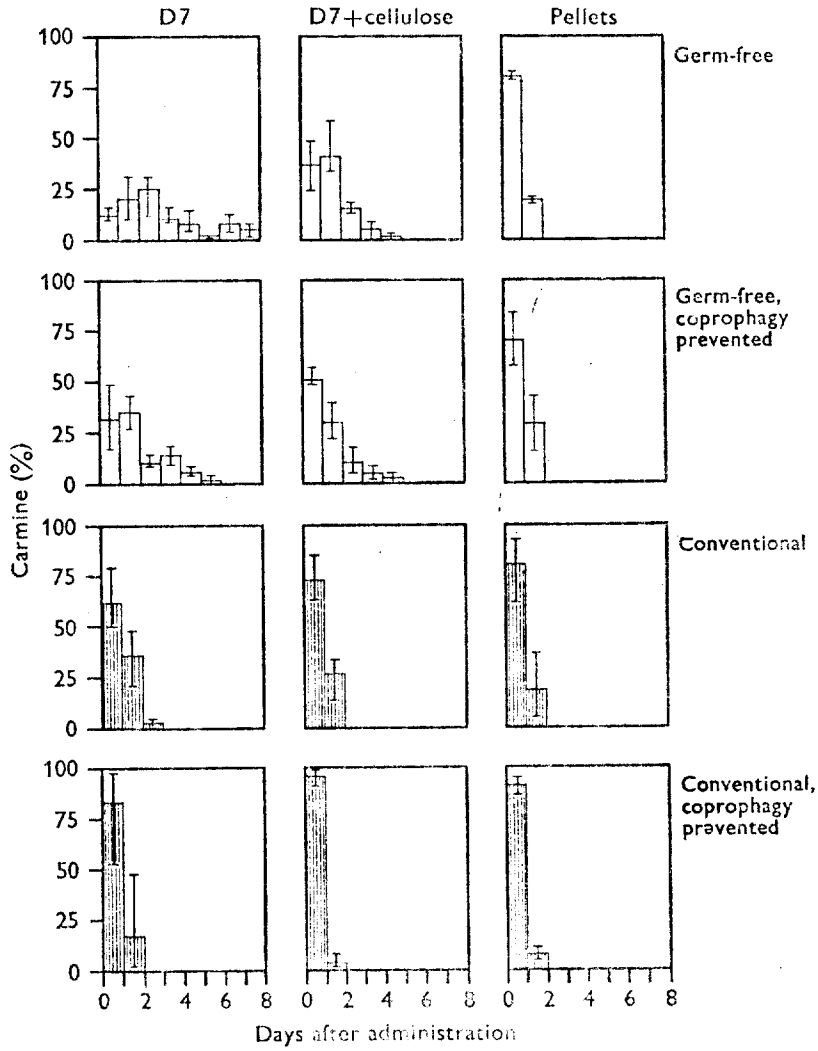

Fig. 6. Carmine excreted, after oral administration, in faeces of rats given diet D7, D7+cellulose, or pellets. Each bar gives carmine excreted daily as a percentage of the recovered amount and the vertical lines indicate the ranges.

The faecal residue after extraction of the labelled bile acids with chloroform methanol 1:1 was homogenized for 5 min in N-NaOH and centrifuged at 25000 *g* for 60 min. The absorption spectrum from 200 to 650 nm of the supernatant fractions was obtained by a recording spectrophotometer. The absorbance at the maximum of 525 nm was used to calculate the concentration of carmine.

The daily faecal excretion of carmine is shown in Fig. 6. The conventional rats excreted more than 50% of carmine in the first 24 h faecal sample. Most of the remainder was excreted within 48 h. In rats reared on diet D7 a trace amount of carmine was detected in faeces 48 h after oral administration of carmine. The rapid excretion of carmine, and the long sampling intervals used, do not allow any conclusions to be made concerning differences in carmine excretion between conventional rats fed on different diets or the effects of prevention of coprophagy on the excretion of carmine.

In germ-free rats the rate of excretion of carmine by those receiving diet D7 was extremely slow when coprophagy was not prevented, since 3–13% of the recovered amount of carmine was present in the intestinal contents of the small intestine, caecum and colon, even after 8 days. However, when coprophagy was prevented, all the carmine administered had been excreted in the faeces by the 7th day. Addition of cellulose to diet D7 only slightly increased the excretion rate of carmine. However, in rats receiving this diet, no differences were observed in excretion rate of carmine between animals in ordinary metabolism cages and animals in which coprophagy was prevented. Carmine was excreted more slowly by germ-free rats than by conventional rats in the groups receiving diet D7 or D7 with cellulose. In germ-free and conventional rats receiving pellets, the same, relatively fast, excretion of carmine was observed.

DISCUSSION

Experimental technique

Several different solvent systems have been described for the extraction of labelled faecal bile acids derived from [24-^{14}C]cholic acid. Eneroth, Hellström & Sjövall (1968) have proved that extraction of human faeces with chloroform–methanol will give complete recovery of bile acids. In our previous investigations in the rat faeces have always been extracted with 80% ethanol as a standard procedure. It has been shown, however, in the present investigation that, although most of the labelled metabolites of cholic acid were extracted with this solvent, a further 2·6–6·7% could be recovered in the subsequent chloroform–methanol extract. It has been suggested (Eneroth *et al.* 1968) that chloroform–methanol is needed for the complete extraction of bile acids, owing to the possibility that they may be bound to micro-organisms. However, this cannot be the only explanation, since chloroform–methanol had also to be used for the complete extraction of faecal bile acids from the germ-free rats.

The percentage recovery of isotope after oral administration of [24-^{14}C]-labelled bile acid has been studied in various animal species, and sometimes a low recovery of isotope has been reported (cf. Danielsson, 1963). A recovery of approximately 90% of the isotope was obtained in the present investigation, after oral administration of [24-^{14}C]cholic acid, and the mean percentage was slightly higher in the conventional than in the germ-free rats. Those investigators, who obtained a low recovery of isotope after oral administration of [24-^{14}C]-labelled bile acids, have suggested that β-oxidation of bile acids might be an explanation for loss of isotope during their experimental studies. Micro-organisms have been isolated from soil that have brought about β-oxidation of the side-chain to form bisnorcholanoic acids, i.e. bile acids without the

carbon atoms C-23 and C-24, after growth in a medium with bile salt as the only carbon source (Severina, Torgov, Skrjabin, Wulfson, Zaretskii & Papernaja, 1968). However, in the present investigation there was a greater percentage recovery of isotope from conventional than from germ-free rats, which makes it very unlikely that β-oxidation due to microbial activity could be an explanation for the loss of isotope observed.

In rats interest has been focused on the turnover of cholic acid, as this is the main bile acid formed from cholesterol (Danielsson, 1963). The quantitative faecal excretion of cholic acid and its metabolites was calculated from determinations of cholic acid and its metabolites remaining in the rat at the end of the experimental period, and the half-life of cholic acid. Owing to the fact that the chemical structures of many faecal metabolites of cholic acid are not known (Gustafsson & Norman, 1969), a direct determination of cholic acid and its metabolites in faeces was not attempted.

Qualitative aspects of bile acid metabolism

In parallel investigations on the same animals described in the present work the physical state of bile acids in the intestinal contents of germ-free and conventional rats (Gustafsson & Norman, 1968) and the excretion of cholic acid metabolites in conventional rats (Gustafsson & Norman, 1969) have been studied. It was shown that approximately the same metabolites of cholic acid were excreted in conventional rats whether reared on pellets or on diet D7 with cellulose. The transformation of the deoxycholate formed was significantly greater in conventional rats on these diets than in those on diet D7 alone. Also, dialysis of faecal homogenates of conventional rats showed that part of the labelled metabolites of cholic acid were excreted in a non-dialysable form. There was a higher excretion of non-dialysable bile acids in rats on pellets than in those on D7 with cellulose or D7 alone. There was a much greater proportion of bile acids in the sediment left after centrifugation of the caecal contents with both the germ-free and conventional rats receiving pellets than with those receiving diet D7. This indicates that a larger fraction of bile acids is adsorbed to intestinal residue in rats fed on pellets than on those fed on diet D7.

Influence of diet on bile acid excretion in germ-free rats

The excretion of bile acids by germ-free rats fed on pellets was found to be markedly greater than that of rats on diet D7. The transit time of intestinal contents, judged from the excretion of carmine, was markedly less in the former rats than in the latter. It is not known which components of pellets cause an increase in bile acid excretion. If the primary cause of this increase were a less efficient absorption of bile acids, and not an increase in their synthesis in the liver, it is possible that either, or both the decrease in transit time of intestinal contents and adsorption of bile acids to intestinal residues may be contributory factors. Increasing the amount of fibre in the intestinal contents by adding cellulose to the semi-synthetic diet D7 did not increase either bile acid excretion, or the transit time, to the same level as that observed in rats fed on pellets. Both the transit time and the amount of bile acids adsorbed to intestinal residue (Gustafsson & Norman 1968) were only slightly different in rats receiving pellets from the values observed in rats on diet D7 alone.

Coprophagy

Previous studies (Gustafsson *et al.* 1957) showed that bile acids were excreted more slowly by germ-free than by conventional rats. One reason for this could be that the germ-free rats practise coprophagy more than the conventional ones. This possibility was, therefore, investigated in the present work by including a group of animals in which coprophagy was prevented. The results show that animals receiving diet D7 practised coprophagy to a rather large extent, since carmine was excreted for more than 8 days when coprophagy was not prevented, and only for 6 days when it was prevented. Carmine excretion was rapid in all groups of conventional rats, irrespective of the diet and was complete in 48 h. During this investigation the interval between samples was not short enough to allow any conclusions to be drawn concerning the possible differences between the various groups of animals within the 24 h periods chosen.

The restraining cages used in the present investigation allowed an almost complete sampling of the faeces from rats in which coprophagy was prevented.

Influence of diet on bile acid excretion in conventional rats

In conventional rats as well as in germ-free rats there was a higher faecal excretion of bile acids in animals receiving pellets than in those receiving diet D7. The addition of cellulose to D7 had only a slight effect on bile acid excretion. Since the composition of the faecal labelled metabolites of cholic acid excreted in conventional rats on both diet D7 with cellulose and on pellets was the same (Gustafsson & Norman, 1969). it is unlikely that the high excretion of bile acids observed in rats on pellets was due to the formation of less absorbable derivatives of bile acids. It is, therefore, reasonable to assume that the greater excretion of bile acids in conventional rats compared with germ-free rats was caused by differences demonstrated in the transit time of intestinal contents, and the physical state of the bile acids in the intestinal contents, rather than by the chemical nature of the excreted bile acids.

Comparison of the amount of bile acids excreted in the faeces from germ-free and conventional rats on the same diet

In previous work (Gustafsson *et al.* 1960) it has been shown that a greater calculated amount of bile acids was eliminated daily by conventional rats than by germ-free rats receiving diet D7. This has been confirmed in the present investigation, but in addition it has been shown that this difference between germ-free and conventional rats did not exist if 20% cellulose was added to diet D7, or if the animals were given pellets. It has also been shown earlier (Gustafsson & Norman, 1962) that most of the bile acids present in the caecal contents and faeces of conventional rats on diet D7 were present in the sediment after centrifugation. The amount of bile acids found in the sediment after centrifugation of the caecal contents was greater in conventional rats than in germ-free rats, in all the dietary groups (Gustafsson & Norman, 1968). Thus the same difference in the physical state of intestinal bile acids existed between conventional and germ-free rats, irrespective of the diet used, and so it is more likely

that the difference in the faecal excretion of cholic acid and its metabolites between germ-free and conventional rats, fed on diet D7, was due to the longer transit time of intestinal contents in germ-free rats, rather than to differences between their physical state in the intestine of the germ-free as compared to the conventional.

Pool size and turnover of [24-^{14}C]*cholic acid*

In previous work we have shown that a reliable estimation of the daily excretion of cholic acid and its metabolites can be performed by determining both the half-life of [24-^{14}C]cholic acid and the total amount of cholic acid and its metabolites in the liver and gastro-intestinal tract (Gustafsson *et al.* 1960). The total amount of bile acids in the rats was found to vary with the diet used. In germ-free rats more than double the amount of cholic acid was present in those receiving pellets than in those fed on diet D7. The same dietary effect was observed in conventional rats, but it was less marked. Owing to the effect of diet on the pool size of bile acids, it is necessary to emphasize that a determination of the biological half-life of cholic acid alone is not sufficient for the study of the effect of diet on the turnover of bile acids.

In the present work it was shown that, irrespective of the diet used, the bile acids in both germ-free and conventional rats were mainly present in the small intestine. Although the large caecum typical of the germ-free rodent was present in the germ-free rats on all three diets, the amount of bile acids in the caecum of germ-free rats was not significantly greater than in that of conventional rats.

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Influence of the diet on the composition of faecal bile acids in rats*

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1. [24-^{14}C]cholic acid was given orally to rats receiving three different diets, a semi-synthetic diet (D7) with and without 20% cellulose, and a commercial, pelleted diet. The labelled metabolites excreted in the faeces in the 8 days following administration of the labelled cholic acid were chromatographically separated, and tentatively identified by means of thin-layer chromatography.

2. In all three dietary groups the faecal bile acids mainly consisted of unconjugated and 7α-dehydroxylated metabolites. In rats receiving pellets or diet D7 with cellulose, there was a higher percentage of monohydroxy-monoketo-cholanoic acids than in those receiving diet D7 alone. Dihydroxy-cholanoic acids, present in approximately the same ratio as were the 3α- and 3β-hydroxy isomers, were excreted by all the rats in the three dietary groups.

The primary bile acids, cholic and chenodeoxycholic acid, are transformed during enterohepatic circulation into secondary bile acids, which are formed initially by the action of intestinal micro-organisms, and are partly absorbed, but which can be further modified in the liver, and partly excreted in the faeces. After isolating and identifying the faecal bile acids from man, Eneroth, Gordon, Ryhage & Sjövall (1966) showed that these acids form a very complicated mixture of various different metabolites. In addition to the hydrolysis of conjugates, the main transformations of the resulting unconjugated bile acids, that take place in the intestinal tract, are: elimination of the hydroxyl groups at C-7; oxidation of hydroxyl groups at C-3, C-7, and C-12 to keto groups which can subsequently be reduced to both α- and β-hydroxyl groups (for a review see Danielsson, 1963).

The transformations of both conjugated and unconjugated bile acids have been studied in vitro in cultures of some microbial strains isolated from the intestinal contents of man and different animal species (Midtvedt & Norman, 1967). It was shown that the hydrolysis of conjugates is a common property of intestinal micro-organisms. The hydroxyl groups at C-3 and C-7 were oxidized by several strains, and those at C-12 by a few of the strains examined. Reduction of the keto groups at C-3 has yielded both α- and β-hydroxyl groups, but reduction of the keto group at C-7 has, as yet, only yielded the 7α-hydroxyl group. Strains capable of 7α-dehydroxylation have been isolated from the intestinal contents of rat and man (Gustafsson, Midtvedt & Norman, 1966). In the blind-loop syndrome, an increased hydrolysis of conjugated bile acids

* This work is part of investigations supported by the Swedish Medical Research Council grants 205, 206 and 602.

takes place in the small intestine (Dawson, 1967). Several strains of intestinal micro-organisms capable of hydrolysing conjugates have been isolated from aspirates of the small intestine of patients with this disease (Draser, Hill & Shiner, 1966; Tabaqchali & Booth, 1966).

It is well known that the diet influences the composition of the gastro-intestinal microflora (for references see Gall, Fenton & Cowgill, 1948). As the 'normal' flora contains strains that differ in their abilities to transform bile acids, it is obvious that the diet may influence the composition of the faecal bile acids through changes in the gastro-intestinal microflora.

The aim of the present investigation was to compare the faecal metabolites of [24-^{14}C]cholic acid excreted by rats on three different diets; a semi-synthetic diet (D7), the same diet with 20% cellulose (D7 with cellulose), and a commercial diet (pellets). This investigation formed part of studies using these diets, on the rate of elimination of [24-^{14}C]cholic acid by germ-free and conventional rats (Gustafsson & Norman, 1969), and on the physical state of the labelled bile acids in the intestinal contents of these rats (Gustafsson & Norman, 1968).

EXPERIMENTAL AND RESULTS

The following systematic names are given to compounds referred to in this report by trivial names: cholic acid, 3α, 7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-monohydroxy-5β-cholanoic acid.

Diet and treatment of animals

The experiments to be reported were carried out at the same time, on the same dietary groups of conventional rats, as those described in a previous paper (Gustafsson & Norman, 1969), where the composition of the diets, housing of the animals and the methods of sampling of faeces and urine are described. Each rat was given 0·1 mg of sodium[24-^{14}C]cholate (5 μc) orally. Faeces were collected daily during 8 days from animals in which coprophagy was prevented by restraining cages (Bollman, Cain & Grindlay, 1948), and also from animals in which coprophagy was not prevented.

Chromatographic technique

Partition column chromatography was used, as described earlier (Norman, 1964), to obtain group separations of [24-^{14}C]cholic acid metabolites. After chromatography with phase system F1 (Fig. 1), cholic and 3,12-dihydroxy-7-keto-5β-cholanoic acid appear with, or just after, the front, and these two acids are separated from deoxycholic acid, 3-hydroxy-12-keto-5β-cholanoic acid and 12-hydroxy-3-keto-5β-cholanoic acid. The last three acids appear in the 40–60 ml, and 80–110 ml effluent fractions, respectively, while 3,12-diketo-5β-cholanoic acid is retained in the stationary phase. However, the α- and β-hydroxy isomers of all these acids cannot be separated by this method. This separation was achieved as follows: the bile acids were methylated and fractionated on columns of aluminium oxide (grade III; Woehn, Schwege, Germany) with a solvent system consisting of increasing concentrations of ethyl acetate in

benzene (Danielsson, Eneroth, Hellström, Lindstedt & Sjövall, 1963). The labelled material isolated in this way was identified by thin-layer chromatography (TLC) in different phase systems (Eneroth, 1963), together with appropriate standards. After TLC, the radioactive spots were located by autoradiography. The steps used for the separation of the various faecal metabolites are outlined in Fig. 1.

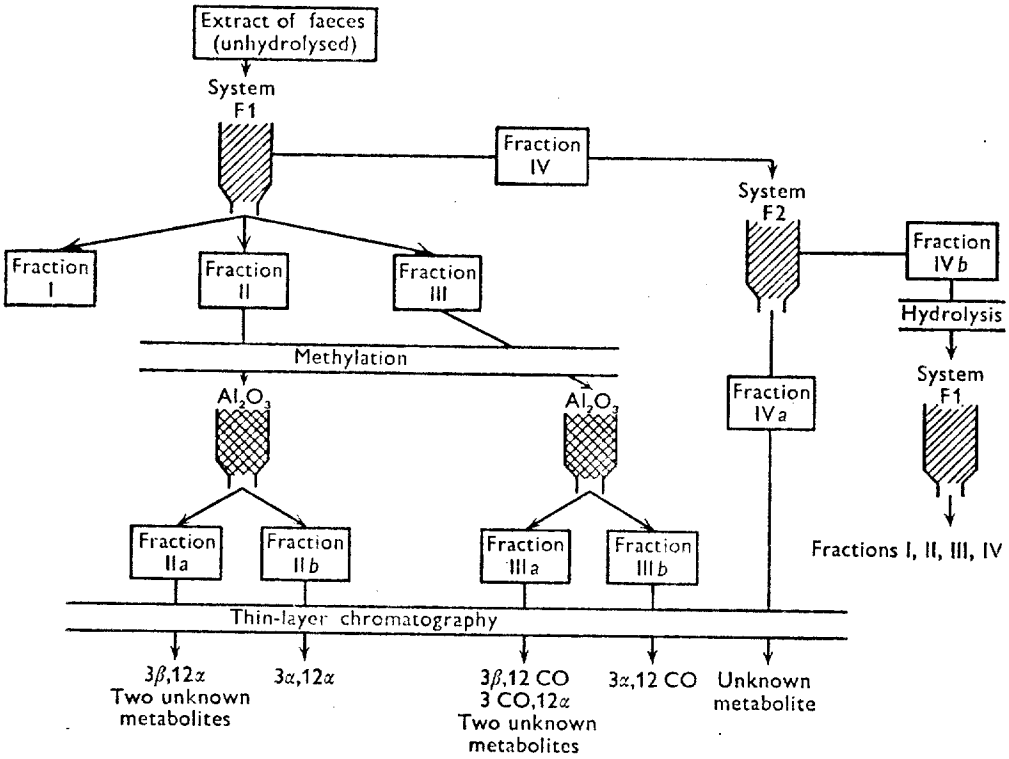

Fig. 1. Outline for the fractionation and separation of metabolites of cholic acid in faeces. The following abbreviations are used: 3β,12α, 3β, 12α-dihydroxy-5β-cholanoic acid; 3α,12α, 3α, 12α-dihydroxy-5β-cholanoic acid; 3β,12 CO, 3β-hydroxy-12-keto-5β-cholanoic acid; 3CO, 12α, 12α-hydroxy-3-keto-5β-cholanoic acid; 3α,12 CO, 3α-hydroxy-12-keto-5β-cholanoic acid.

Extraction of faecal bile acids

The faeces were collected separately for each day from individual rats for 8 days after the administration of [24-^{14}C]cholic acid, and stored at −17° until they were analysed. Each of these daily samples was then homogenized in water, ethanol was added to give a final concentration of 80%, and the mixture was boiled for 12 h and filtered. Equal portions of the filtrates obtained from each of these samples were combined, evaporated, dissolved in water, acidified to pH 1 with hydrochloric acid, and then extracted with diethyl ether followed by butanol. The labelled material in the faeces was separated into two groups by the ether extraction. Unconjugated bile acids and glycine conjugates of mono- and dihydroxy-cholanoic acids were recovered in the ether extracts, whereas glycine conjugates of trihydroxy-cholanoic acids, and all taurine conjugates, appeared in the subsequent butanol extracts. The labelled material

in the butanol extract did not exceed 2% of the total recovered isotope in any of the experiments. Thus a maximum of 2% cholic acid was excreted in faeces either as taurine conjugates or glycocholic acid.

Chromatographic separation of labelled faecal metabolites of [24-^{14}C]cholic acid

The ether extracts were chromatographed with phase system F 1, and a representative chromatogram is shown in Fig. 2. Labelled material representing four bile acid regions, three in the eluate and one retained in the column, was obtained. This was further separated as shown in Fig. 1, and the following fractions were recognized.

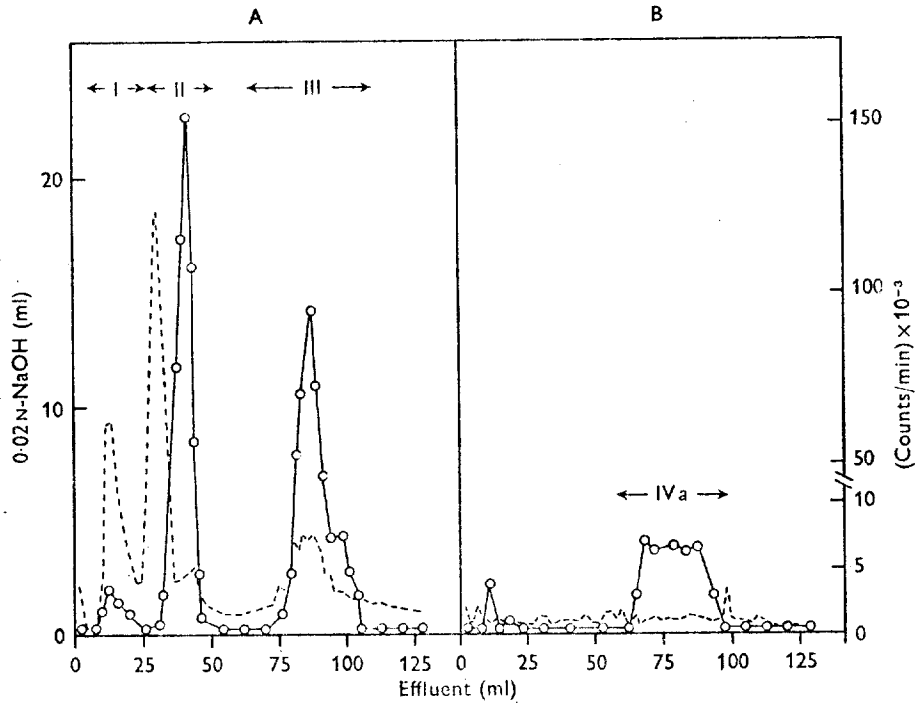

Fig. 2. Separation by chromatography of labelled metabolites from faeces during 8 days after administration of [24-^{14}C]cholic acid to one rat reared on diet D7.
A—ether extract (phase system F 1); B—rechromatography of labelled compounds retained in the column after chromatography A (phase system F2). ---, titration values; O–O, radioactivity.

Phase system	Moving phase	ml	Stationary phase	ml
F 1	Methanol:water	165:135	Chloroform:heptan	45:5
F 2	Methanol:water	130:220	Chloroform:heptan	45:5

Columns: 4 ml stationary phase. Supported on 4·5 g hydrophobic Hyflo Super-Cel (Johns-Manville, New York).

Fraction I. This fraction consisted of minor amounts of labelled compounds appearing with, or just after, the front. Subsequent TLC analysis showed that this fraction contained several unconjugated metabolites of cholic acid in addition to small amounts of unchanged cholic acid.

Fraction II. This fraction was methylated and further fractionated on a column of aluminium oxide. Two labelled fractions were obtained (Fractions II*a* and II*b*, Fig. 3). TLC showed that the major fraction (II*b*) consisted of methyl 3α, 12α-dihydroxy-5β-cholanate, and the minor fraction (II*a*) mainly of methyl 3β, 12α-dihydroxy-5β-cholanate with trace amounts of two unidentified metabolites.

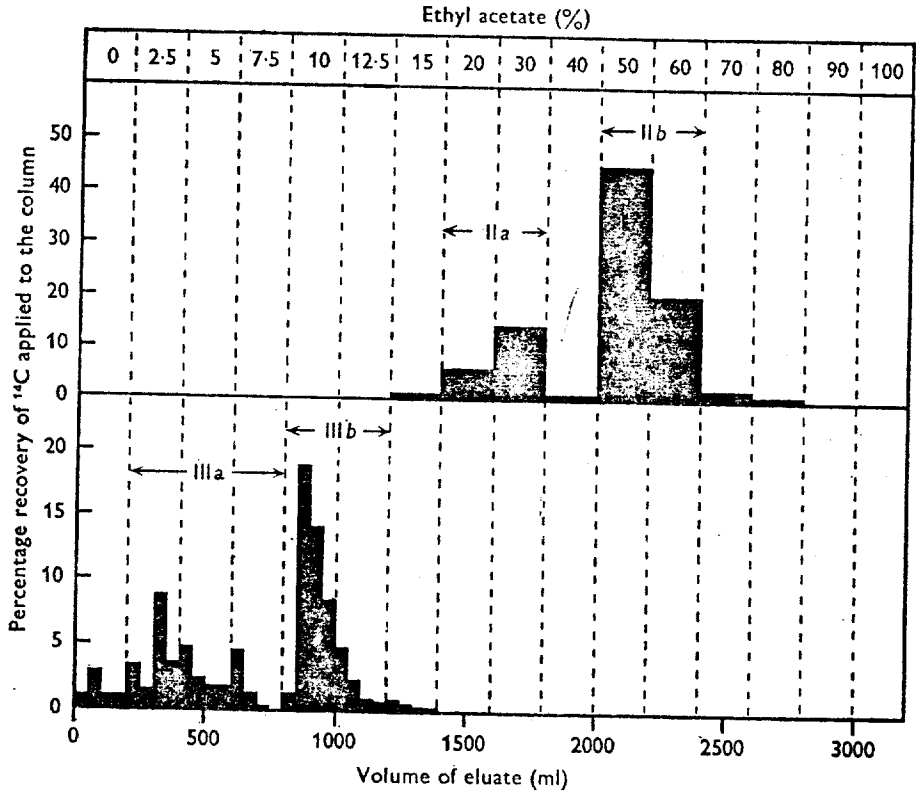

Fig. 3. Alumina fractionation of Fractions II and III (see Fig. 1). Column: 20 g aluminium oxide. The composition of the eluate is given as the percentage of ethyl acetate in benzene.

Fraction III. After methylation, this fraction was further separated on a column of aluminium oxide into two fractions (III*a* and III*b*, Fig. 3). Subsequent TLC showed that Fraction III*b* consisted of methyl 3α-hydroxy-12-keto-5β-cholanate and that Fraction III*a* consisted of four compounds: methyl 3β-hydroxy-12-keto-5β-cholanate, methyl 12α-hydroxy-3-keto-5β-cholanate, and two unidentified metabolites.

Fraction IV. This fraction represented the labelled material retained in the stationary phase after chromatography with phase system F1. Rechromatography with phase system F2 (Fig. 2) showed that only minor amounts of a metabolite were eluted at the position of 3,12-diketo-5β-cholanoic acid (Fraction IV*a*), but the TLC mobility of this metabolite revealed a difference between these two compounds. The major part of this fraction (IV*b*) remained in the stationary phase and consisted of saponifiable derivatives of labelled bile acids, which after hydrolysis (refluxing for 2 h in N-NaOH

in 90% methanol), and subsequent chromatography, were shown to be identical to the labelled bile acids found in Fractions I to IV.

Labelled, faecal metabolites in rats receiving different diets

Fig. 4 gives the percentage distribution of the labelled bile acid metabolites in Fractions I to IV, obtained after chromatography with phase system F1 (Fig. 1), of the ether extracts of faeces of rats in the different dietary groups. Comparison of the values for the percentage distribution of bile acids in the faeces of rats with and without prevention of coprophagy, showed that there were no significant differences between the six different animals in the same group, and so the means and ranges of these six combined values are given together in Fig. 4.

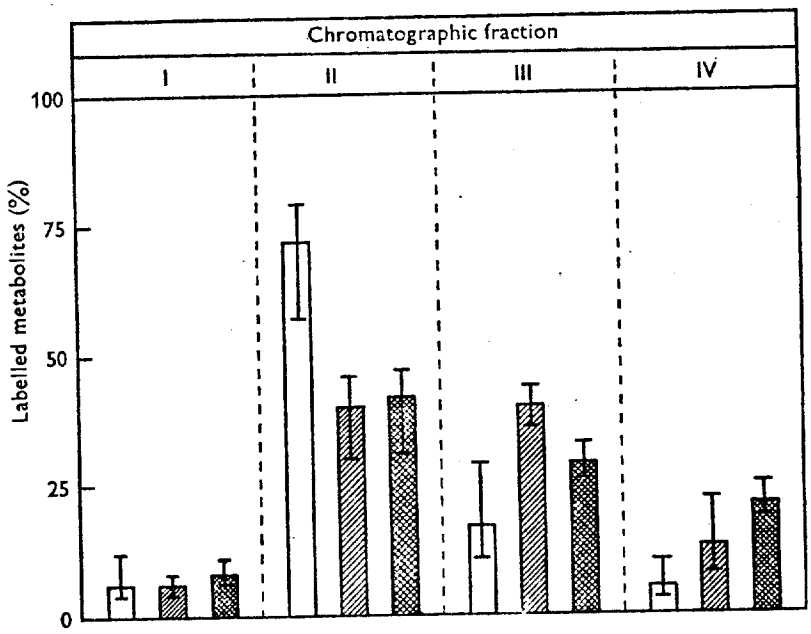

Fig. 4. Percentage distribution of labelled metabolites in Fractions I, II, III and IV (see Fig. 1), obtained after chromatography of faecal bile acids from rats reared on diets D7, □, D7 with cellulose, ▧, and pellets, ▩. Each bar represents the mean value for six rats and the vertical lines indicate the range.

Fraction I, which contained the unchanged cholic acid, contained only 6–8% of the labelled metabolites. The other labelled metabolites were recovered in Fractions II to IV, and thus lacked hydroxyl or keto groups at C-7. Fig. 4 also shows that the labelled bile acid metabolites from rats in the different dietary groups were distributed between the fractions in a similar manner, although, in rats receiving diet D7, a greater proportion of metabolites was recovered in Fraction II. Approximately the same amount of labelled bile acids was recovered in Fractions II and III from rats receiving diet D7 with cellulose. In these rats and in those receiving pellets, a larger proportion of the dihydroxy-cholanoic acids (Fraction II) had been oxidized to monoketo-monohydroxy-cholanoic acids (Fraction III) than in those receiving diet D7 alone.

The labelled material in Fraction II was further analysed according to the scheme shown in Fig. 1. The results, summarized in Table 1, indicate that more than 90% of the labelled material consisted of two metabolites with the TLC mobilities of 3α, 12α-dihydroxy-5β-cholanoic and 3β,12α-dihydroxy-5β-cholanoic acid. The ratio between these two isomers was roughly 3:1 in all the rats from the different dietary

Table 1. *Percentage distribution of labelled faecal metabolites of bile acids in Fraction II from groups of six rats reared on three different diets, D7, D7+cellulose or pellets*

	D7		D7+cellulose		Pellets	
TLC mobility of metabolite	Mean	(Range)	Mean	(Range)	Mean	(Range)
3α, 12α-dihydroxy-5β-cholanoic acid	73	(67–79)	75	(71–81)	74	(71–80)
3β, 12α-dihydroxy-5β-cholanoic acid	22	(14–28)	18	(12–22)	20	(15–23)
Unknown metabolites	5	(2–7)	7	(3–9)	6	(2–8)

Table 2. *Percentage distribution of labelled faecal metabolites of bile acids in Fractions III and IV from rats reared on three different diets*

(Mean values for the pooled faecal metabolites from six rats)

Diet	Metabolites with TLC mobilities of 3α-hydroxy-12-keto-5β-cholanoic acid and 3β-hydroxy-12-keto-5β cholanoic acid in Fraction III (% of labelled compounds in Fraction III)	Unknown metabolites recovered in Fraction IVa (% of labelled compounds in Fraction IV
D7	82	2
D7+cellulose	85	3
Pellets	80	3

groups. The percentage distribution of isotope between the other, unknown, metabolites in Fraction II was not determined. Fraction III, from each of the rats in the same dietary group, was combined, and the labelled material was analysed in the manner shown in the scheme in Fig. 1. The results (Table 2) show that, irrespective of the diet received, Fraction III mainly consisted of metabolites with the TLC mobilities of 3α-hydroxy-12-keto-5β-cholanoic and 3β-hydroxy-12-keto-5β-cholanoic acid. Only trace amounts of 12-hydroxy-3-keto-cholanoic acid were found. Fraction IV from each of the rats in the same dietary group was also combined. The same small percentage amount of an unknown metabolite was recovered in Fraction IV*a*, from rats in all dietary groups (Table 2). Fraction IV, therefore, mainly consisted of saponifiable derivatives of labelled bile acids.

DISCUSSION

In the present investigation the nature of the labelled faecal metabolites of [24-^{14}C]-cholic acid has been studied in rats on three different diets. The rats receiving the two semi-synthetic diets (D7 and D7 with cellulose) gave a low excretion of bile acids, whereas this was high in rats receiving the commercial diet (Gustafsson & Norman, 1969). The labelled faecal bile acids were excreted mainly in an unconjugated and 7α-dehydroxylated form by rats in all three dietary groups. There was a lower excretion of monohydroxy-monoketo-cholanoic acids by rats fed on diet D7 than by those fed on either of the other diets. No significant difference in the ratio of the 3α- and 3β-hydroxy isomers was observed in the faecal metabolites from rats in the different dietary groups. 3,12-diketo-5β-cholanoic acid was not isolated from the faeces of any of the rats. The large amount of monoketo-mono-hydroxy-cholanoic acids excreted by rats on the commercial diet cannot be considered to be the main cause for the previously observed high excretion of bile acids by these rats (Gustafsson & Norman, 1969), since an even greater amount of these acids was formed in rats on diet D7 with cellulose, whose faecal excretion of bile acids had been shown to be low.

It is not possible to state whether the increased formation of keto derivatives that was observed in rats fed on diet D7 with cellulose, or on the commercial diet, had been brought about by a change in the composition of the gastro-intestinal microflora, or by an influence of the diet on the activity of the micro-organisms that transformed the bile acids.

Fitzgerald, Gustafsson & McDaniel (1964) have shown that, in rats where coprophagy was prevented with tail cups, there was a numerical change within certain bacterial groups in the faeces. In the present investigations, however, the bacteria were not enumerated, and the fact that the same composition of faecal bile acid metabolites was observed in animals with and without prevention of coprophagy seems to indicate that coprophagy prevention had not had any influence on that part of the intestinal microflora which is responsible for the transformation of bile acids.

In the rat, bile acids are mainly absorbed from the distal part of the ileum (Dietschy, Salomon & Siperstein, 1966), where transformations of bile acids by micro-organisms occur only to a minor extent (Norman & Sjövall, 1958). An extensive transformation of bile acids by microbial enzymes starts in the caecum, where various quantities of the metabolites formed are absorbed, and continues in the colon, before excretion in the faeces (Norman & Sjövall, 1958). Various methods have been used for studying the extent of absorption of microbial metabolites of bile acids. The radioactive material in the portal blood has been analysed by means of reversed-phase-partition chromatography after the administration of tritium-labelled cholic acid to rats (Olivecrona & Sjövall, 1959), and radioactive bands have been found at the positions corresponding to the main cholic acid metabolites known to be formed in the caecum. Approximately 15% of the radioactive bile acids in the portal blood have been shown to be unconjugated, and deoxycholic acid formed approximately 50% of these unconjugated acids. It has also been found, in investigations using a gas chromatographic technique

(Cronholm & Sjövall, 1967), that the composition of the bile acids in the portal blood of rats could be influenced by the diet. Using two of the diets employed during the present investigation, they showed that the portal blood of rats fed on pellets contained more unconjugated bile acids (36%) than that of rats receiving diet D7 alone (10%). Nevertheless, no significant differences were observed between the groups of rats on these two diets in the proportion of cholic and deoxycholic acid. However, the only microbial metabolite of cholic acid they determined was deoxycholic acid. Up to the present, there has been no report on the identification of the other cholic acid metabolites that may occur in portal blood. It is, therefore, not known whether the large amount of monoketo-hydroxy-cholanoic acids formed in the intestinal tract and appearing in the faeces, observed in the present investigation, may affect the composition of the bile acids in the enterohepatic circulation.

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METABOLISM OF CHOLIC ACID IN GERMFREE ANIMALS AFTER THE ESTABLISHMENT IN THE INTESTINAL TRACT OF DECONJUGATING AND 7α-DEHYDROXYLATING BACTERIA

By

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The effect *in vivo* of the intestinal flora upon bile acid metabolism has been investigated by comparative work on conventional and germfree rats. No degradation of the conjugated primary bile acids, cholic and chenodeoxycholic acids, takes place in the intestinal tract of the germfree rat (6). The half-life of cholic acid in germfree rats is more than twice that found in conventional animals (6), and the amount of bile acids excreted is less than half that excreted in conventional rats (7).

The ability to transform bile acids is widely spread amongst several genera represented in the intestinal microflora (13). Several strains are able to split bile acid conjugates and to oxidize the hydroxyl group at C-3 and C 7 in cholic acid and chenodeoxycholic acid, but so far only one group of microorganisms has been found capable of removing the hydroxyl group at C 7. The 7α-dehydroxylation is a major feature in intestinal bile acid metabolism. The microorganisms performing this reaction were isolated from rat and human faeces (11), and tentatively identified as anaerobic members of the tribe *Lactobacilleae* (12). One of these strains, Strain H, has been further studied *in vitro* (14). It was shown to be capable of 7α-dehydroxylating both cholic and chenodeoxycholic acid to form deoxycholic and lithocholic acid, respectively, and to oxidize the hydroxyl groups at C 3 and C 7 to keto groups. However, Strain H was unable to split conjugated bile acids or to remove the 7α-hydroxyl group from the conjugated bile acids. The

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The following systematic names are given to compounds referred to in this report by trival names: cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-monohydroxy 5β-cholanoic acid.

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present investigation deals with the problem whether, and to what extent, deconjugation and 7α-dehydroxylation would occur when bacteria capable of such reactions *in vitro* had been established in the intestinal tract of germfree animals.

MATERIALS AND METHODS

Animals

Contamination of germfree mice. In a preliminary screening trial to contaminate germfree animals, 9 male mice, 220 days old, from the germfree N.M.R.I. strain on diet D 7 (5) were transferred from the breeding unit in single animal isolators used by *Gustafsson et al.* (10). The bacteria were introduced by spraying approximately 1 ml of a 72 hour culture in Todd Hewitt broth (Oxoid) (TH broth) into the isolator by means of a sterile syringe which pierced the air filter. At the end of the experiments, *i.e.* after 14 days, the mice were killed by exsanguination under ether anaesthesia. The last faecal pellet in the rectum was collected and examined bacteriologically. The presence of Strain H was also controlled biochemically using *in vitro* methods (11).

Contamination of germfree rats. The rats used were of the Swedish germfree strain and reared according to the technique described by *Gustafsson* (4). They were given semisynthetic diet D 7 and water *ad libitum* (5). Cholic acid-24-C^{14} was administered *per os* as sodium salt dissolved in saline. The rats were kept in metabolism cages and faeces were collected and frozen every 24 hours. The faeces collected for 4 days were pooled combined and analysed for the labelled compounds excreted.

The bacterial strains were grown in TH broth or Fluid Thioglycollate medium (Difco) (FT medium). Five ml of the cultures were transferred into sterile glass ampules and put into the isolator using techniques described elsewhere (4). The rats were contaminated by giving each animal 1-2 ml of the broth cultures both *per os* and *per rectum*. The remaining broth was swabbed on to the skin of the animals as well as being distributed in the diet.

Bacteriological Procedures

The strains used for experiments are listed in Table 1 together with their sources and the transformation *in vitro* of bile acids. Quantitative determinations of deconjugating and 7α-dehydroxylating microorganisms were performed on faecal samples taken at intervals as described earlier (15).

Chemical Procedures

Extraction methods. Faeces were homogenized in 40 ml water. Ethanol was added to give a final concentration of 80 per cent. and the mixture boiled for 12 hours and filtered. The filtrate was evaporated, dissolved in water, acidified to pH 1 with hydrochloric acid and extracted first with ethyl ether and then with butanol. Unconjugated bile acids and glycine conjugates of dihydroxycholanoic acids can be extracted with ether from an acidified aqueous solution, whereas glycine conjugates of trihydroxycholanoic acids and all taurine conjugates must be extracted with butanol. Therefore, the labelled material in faeces was separated into two groups according to extractibility with ether. The labelled compounds in these groups were further separated with chromatography.

Chromatographic technique. For the description of the chromatographic technique and the phase systems used for column chromatography and thin-layer chromatography (TLC), see (14).

Fractionation of labelled compounds in ether extracts. Preliminary fractionation of labelled metabolites was performed by reversed phase partition chromatography on columns with phase system C1. When 200 ml of moving phase had passed the columns, the glycine conjugates, trihydroxycholanoic acids and their keto derivatives were eluted. Dihydroxycholanoic acids and their keto derivatives were retained in the stationary phase and were rechromatographed using phase system F1. The isolated labelled fractions were further analysed with TLC to establish the identity of the labelled compounds.

Fractionation of labelled compounds in butanol extracts. The labelled taurine con-

TABLE 1

Names and Sources of Bacteria Used and Their Transformation of Bile Acids in vitro.

Bacterial strains*	Source†	Transformation of bile acids§				
		Splitting of conjugated bile acids	Removal of 7α-hydroxyl group	Oxidation of hydroxyl group at		
				C-3	C-7	C-12
Strain H	DGR	+	+	+	+	+
Bacillus cereus, 14/11 63	SBL	+	+	+	+	+
Bacillus subtilis, ATCC 6633	ATCC	+	+	+	+	+
Clostridium, G62‡	DGR	+	+	+	+	+
Clostridium perfringens, type B	RVC	+	+	+	+	+
Clostridium perfringens, CN 1451	SBL	+	+	+	+	+
Escherichia coli, 3201	WW	+	+	+	+	+
Lactobacillus casei, ATCC 7469	ATCC	+	+	+	+	+
Lactobacillus delbrückii, ATCC 9595	ATCC	+	+	+	+	+
Proteus mirabilis, 4198	WW	+	+	+	+	+
Streptococcus faecalis, M 19	SBL	+	+	+	+	+
Streptococcus faecalis, ATCC 8043	ATCC	+	+	+	+	+

* The names of the strains are those given by the reference laboratories.

† Abbreviations: *ATCC:* American Type Culture Collection, Washington DC, USA; *DGR:* Department of Germfree Research, Stockholm, Sweden; *RVC:* Royal Veterinary College, Stockholm, Sweden; *SBL:* State Bacteriological Laboratory, Solna, Sweden; *WW:* Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Oslo, Norway.

§ The transformation of different bile acids is published elsewhere (13).

‡ See (8).

jugates were separated as one group from the glycine conjugates by chromatography on columns with phase system C1. The taurine conjugates were hydrolysed in N NaOH for 6 hours at 120° C in a sealed tube. After acidification the free bile acids were extracted with ether and analysed with column chromatography and TLC as described above.

RESULTS

Establishment of Strain H

Mice. The results of the screening trial with contaminations of germfree mice are listed in Table 2. They indicated that it was not possible to establish the fastidious, obligately anaerobic 7α-dehydroxylating Strain H as a monocontaminant. Strain H might be established, however, when introduced together with a second contaminant.

TABLE 2
Establishment of Strain H in Germfree Mice.

Bacterial strains		Strain H in faeces after 14 days
Strain H		—
Strain H +	*Bacillus cereus*, 14 11 63	+
Strain H +	*Bacillus subtilis*, ATCC 6633	+
Strain H +	*Clostridium perfringens*, CN 1491	+
Strain H +	*Escherichia coli*, 3201	+
Strain H +	*Lactobacillus delbrückii*, ATCC 9595	+
Strain H +	*Proteus mirabilis*, 4198	+
Strain H +	*Streptococcus faecalis*, M 19	—
Strain H +	*Streptococcus faecalis*, ATCC 8043	—

Rats. As shown in Fig. 1, establishment of Strain H in germfree rats was not always obtained. In trials 1, 2 and 3, cultures of faeces were all negative 3 days after contamination, except in animal number 1 when a 3 day old culture in TH broth was used. However, even in this rat, all control cultures turned out to be negative after 14 days. In trials 4–7 an immediate establishment was obtained in all the animals after introduction of a 3 day old culture in TH broth, and Strain H could be recovered for several months as a monocontaminant in these animals.

Strain H was easily established in germfree rats when it was introduced with *Streptococcus faecalis*, ATCC 8043, or with *S. faecalis*, ATCC 8043, and other bacterial strains (Fig. 1, trials 8 and 9).

In another series of contamination experiments, 2 rats, placed in metabolism cages on one side of a large 6 × 6 foot jacket isolator (Fig. 2) were infected with faeces from a rat harbouring Strain H as a monocontaminant. On the other side of the isolator, 2 germfree rats were infected with a 7-day-culture of Strain H in TH broth. One uninfected animal served as a control on each side of the isolator. Strain H became established in both the animals infected with faeces and in the control rat on the same side of the isolator. However, all the animals in-

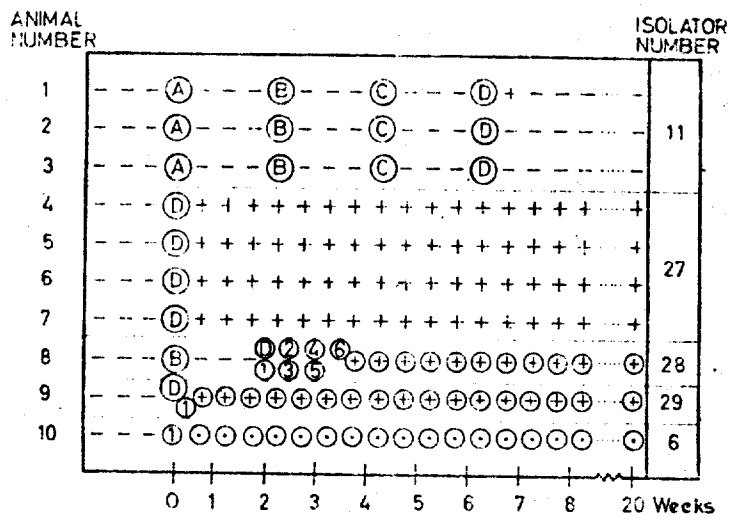

Fig. 1.

Examination of germfree rats with different cultures of Strain H either alone or together with other bacteria.

Animals no. 1-10 were voluntarily contaminated with
(A) = 7 day culture of Strain H in FT medium.
(B) = 3 day culture of Strain H in FT medium.
(C) = 7 day culture of Strain H in TH broth.
(D) = 3 day culture of Strain H in TH broth.
(1) = *Streptococcus faecalis*, ATCC 8043
(2) = *Lactobacillus casei*, ATCC 7469
(3) = *Bacillus cereus*, 14.11.63
(4) = *Clostridium perfringens*, CN 1491
(5) = *Clostridium*, G92
(6) = *Escherichia coli*, 3201

Examination of faeces was made twice a week. (−), no growth, (+) growth of Strain H, ⊙, growth of microorganisms 1-6.

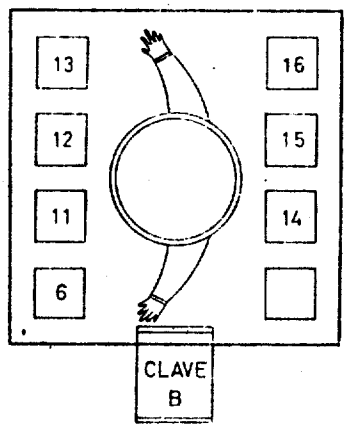

Fig. 2.

Plan of jacket isolator with metabolism cages.
Numbers refer to animals in Fig. 3.

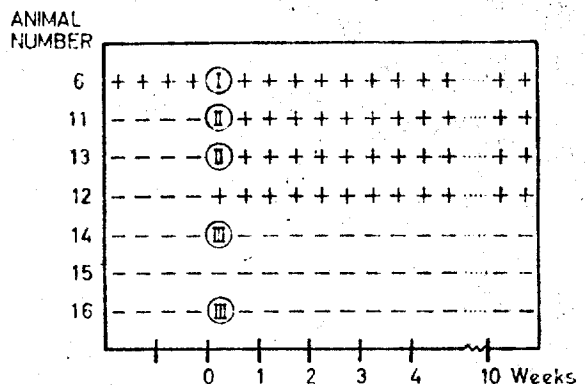

Fig. 3.

Contamination of germfree rats with Strain I in jacket isolator (Fig. 2).

(I) = Rat no. 6 (Fig. 1) with Strain II established before transfer to isolator. Animals no. 11, 12, 14 and 16 were voluntarily contaminated with

(II) = Faeces from rat no. 6.

(III) 7 day culture of Strain II in TH broth.

Examination of faeces was made twice a week.

(—) = no growth. (+) = growth of Strain II.

fected with the TH broth culture of Strain II and the control rat on the opposite side of the isolator remained germfree for 5 months, although these animals were handled by the same jacket-attired attendant as those in which Strain II had been established with the faecal culture (Fig. 2 and 3).

Quantitative Determination of 7α-dehydroxylating and Deconjugating Microorganisms

The number of the 7α-dehydroxylating microorganism, (Strain II) present in the faeces of all mono-, di-, and polycontaminated rats was of the same order of magnitude (Table 3). Strain II and the deconjugating microorganisms reached approximately the same levels (10^9-10^{10}) in the faeces of both dicontaminated and polycontaminated rats.

Transformation of cholic acid *in vivo*. Cholic acid-24-C^{14} was administered orally to conventional, germfree, mono-, di- and polycontaminated germfree rats and the nature and amounts of labelled metabolites excreted in faeces were determined. The results are summarized in Table 4.

Splitting of conjugates. The orally administered cholic acid is absorbed and appears in bile in a conjugated form, mainly as taurocholic acid. In germfree animals the bile acids are excreted in faeces as conjugates, whereas in conventional rats the faecal bile acids are mainly unconjugated due to splitting by intestinal microorganisms (6). No splitting was observed in the rat monocontaminated with the 7α-dehydroxylating bacterium (Strain II). Splitting occurred in the

three rats contaminated with bacteria known to split conjugates, *faecalis*, *Clostridium perfringens* and *Clostridium*, G62). The ex- of hydrolysis (15–25 per cent), however, was much smaller than observed in conventional rats on the same diet.

TABLE 3

Establishment of Strain H in Germfree Rats.

Contaminating bacterial strains	Animal no.	No. of bacteria per gm of faeces*	
		Deconjugating	7α-dehydroxylating
H	7	—	10^{10}, 10^{10}
H + *Streptococcus faecalis*, ATCC 8043	9	10^{9}, 10^{10}	10^{9}, 10^{10}
H + *Bacillus cereus*, 14/11 63 *Clostridium*, G62 *Clostridium perfringens*, CN 1491 *Escherichia coli*, 3201 *Lactobacillus casei*, ATCC 7469 *Streptococcus faecalis*, ATCC 8043	8	10^{9}, 10^{10}	10^{10}, 10^{10}

* separate readings 5 months after contamination.

7α-dehydroxylation. In the germfree rat with the *7α-dehydroxylating* bacterium as a monocontaminant no 7α-dehydroxylation was observed. In the polycontaminated rats, Strain H was able to 7α-dehydroxylate the formed unconjugated bile acids. No deoxycholic acid was found among the conjugated bile acids in any of these rats.

Oxidation of hydroxyl groups. In the rat monocontaminated with the 7α-dehydroxylating bacterium, analysis of the conjugates showed the presence of two labelled metabolites of cholic acid. The major metabolite had the TLC mobility of 3, 12-dihydroxy-7-keto-5β-cholanoic .

DISCUSSION

It has previously been shown that establishment of strictly anaerobic microorganisms such as *Bacteroides* or anaerobic lactobacilli often failed in germfree animals (3, 16). Our difficulties concerning the establishment of cultures of Strain H are in agreement with these observations.

Strain H was never established when 7-day-cultures in TH broth were introduced. Our findings indicate that cultivation of Strain H in TH broth for 3 days gives the best result if an establishment as a monocontaminant in the animals is achieved although this method failed in 3 out of the 7 rats investigated. Strain H was, however, easily established when accompanied by other bacteria. The fact that Strain H is not able to 7α-dehydroxylate conjugated bile acid (14) does not seem to be of any importance for its establishment, as it could easily be established in germfree mice with *Escherichia coli, Bacillus cereus,*

TAB

Faecal Metabolites of Cholic Acid in Conventional, Germfree and Ex-Germfree Rat. Polycontaminant. The Pooled Faeces excreted 0 4 Da

Animal no.	Rearing* conditions	Contaminating bacterial strains	Unconjugated bile acids as per cent of total excreted labelled bile acids
C 1	C		97
G 1	G		0
7	Ex-G	Strain H	0
Ex-G1	Ex-G	*Streptococcus faecalis*, ATCC 8043	15
9	Ex-G	Strain H *Streptococcus faecalis*, ATCC 8043	22
8	Ex-G	Strain H *Bacillus cereus*, 14 11 63 *Clostridium*, G62 *Clostridium perfringens*, CN 1491 *Escherichia coli*, 3201 *Lactobacillus casei*, ATCC 7469 *Streptococcus faecalis*, ATCC 8043	25

* Conventional (C), Germfree (G), Ex-germfree (Ex G). — Not determined.

B. subtilis and *Lactobacillus delbrückii*, all of which do not deconjugate bile acids. As organisms of such different types are effective for the establishment of Strain H, the rôle of the additional contaminants seems to be unspecific. A probable influence might be a reduction in the redox potential of the intestinal contents to levels more suitable to Strain H, which is killed *in vitro* under aerobic conditions (12).

When established alone, or together with other bacteria, Strain H could easily be recovered for several months in ex-germfree rats. Feeding with faeces from a monocontaminated rat was found to be an effective method for introducing Strain H into other animals within the same isolator. However, it is astonishing that some of the germfree rats placed within the same isolator as the monocontaminated ones (Fig. 2 and 3) remained germfree throughout the experimental period (5 months). This stresses the need of contact between animals for the propagation of certain bacteria. The conditions are similar to those that might exist in an animal room where caries-active animals harbouring caries-inducing microorganisms can be kept in the same cage-rack as inactive animals without the bacteria (2).

-dehydroxylating Bacterium (Strain II) was Established as a Mono-, Di- or inistration of Cholic Acid-24-C[illegible] was Analysed.

	e cent of labelled compounds in different fractions after chromatography of							
	excreted unconjugated bile acids				excreted conjugated bile acids after hydrolysis *in vitro*			
Unchanged cholic acid	Metabolites of cholic acid with hydroxyl or keto group at C-7	Deoxycholic acid	Metabolites of deoxycholic acid	Unchanged cholic acid	Metabolites of cholic acid with hydroxyl or keto group at C-7	Deoxycholic acid	Metabolites of deoxycholic acid
2	6	72	20	—	—	—	—
—	—		—	100	0	0	0
—	—		—	87	13	0	0
	0	0	0	100	0	0	0
5[illegible]	18	27	0	82	18	0	0
11	16	40	0	83	17	0	0

In germfree rats, bile acids are excreted in faeces in conjugated orms, mainly as taurine conjugates (1, 6). Strain II is unable to split onjugated bile acids and to form taurodeoxycholate *in vitro*, and the resent investigation demonstrates the same inability *in vivo*. An additional contamination with deconjugating microorganisms shows that Strain II is able to 7α-dehydroxylate the free bile acids formed by this second contaminant. However, transformation of the labelled cholic acid administered to different metabolites was considerably less than in conventional rats on the same diet (9). This could not be due to the fact that the bacteria had not been established in sufficient large numbers. On the contrary, the numbers of Strain II in the faeces from the ex-germfree rats in this investigation were found to be higher (10^{10}, versus 10^{1}–10^{8} per gm of faeces) than in the conventional rats on the same diet D7 (15).

Di- and polycontaminated rats have not been conventionalized with regard to the quantitative extent of deconjugation and 7α-dehydroxylation of bile acids. This has not been obtained even though the 7α-dehydroxylating bacterium in the intestinal tract of these rats has

attained equal or higher numbers as compared with those in the conventional rat on the same autoclaved diet. Other factors such as intestinal pH, redox potential, symbiosis with other intestinal microorganisms etc., may play important rôles for the extent of the intestinal microbial transformation of bile acids.

SUMMARY

A bacterium, (tentatively identified as a member of the tribe *Lactobacilleae*) with the *in vitro* ability to 7α-dehydroxylate bile acids, was established as a monocontaminant in germfree rats. Examination of the faeces of these rats after administration of cholic acid (24 C^{14}), showed that bile acid conjugates had not been split or 7α-dehydroxylated, but had been oxidized to compounds with keto groups at C 7. When deconjugating bacteria had been established in the digestive tract of germfree rats, 15–25 per cent of the excreted bile acids were unconjugated. In rats, polycontaminated with both deconjugating and 7α-dehydroxylating bacteria, 7α-dehydroxylation of the formed unconjugated bile acids was observed.

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Metabolism of deoxycholic acid in bile fistula patients

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ABSTRACT Although it has been assumed that the secondary bile acid deoxycholic acid is not rehydroxylated by the human liver, little direct evidence is available to support this assumption. To investigate the metabolism of deoxycholic acid in man, deoxycholic acid-^{14}C was given intravenously to two patients with complete external bile fistulas. After hydrolysis of the bile salts and chromatographic separation of bile acids, more than 94% of the radioactivity was found in deoxycholic acid and the remainder was scattered in several small unidentified peaks, none of which was cholic acid.

Approximately 85% of deoxycholate was excreted as glycine conjugates and 13% as taurine conjugates in this experiment. No detectable sulfate esters were found. These results indicate that the metabolism of deoxycholic acid in man involves only the reconjugation with glycine and taurine without rehydroxylation to cholic acid or sulfation.

SUPPLEMENTARY KEY WORDS conjugation · sulfation

Deoxycholic acid is one of the major bile acids derived from 7-dehydroxylation of cholic acid by bacterial enzymes in many animals, including man (1). When deoxycholic acid undergoes enterohepatic circulation in the rat, much of it is hydroxylated by the rat liver to either cholic acid (2) or 3α,6β,12α-trihydroxy-5β-cholanoic acid (3). The boid snakes hydroxylate deoxycholic acid at position 16 to give pythocholic acid (4). The rabbit, on the other hand, does not rehydroxylate deoxycholic acid (5).

In man, deoxycholate ranges from 11% to 30% of the total bile salts present in duodenal aspirates (6, 7). It has been assumed that no appreciable rehydroxylation of deoxycholate takes place in the human liver; however, there is little direct evidence to support this assumption. We therefore administered sodium deoxycholate-24-^{14}C to two patients with complete external bile fistulas to determine if this compound was converted to other products by the human liver.

METHODS AND RESULTS

Solvents and Compounds

All solvents were reagent grade or distilled just prior to use. Deoxycholic acid was purchased from Mann Research Chemicals, Orangeburg, N.Y., and recrystallized from acetone. The melting point was 174–177°C, reported 177°C (8). Taurolithocholate sulfate was received from Professor G. A. D. Haselwood, Guy's Hospital Medical School, London. Methylation of bile acids was done with diazomethane.

Labeled deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-^{14}C-oic acid) with a specific activity of 2.07 μCi/μmole was purchased from Tracerlab, Waltham, Mass., and was purified by column partition chromatography on Celite; this gave a single peak of radioactivity. When a small amount of labeled material was added to unlabeled deoxycholic acid and chromatographed on Celite, single coincident peaks were obtained from the radioactivity and from the mass of deoxycholic as measured by titration. A constant specific activity was ob-

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Systematic names of bile acids referred to in the text by their trivial names are as follows: deoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; pythocholic acid, 3α,12α,16α-trihydroxy-5β-cholanoic acid; taurolithocholate sulfate, 3α-hydroxy-5β-cholanoyl-taurine-3-sulfate; glycodeoxycholic acid, 3α,12α-dihydroxy-5β-cholanoyl-glycine; taurodeoxycholic acid, 3α,12α-dihydroxy-5β-cholanoyl-taurine.

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served when 2.58 × 10^5 dpm of ^{14}C-labeled deoxycholic acid was added to 100 mg of unlabeled deoxycholic acid and recrystallized with acetone (2.09 × 10^3 dpm/mg), ethyl acetate (2.18 × 10^3 dpm/mg), and acetone–water (2.18 × 10^3 dpm/mg). The specific activity of methyl deoxycholate prepared from the final acetone–water recrystallization was 2.31 × 10^3 dpm/mg when recrystallized from acetone–water.

Additional evidence of radiopurity was obtained by chromatography of the ^{14}C-labeled deoxycholic acid used in these studies on thin-layer chromatography. Isooctane–ethyl acetate–acetic acid 5:5:1 (Eneroth S11, Ref. 9) was used, and after development the deoxycholic acid standard was located with I_2 vapor. The silica gel was divided into six sections and counted. All of the recovered radioactivity was in the section corresponding to the deoxycholic acid standard.

Approximately 10 μCi of ^{14}C-labeled deoxycholic acid was dissolved in a small volume of ethanol (0.5 ml) and slowly injected into two patients by an intravenous saline drip.

Patients

The first patient was a 69-yr-old man who had a T tube placed in his common bile duct at the time of cholecystectomy for cholecystitis. The second patient was a 63-yr-old female who had a T tube placed in her common bile duct to relieve an obstruction due to a carcinoma of the head of the pancreas. Both patients were studied approximately 10 days after surgery; they were eating a regular diet. The first patient had normal liver function tests (bilirubin, alkaline phosphatase), and the second patient had evidence of abnormal liver function with a serum bilirubin of 2.3 mg per 100 ml/4.7 mg per 100 ml (direct/total) and a serum alkaline phosphatase of 69 K.A. units, at the time of the study. The bile fistulas were virtually complete, as judged by the absence of the secondary bile salts in the fistula bile and excretion of less than 0.5% of the administered radioactivity in the feces collected over a 6-day period after the start of the study. Presumably, in the first patient a complete biliary diversion was produced by the siphoning action of the bile in the T tube.

Collection and Analysis of Bile

Complete bile collections were obtained at intervals ranging from 2 to 12 hr; the bile was mixed with an equal volume of ethanol and stored at 5°C. Radioactivity was determined by counting in a scintillation counter using Biosolv 3 Solubilizer and Fluorally TLA Counting Mixture (Beckman Instruments, Inc., Fullerton, Calif.). The amount of quenching was estimated after the addition of an internal standard of ^{14}C-labeled toluene to the counting vials containing the bile.

TABLE 1 RECOVERY OF DEOXYCHOLIC ACID-^{14}C

Patient	dpm Given	% Recovered in Bile, First 24 hr	% Recovered in 6 days
1	2.5 × 10^7	72.3	77.6
2	1.67 × 10^7	71.7	83.0

As shown in Table 1, approximately 72% of the total radioactivity recovered in bile was excreted in the first 24 hr. Hydrolysis and chromatographic separation of the bile acids were carried out as follows. Aliquots from the fractions of bile collected during the first 24 hr were combined and evaporated to near dryness on a steam bath under a nitrogen stream. The residue was hydrolyzed with 4.5 N NaOH in a steel bomb at 130°C for 24 hr. The hydrolysate was diluted with water, adjusted to pH 1 with concentrated HCl, and extracted three times with equal volumes of diethyl ether. The evaporated ether extract was chromatographed on Celite using 70% aqueous acetic acid (v/v) as the stationary phase and increasing concentrations of benzene in petroleum ether (bp 60–70°C) as the moving phase. Radioactivity in each fraction was measured, and similar results were obtained in both patients. The distribution of radioactivity in the eluate of this column is shown for the first patient in Fig. 1. The small peak of radioactivity in the 0% benzene fraction appeared with the solvent front and probably represents radioactivity which did not equili-

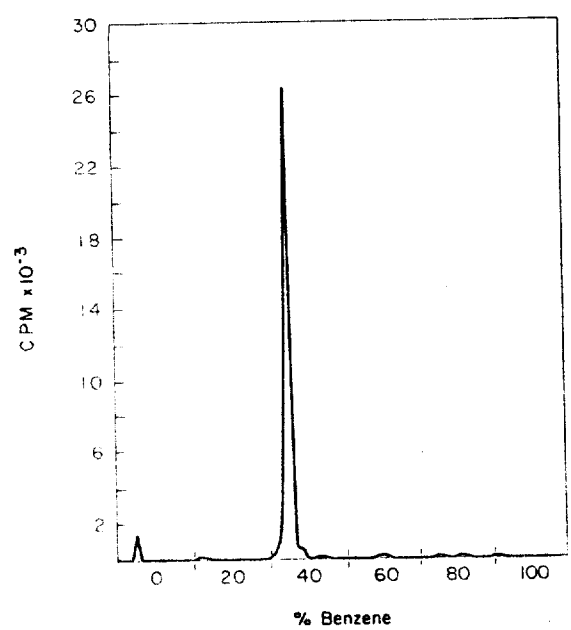

FIG. 1. Chromatographic distribution of radioactivity in bile after intravenous administration of ^{14}C-labeled deoxycholic acid. Celite column: stationary phase, 70% aqueous acetic acid; moving phase, increasing concentrations of benzene in petroleum ether. 5-ml fractions were collected.

brate with the stationary phase. Over 94% of the radioactivity placed on the column was eluted in a single peak in the 40% benzene fraction; this is the dihydroxy acid zone. The remainder of the radioactivity was distributed as very small peaks, none of which contained more than 1% of the radioactivity placed on the column.

The endogenous cholic acid in the 80% benzene fraction was titrated with 0.01 N NaOH and compared with the small amounts of radioactivity found in the fraction (Fig. 2). The small peaks of radioactivity did not appear to migrate with the mass of cholic acid.

Identification of Deoxycholic Acid

From the Celite column (Fig. 1), aliquots taken from the three tubes of peak radioactivity found in the 40% benzene fraction were chromatographed on Whatman No. 3 paper using isopropyl ether–petroleum ether (bp 60–70°C) 20:80 as solvent (10). Unlabeled deoxycholic, cholic, and chenodeoxycholic acids were used as standards. Bile acids were identified using ultraviolet light after wetting the paper with antimony trichloride (11). The paper was cut into 30 sections, each of which was placed in a vial with scintillation fluid and counted. Over 93% of the radioactivity placed on the paper was accounted for. All radioactivity migrated with deoxycholic acid, as shown in Fig. 3.

A second aliquot was taken from the same tubes, and thin-layer chromatography was carried out using isooctane–ethyl acetate–acetic acid 5:5:1 as solvent, Eneroth S11 (9). Three developments were done in the same direction to separate deoxycholic acid from chenodeoxycholic acid (12). This technique gave final R_F values of 0.64 and 0.74 for chenodeoxycholic acid and deoxycholic acid, respectively. After the last development, the silica gel was cut into sections and the section corresponding to the deoxycholic acid standard was eluted with methanol. Unlabeled deoxycholic acid (100.0 mg) was added to the eluate which contained 3.16 × 10^4 dpm. The acid was recrystallized with different solvents (Table 2) and the specific activity remained constant. The specific activity of methyl deoxycholate prepared from the material recovered from the ethyl acetate recrystallization was unchanged from the specific activity of deoxycholic acid.

Conjugation of Deoxycholic Acid

An aliquot of unhydrolyzed bile from the first patient was placed on a silica gel G plate, which was developed in isoamyl acetate–propionic acid–*N*-propanol–water 4:3:2:1 (v/v/v/v), Hofmann System II (13). Glyco- and taurodeoxycholate were used as standards. After visualization of the spots with iodine vapor, the silica gel from each of six approximately equal zones was scraped off and heated at 60°C for 60 min in 4 ml of methanol. The samples were counted in a scintillation counter after addition of 12 ml of counting fluid. Of the recovered radioactivity, 85% was found in the glycodeoxycholate zone, and 13.3% in the taurodeoxycholate zone.

In order to determine whether or not ^{14}C-labeled deoxycholate had undergone sulfation, a reaction known to occur with lithocholate (14), another aliquot of the hydrolyzed bile was chromatographed on thin-layer plates using butanol–Tris buffer–propionic acid (pH 3.

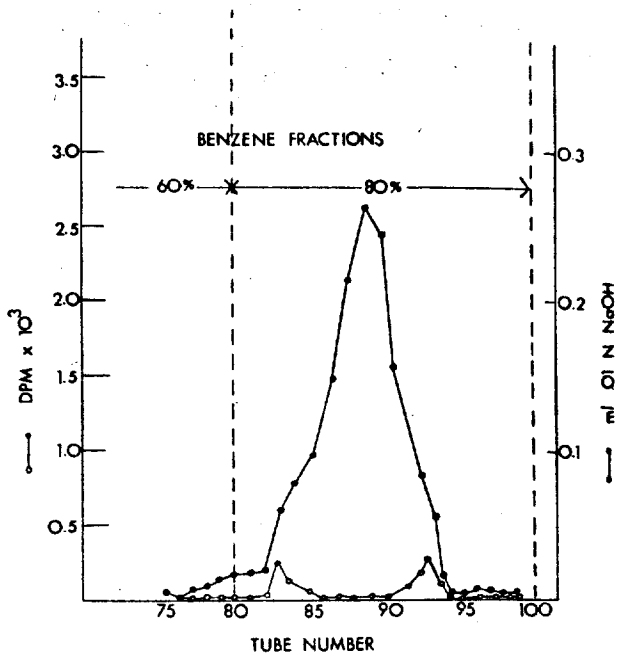

FIG. 2. Chromatography of the 80% benzene fraction of Fig. 1 (cholic acid). Titration, closed circles; radioactivity, open circles.

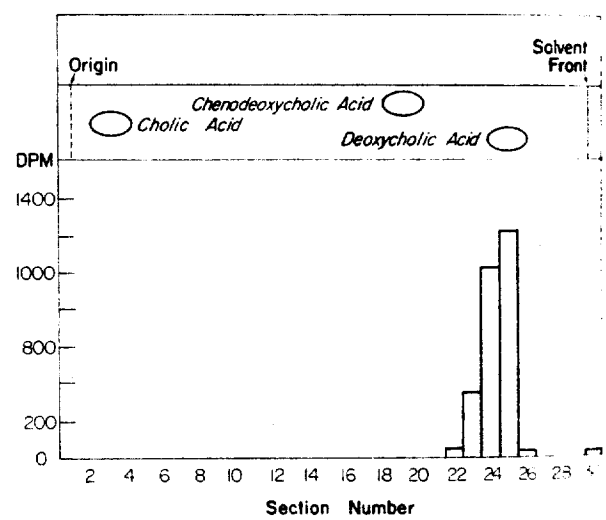

FIG. 3. Paper chromatography of an aliquot of the radioactivity found in the 40% benzene fraction of Fig. 1, along with standards chenodeoxycholic, cholic, and deoxycholic acids. The developing solvent was isopropyl ether–petroleum ether 20:80. After development and identification of the standards, the paper was cut into 30 sections and assayed for radioactivity.

TABLE 2 SPECIFIC ACTIVITY OF RECRYSTALLIZED DEOXYCHOLIC ACID

Number	Solvent	Weight	Specific Activity
		mg	*dpm/mg*
1	Acetone	49.0	282.0
2	Ethyl acetate	31.5	278.5
	Methyl deoxycholate		
3	Acetone-water	10.8	288.6

50:9.25:0.75 as the developing solvent (14). This solvent system separates the more polar sulfate conjugates from the less polar unsulfated conjugates of lithocholate. Presumably, the sulfate conjugates of deoxycholate would be even more polar than the sulfates of lithocholate. Taurolithocholate sulfate and the taurine and glycine conjugates of deoxycholate were used as standards. The plate was developed and counted as described above. All radioactivity was found in the area of glycine and taurine conjugates of deoxycholate, and no appreciable radioactivity was in the more polar zones where sulfated esters of deoxycholate would have appeared.

DISCUSSION

This study provides direct evidence that the human liver is not able to rehydroxylate deoxycholate to any significant extent. More than 94% of the administered radioactivity was excreted as conjugated deoxycholate. Lower forms of animals, such as the rat, can add hydroxyl groups at positions 6 or 7 of the sterol and excrete these in the bile as secondary bile salts. The human liver has less ability to rehydroxylate secondary bile acids. As shown in Fig. 1, there were three or four small peaks that were more polar than deoxycholate. Two of these peaks were located in the 80% benzene fraction where cholic acid was eluted from the column, but neither was cholic acid, as shown in Fig. 2.

Previous work has shown that the other major secondary bile acid in man, lithocholic acid, is also not rehydroxylated by the human liver (15). Conjugated lithocholate, however, can undergo esterification with sulfate at position 3 and approximately 50% of the lithocholate in human gallbladder bile has been demonstrated to be present as the sulfate ester (14). The possibility that conjugated deoxycholate can also undergo sulfation has not previously been investigated in man. The present study suggests that no appreciable sulfation has taken place.

Some patients with cirrhosis of the liver have been found to excrete very little deoxycholate in their bile (6, 16). One of the suggested explanations for this finding is a rapid rehydroxylation of deoxycholate to cholate in the liver (16). If this phenomenon exists, it must be peculiar to the cirrhotic liver.

We are indebted to Dr. James B. Carey, Jr. (deceased), who initiated this study.

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Studies on the Interrelationships Between Dietary Magnesium, Quality and Quantity of Fat, Hypercholesterolemia and Lipidosis[1]

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Many studies have demonstrated that the quantity and quality of the dietary fat have a profound effect upon the serum cholesterol level in experimental animals and in human beings (Portman and Stare, '59). Generally, diets high in unsaturated fats tend to lower serum cholesterol levels whereas those which are saturated result in hypercholesterolemia. The mechanism by which fats influence serum cholesterol levels is obscure.

Recent studies from this laboratory have demonstrated that high dietary magnesium has an "anti-sudanophilic" effect in cholesterol-cholic acid-fed rats. Young weanling rats fed cholesterol and cholic acid developed lipid deposition in the left ventricular valves and aorta which could be partially prevented by increasing the level of dietary magnesium (Vitale et al., '57). This "anti-sudanophilic" effect of magnesium was not correlated with differences in serum cholesterol levels. All of these studies in which the "anti-sudanophilic" effect of magnesium was demonstrated were carried out with animals fed a hydrogenated cottonseed oil[3] as the sole source of fat.

The present experiment deals with the interrelationships between the amount of either a relatively saturated or an unsaturated dietary fat and varying levels of dietary magnesium upon the serum magnesium and cholesterol levels, vascular sudanophilia and other parameters of interest.

METHODS

Experiment 1. Young albino male rats, weighing 50 to 55 gm, obtained from the Charles River Breeding Laboratories, were fed a diet of the following constituents per hundred grams of diet: casein (purified), 10; glucose, 73.1; salt mixture, 5.0; celluflour,[4] 5.0; $CaCO_3$, 1.5; choline chloride, 0.3; a vitamin A, D and E mixture, 0.1; and fat, 5. The fats used were a hydrogenated cottonseed oil[5] (HCSO) or corn oil[6] (CO). When cholesterol and cholic acid as well as the higher level of fat (20%) were added to the diet, this was done at the expense of the glucose, gram per gram. The salt mixture was that of Jones and Foster ('42) with the calcium carbonate and magnesium sulfate removed. The vitamin A, D and E mixture contained 5 gm of halibut liver oil,[7] 1 gm of α-tocopheryl acetate and 34 gm of corn oil. The amounts of the water-soluble vitamins used in the diets have been listed elsewhere (Vitale et al., '57). Cholesterol was added at a level of 1.0% and cholic acid at 0.3%. The magnesium was added to the diet in the form of MgO to supply 24, 96 and 192 mg of magnesium per hundred gm of diet. The animals were fed ad libitum and housed in individual cages. At the end of 28 days, 6 animals from each group were killed and serum cholesterol levels determined by the method of

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[2] Present address: Kyushu University Medical School, Fukuoka, Japan.

[3] Spry.

[4] Cellu Flour, Chicago Dietetic Supply House.

[5] See footnote 3.

[6] Mazola.

[7] Haliver oil.

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Carpenter et al. ('57). Total serum protein was determined by the standard Kjeldahl method. Serum proteins and lipoproteins were determined by paper electrophoresis according to the method described by Moinat and Tuller ('57, '58). Serum magnesium was determined by the method of Orange and Rhein ('51).

Sections of kidney, heart and lesser curvature of the stomach from each animal were fixed in 10% formalin and then stained with hematoxylin and eosin. After the heart and aorta were opened so as to expose the left ventricular valves and aortic intima, they were fixed in formalin, stained with Sudan IV and graded as to the extent of sudanophilia of left ventricular valves and aorta (heart score). The left kidney of each animal was decapsulated and weighed. The extent of the microscopic calcium deposition in tubular lumens, mainly in the zona intermedia, the outer portion of the medulla, of the kidney was scored on the basis of zero to +4 (kidney calcium score) (Hellerstein et al., '57).

Experiment 2. At a later date, two groups of rats housed in individual cages were fed a diet containing 20% of corn oil but with the dietary cholesterol and cholic acid at considerably higher levels, 3 and 1%, respectively. Dietary magnesium was supplied at 24 or 192 mg/100 gm. The remainder of the diet and the methodology for serum cholesterol and "heart score" were the same as in experiment 1.

RESULTS

Experiment 1. Tables 1A and 1B illustrate the results of variations in the amount and kind of dietary fat and magnesium, with or without added cholesterol and cholic acid. The rate of weight gain appeared to be independent of the quality and quantity of fat fed and of the dietary level of magnesium. The addition of cholesterol and cholic acid to the diet resulted in decreased rates of weight gain. With the exception of the high-corn-oil group, the addition of cholesterol and cholic acid to the diet resulted in significantly lower serum magnesium levels when the dietary magnesium was 24 mg/100 gm. Increasing the level of dietary magnesium to 96 or 192 mg/100 gm resulted in a rise in the serum magnesium to approximately 2 mg/100 ml.

In the groups not consuming cholesterol-cholic acid containing diets (table 1A), increasing the level of dietary magnesium from 24 to 192 mg/100 gm resulted in decreased serum cholesterol levels in the groups fed 5 and 20% of HCSO. No such effect of magnesium was noted in either of the groups fed corn oil. The serum cholesterol levels of the groups fed 5% of HCSO were significantly lower than those of the groups fed 5% of CO, at each level of dietary magnesium. On the other hand, there was no difference in the serum cholesterol levels at any level of magnesium between the groups fed 20% of HCSO and 20% of CO.

When the animals were made hypercholesterolemic by the inclusion of cholesterol and cholic acid in the diet (table 1B), no consistent effects of magnesium upon the serum cholesterol levels were apparent. The serum cholesterol level in group 10 was significantly lower than the levels in groups 8 and 22, and the level in group 24 was significantly higher than in group 20 or 22. There are no consistent trends to suggest a logical interpretation. With the exception of group 10 and 24, the effects of HCSO and CO were those expected from previous work; i.e., increasing the level of the hydrogenated oil from 5 to 20% usually resulted in increased hypercholesterolemia; the reverse was true with CO, and animals fed CO had lower cholesterol values than those fed the HCSO.

The "anti-sudanophilic" effect of dietary magnesium was seen only in the groups fed the diets containing HCSO. At the 5% level of HCSO, the heart score of the animals in the group fell from 9 to 6 as the level of dietary magnesium was raised from 24 to 192 mg/100 gm. At the high level of HCSO, the heart score fell from 6 to 4 with increased dietary magnesium.

At the low level of dietary magnesium (24 mg%), rats fed the 20% HCSO diet had significantly higher serum cholesterol levels than rats fed the 5% HCSO diets. It should be emphasized, however, that the group fed 20% of HCSO had a significantly lower heart score ($P < 0.05$) than the 5% HCSO group. These observations hold true for the high level of dietary magnes-

TABLE 1A

Effect of dietary fat and magnesium upon serum cholesterol, kidney profile and lipidosis of heart valves and aorta (no cholesterol or cholic acid added)

Group[1]	Dietary		Weight gain	Serum magnesium	Serum cholesterol	Kidney		Heart score
	Fat	Magnesium				Calcium score	KW/BW	
		mg/100 gm	*gm*	*mg/100 ml*	*mg/100 ml*		%	
1	5% HCSO[2]	24	78	1.08	105	1.2	0.48	0
3	5% HCSO	96	66	2.21	99	0	0.37	0
5	5% HCSO	192	71	2.56	83	0	0.38	0
7	20% HCSO	24	65	1.51	115	1.0	0.48	0
9	20% HCSO	96	78	1.89	113	0.2	0.38	0
11	20% HCSO	192	76	2.09	97	0	0.38	0
13	5% CO[3]	24	64	1.25	127	0.9	0.48	0
15	5% CO	96	63	2.03	138	0.2	0.40	0
17	5% CO	192	60	1.91	116	0.2	0.36	0
19	20% CO	24	76	0.96	115	1.0	0.50	0
21	20% CO	96	51	1.99	123	0	0.46	0
23	20% CO	192	76	1.95	102	0	0.38	0

[1] Six rats per group (28-day experiment).
[2] HCSO = Hydrogenated cottonseed oil.
[3] CO = corn oil.

TABLE 1B

Effect of dietary fat and magnesium upon serum cholesterol, kidney profile and lipidosis of heart valves and aorta (cholesterol and cholic acid added)

Group	Dietary		Weight gain	Serum magnesium	Serum cholesterol	Kidney		Heart score
	Fat	Magnesium				Calcium score	KW/BW	
		mg/100 gm	*gm*	*mg/100 ml*	*mg/100 ml*		%	
2	5% HCSO	24	56	0.88	724	0.8	0.54	9.0
4	5% HCSO	96	46	2.29	794	0.2	0.44	8.8
6	5% HCSO	192	51	2.22	821	0	0.41	6.2
8	20% HCSO	24	44	1.08	1086	0.8	0.50	6.0
10	20% HCSO	96	46	2.08	653	0	0.41	3.8
12	20% HCSO	192	53	1.91	1085	0	0.40	4.1
14	5% CO	24	42	0.98	397	0.8	0.58	3.6
16	5% CO	96	61	1.89	341	0.5	0.43	2.8
18	5% CO	192	53	2.15	385	0	0.40	5.3
20	20% CO	24	53	1.11	210	0.6	0.44	2.0
22	20% CO	96	45	1.99	270	0	0.42	2.3
24	20% CO	192	58	2.14	355	0	0.43	2.3

ium (192 mg/100 gm) groups, although the decrease in heart score was not statistically significant ($0.1 > P > 0.05$).

No "anti-sudanophilic" effect of magnesium was observed in the CO, cholesterol-cholic acid-fed groups. The overall average heart score for the low CO groups was 4. Increasing the level of CO to 20% resulted in a lower heart score of two regardless of the level of dietary magnesium.

The kidneys of the low dietary magnesium groups appeared speckled, pale, edematous and swollen. Increasing the level of dietary magnesium resulted in smaller kidneys which in the gross appeared normal. These gross signs of changes in kidney morphology appeared to be pronounced in the cholesterol-cholic acid-fed groups. Associated with this difference in gross appearance, there was a drop in the percentage of the body weight represented by the left kidney as the dietary magnesium was increased; i.e., group 1 versus group 3. The increased kidney weight-body weight ratio in the low-magnesium-fed animals did not appear to be a function of final weight or weight gain. In the group consuming 5% of HCSO and cholesterol and cholic acid, the weight gains of the low- and high-magnesium groups were similar but the kidney weight-body weight ratio was 0.54 at the low level of dietary magnesium and 0.41 at the high level of dietary magnesium. Thus, there was an absolute increase in kidney weight with low dietary magnesium. The absolute and relative drop in kidney weight with increased dietary magnesium was seen in all the groups with the exception of those animals consuming 20% of CO, cholesterol- and cholic acid-containing diets.

The microscopic appearance of the kidneys of the groups consuming high magnesium was always clearly differentiated from that of the low-magnesium-fed groups. This is expressed by the "calcium score." This score was approximately one in the animals fed a diet containing 24 mg/100 gm of magnesium and zero at the higher levels of magnesium. The histologic appearance of the kidneys and the deposition of fat in the aortic media have been described previously (Hellerstein et al., '57). A few animals among the various groups consuming low-magnesium diets (24 mg/100 gm) showed metastatic calcification of the gastric subserosal small arteries. A variable but small minority of rats fed the low magnesium diets have shown such calcification in our previous acute experiments.[8]

Tables 2A and 2B illustrate the effect of the type and level of fat and varying levels of magnesium on total serum protein and protein fractions as determined by paper electrophoresis. In general, in the HCSO-fed groups, the albumin levels decreased with the inclusion of cholesterol and cholic acid in the diet. Dietary cholesterol and cholic acid tended to increase the α-1 and β-globulin group (e.g., group 4 versus group 3).

Increasing the level of dietary magnesium resulted in decreased levels of both α- and β-lipoproteins in groups fed 20% of CO but not in the 5% CO, cholesterol-free groups. This effect of dietary magnesium was also noted in the 20 and the 5% HCSO group, although the absolute values were higher in the former.

The α- and β-lipoproteins were determined by paper electrophoresis in the cholesterol-cholic acid-fed rats but are not reported because of serious questions of methodology (see discussion section).

In experiment 2, increasing the level of dietary magnesium decreased the heart score from 5.2 to 2.9 ($P < 0.05$), at serum cholesterol levels of 440 and 385 mg/100 ml ($P > 0.05$) respectively. The weight gains of both groups of rats were identical.

Although experiments 1 and 2 were done at different times, it might be illegitimate to compare results of each.

The data in table 3 demonstrate, when compared with those in table 1B, that increasing the level of dietary cholesterol and cholic acid to 3 and 1%, respectively in 20% CO diets, resulted in an increased serum cholesterol and heart score in the group fed 24 mg/100 gm magnesium diets but not in those animals fed the 192 mg/100 gm magnesium diets. The rats fed a 20% CO diet containing 1% of cholesterol and 0.3% of cholic acid and 24 mg/100 gm of magnesium had a serum cholesterol level of 210 and a heart score of 2.0 (table 2B). The animals receiving the dietary cholesterol and cholic acid at 3 and 1%

[8] Unpublished observations.

TABLE 2A

Effect of dietary magnesium and quality and quantity of fat on serum protein and lipoprotein fractions (no cholesterol or cholic acid added)

Group	Dietary		Total protein	Albumin	Globulins				Serum lipoprotein[1]	
	Fat	Magnesium			α_1	α_2	β	γ	α	β
		mg/100 gm	%	%	%	%	%	%	*cm²*	*cm²*
1	5% HCSO	24	6.4	3.1	0.7	0.6	1.4	0.7	9.7	11.7
3	5% HCSO	96	6.1	2.9	0.9	0.5	1.1	0.7	8.5	10.3
5	5% HCSO	192	5.8	2.8	0.9	0.6	1.0	0.6	4.1	4.8
7	20% HCSO	24	6.3	2.8	1.1	0.6	1.2	0.7	15.9	11.8
9	20% HCSO	96	6.0	2.5	0.8	0.6	1.2	0.8	16.3	12.6
11	20% HCSO	192	5.9	2.6	0.8	0.6	1.2	0.8	7.7	6.3
13	5% CO	24	6.0	2.5	1.0	0.5	1.4	0.7	6.9	8.8
15	5% CO	96	6.3	2.9	0.9	0.6	1.3	0.7	10.0	10.6
17	5% CO	192	6.1	2.5	0.9	0.6	1.3	0.7	7.3	7.7
19	20% CO	24	5.8	2.3	0.9	0.6	1.4	0.7	8.1	8.5
21	20% CO	96	6.2	2.8	0.9	0.6	1.1	0.8	6.9	5.4
23	20% CO	192	5.7	2.3	0.9	0.5	1.0	0.8	4.0	4.8
Average			6.05	2.66	0.891	0.58	1.2	0.73		

[1] These values represent the total stainable lipid of the α- and β-lipoproteins determined by paper electrophoresis.

TABLE 2B

Effect of dietary magnesium and quality and quantity of fat on serum protein (cholesterol and cholic acid added)

Group	Dietary		Total protein	Albumin	Globulins			
	Fat	Magnesium			α_1	α_2	β	γ
		mg/100 gm	%	%	%	%	%	%
2	5% HCSO	24	6.7	2.8	1.1	0.6	1.4	0.8
4	5% HCSO	96	7.0	2.7	1.1	0.7	1.5	1.0
6	5% HCSO	192	6.6	2.6	1.1	0.7	1.4	0.9
8	20% HCSO	24	6.6	2.5	1.1	0.6	1.5	0.8
10	20% HCSO	96	6.3	2.4	1.1	0.6	1.4	0.8
12	20% HCSO	192	6.0	2.3	1.0	0.5	1.4	0.7
14	5% CO	24	6.7	2.6	1.2	0.6	1.7	0.7
16	5% CO	96	6.0	2.4	1.1	0.6	1.3	0.6
18	5% CO	192	6.3	2.5	0.9	0.6	1.5	0.8
20	20% CO	24	6.6	2.3	1.3	0.7	1.5	0.8
22	20% CO	96	6.0	2.3	1.0	0.6	1.3	0.7
24	20% CO	192	6.2	2.1	1.1	0.7	1.5	0.7
Average			6.42	2.46	1.09	0.62	1.45	0.78

TABLE 3

The effect of feeding 3% of cholesterol and 1% of cholic acid on serum cholesterol and heart score

Dietary		Weight gain	Serum cholesterol	Heart score
Magnesium	Fat			
mg/100 gm		*gm*	*mg/100 ml*	
24[1]	20% CO	46	440	5.2
192[1]	20% CO	46	385	2.9

[1] Fifteen rats per group.

respectively, had a significant increase in the serum cholesterol (440 mg/100 ml) and heart score (5.2). On the other hand, there was no effect of the increased dietary cholesterol and cholic acid either on serum cholesterol (387 mg/100 ml) or heart score (2.9) when the diet contained 192 mg/100 gm of magnesium.

DISCUSSION

High dietary magnesium may be "anti-sudanophilic" in the rat only where the serum cholesterol level is markedly elevated, somewhere in the range of 400 mg/100 ml or above, and where the heart score is high (above 3). In a recent study (Vitale et al., '57), in which thyroxine was fed to animals consuming a 10% protein and cholesterol-cholic acid containing diet, the low heart scores (approximately two) were not reduced further by the addition of dietary magnesium. Again the serum cholesterol level concentrations were below 400 mg/100 ml. This finding held in the present study in which the "anti-sudanophilic" effect of high dietary magnesium was observed only in those groups of animals in which the level of serum cholesterol was above 400 mg/100 ml. As in previous experiments (Vitale et al., '57; '59), the "anti-sudanophilic" effect of magnesium was not correlated with serum cholesterol levels.

The effect of CO on lowering serum cholesterol levels in humans and in animals is well known. However, not many studies have been conducted in which the effect of low and high levels of corn oil on the serum cholesterol levels have been studied. Recently, Hegsted et al. ('59) studied the interrelationships between the kind and amount of dietary fat and dietary cholesterol in experimental hypercholesterolemia. These investigators found that when the level of CO was 20% of the diet in which 0.45% of cholesterol and 0.45% of cholic acid had been added, there was, after 4 weeks, a significantly lower serum cholesterol level of 162 mg/100 ml compared with 310 mg/100 ml for the 5% CO-fed group. The difference between the high serum cholesterol levels seen in the present experiments (table 1B) and those recorded by Hegsted et al. ('59) is explained in part by the size of the animals used in their experiments. (In the present experiments 21-day-old male rats were used, whereas in those experiments recorded by Hegsted et al. ('59) young adult male rats, weighing 250 to 300 gm, were used.) These investigators further found that when the cholesterol was fed at a level of 1.35%, the cholic acid being held at 0.45%, there was no apparent effect upon serum cholesterol when the higher level of CO was fed (20 versus 5%).

In the present study, increasing the level of dietary cholesterol and cholic acid in animals fed 20% of CO resulted in no apparent increase in serum cholesterol of the high dietary magnesium group. There was, however, a significant rise in serum cholesterol of the rats fed low dietary magnesium. It is apparent that the level of cholesterol and cholic acid as well as the level of CO affects the serum cholesterol level. As Hegsted et al. ('59) have pointed out, the effect of most of these variables may be definable only in relation to many or all of the other variables.

In our previous studies (Vitale et al. '57; '59) and in this study, the "anti-sudanophilic" effect of high dietary magnesium in rats fed 20% of HCSO was not associated with a decrease in the hypercholesterolemia. In the present experiment, there was, at a constant level of magnesium (24 mg/100 gm), a higher lipidosis with 5% of HCSO than with 20% of HCSO, although the serum cho-

sterols were significantly higher with the 20% HCSO group.

Paper electrophoresis does not appear to be an adequate method for quantitating α- and β-lipoproteins in hyperlipemic, hypercholesterolemic serum. In our hands, the amount of lipid stained, quantitated by the use of a photoelectric densitometer, is not proportional to the amount of serum used to spot the paper. Secondly, reproducibility is extremely poor when hyperlipemic and hypercholesterolemic serum is used. Serum lipoproteins determined in animals fed diets devoid of cholesterol and cholic acid do not present these problems. Perhaps if one were to dilute the serum of cholesterol-cholic acid-fed animals with "mock serum" to a point where the concentrations of the lipoproteins were of the same order of magnitude found in the control animals, the values obtained might be more reliable and meaningful.

The α- and β-lipoproteins of the cholesterol-cholic acid-fed animals in the present experiment ranged from 2.5 to 7.5 and from 25 to 50 cm,² respectively, regardless of the fat and level of magnesium. The values for these fractions are of the same order of magnitude previously reported (Vitale et al., '57).

Despite the unreliability of the method, the data would indicate that the inclusion of cholesterol and cholic acid in the diet resulted in decreased α- and increased β- lipoproteins. The results of the present and previous experiment (Vitale et al., '57), in which high dietary magnesium lowered the α- and β-lipoproteins in non-cholesterol-cholic acid-fed animals, suggest that one mechanism through which magnesium might exert an "anti-sudanophilic" effect could be related to lipoprotein metabolism. The results of an experiment dealing with the effects of dietary magnesium on serum lipoproteins determined by the ultracentrifuge method will be reported.

Many factors play an important role in the production of hypercholesterolemia; so, too, the "anti-sudanophilic" effects of thyroxine, magnesium, or other compounds may well be affected by the level and type of dietary fat consumed as well as by the quality and quantity of other nutrients.

The variation in vascular sudanophilia caused by various dietary constituents may rest not only on their influence upon serum lipids but also on their effects on the vascular wall. There are investigators, a minority, who believe that the primary lesion in arteriosclerosis or atherosclerosis lies within the media and is related to changes in the elastica or in the metabolism of mucopolysaccharides or collagen (Gillman et al., '55). Gillman ('59) has demonstrated changes in mucopolysaccharide metabolism in human atherosclerotic aortas and suggested the appearance of "abnormal" mucopolysaccharides. Shimamoto et al. ('59) demonstrated in the rabbit that infusion of large bacterial polysaccharides result in arteriosclerosis which could be prevented by the addition of dietary magnesium. In limited studies from this laboratory, we have observed changes in mucopolysaccharides from magnesium-deficient animals. Studies are now underway to test the effect of magnesium deficiency and excess upon mucopolysaccharides and collagen formation in normocholesterolemic and hypercholesterolemic animals.

SUMMARY

Young weanling rats were fed one of several diets which varied in the amount of magnesium (24, 96 or 192 mg/100 gm) and corn oil or a hydrogenated cottonseed oil (HCSO) (5 or 20%) added. The calcium content of the diet was 600 mg/100 gm. When used, cholesterol and cholic acid were added at one and 0.3%, respectively. The experimental period was 28 days. Increasing the level of HCSO from 5 to 20% usually resulted in increased hypercholesterolemia in rats fed cholesterol and cholic acid, whereas lowered cholesterol values were observed in rats fed a similar diet containing corn oil. The hypercholesterolemic response to cholesterol and cholic acid feeding was less marked in the animals fed corn oil. The "anti-sudanophilic" effect of high dietary magnesium was not related to decreased serum cholesterol concentrations. High dietary magnesium appeared to be "anti-sudanophilic" only when the serum cholesterol level was elevated and somewhere in the range of 400 mg/100 ml or above. Thus, no "anti-sudanophilic" effect of magnesium was noted in animals fed corn oil (5 or 20%) and 1% of cholesterol—0.3% of cholic acid. However, high magnesium

was "anti-sudanophilic" with 20% of corn oil when the serum cholesterol level was increased by feeding higher levels of cholesterol and cholic acid. The relationship of hypercholesterolemia and high dietary magnesium to lipidosis is discussed.

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THE TOXIC FACTOR IN BILE

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That bile in sufficient quantities is toxic by the oral, the subcutaneous, the intravenous and the intraperitoneal routes is well established. The pathological physiology attendant upon clinical and experimental obstructive jaundice is not so clearly referable to bile itself, because of the probable impairment of the liver and the absence of bile in the intestinal tract. The peritonitis and death following the introduction of large quantities of bile in the peritoneal cavity have been ascribed to bacteria as well as to the toxic constituents in the bile itself. We (1) have recently reported experiments showing that sterile bile in sufficient quantity in the peritoneal cavity produces all the symptoms of bile peritonitis.

On experimental evidence, toxic actions have been ascribed to the following bile constituents: the pigments, the cholesterol and the bile acids. We will refer only to the most recent and significant of these reports. Bouchard (2), King and Stewart (3), King, Biglow and Pearce (4) and de Bruin (5) conclude that the pigment acts as the toxic agent in bile or is more toxic than the other constituents. On the other hand, Raywoch (6) and Gilbert and Herscher (7) believe that if the pigment has any poisonous action at all it is very slight. Danilewsky (8) and Flint (9) attribute a toxic action to cholesterol. Fasciani (10) was unable to confirm this, even though he injected large amounts. Rohrig (11), Traube (12), de Bruin (5) and Sellards (13) attribute various poisonous actions to the bile acids. Landois (14), King and Stewart (3) and others conclude that the bile salts are either feeble poisons or non-poisonous. Boisson (15) thought that the toxicity of bile was due to "impurities," which could be removed by filtering.

Intraperitoneal injections of whole gall-bladder bile of dogs in dogs in amounts of 5 cc. or more per kilogram caused the death of all dogs within twenty-four hours. In smaller quantities the effects were variable, but when less than 3 cc. per kilogram was used, all the dogs lived. The symptoms and autopsy findings are those typical of bile peritonitis, viz., vomiting, diarrhea, anuria or albuminuria, bradycardia, hypotension, irregular and slow respiration, coma, in many instances edema and slight jaundice,

bloody fluid in peritoneal cavity, petechia of brain, lungs, stomach and intestines, and congestion of liver, kidneys and spleen.

A quantity of dog gall-bladder bile was divided into three portions. The first portion was sterilized in the autoclave at twenty pounds' steam pressure for thirty minutes. This sterilization was repeated three successive days before the bile was used. This bile was filtered before using intravenously, to remove the coagulated material which was caused by heating. The second portion was frozen for three days before use. The third portion was evaporated to dryness on a steam bath, then in an oven at 100°C. to constant weight. Alcohol (95 per cent) extracts were made of one-half of this and again evaporated to dryness and then dissolved in water ready for injection. Water extracts were made directly from the other half of the dried bile. All of these extracts were made up in volume to the strength of the original bile. The alcoholic extractions could not be made quantitatively, probably due to the difference in solubility of the bile salts in alcohol, and also the heating caused coagulation of the protein, so after alcoholic or water extraction of dried bile there remained an insoluble residue.

Fig. 1. Dog weighing 12.8 kgm. was injected intravenously with 12.8 cc. of whole gall-bladder bile from dog.

Resp.—Respiration. *B. P.*—Blood pressure. *B. L.*—Base line.

The figure shows the effect of bile on the respiration, giving the typical Cheyne-Stokes type, the respiration period begins when the heart stops, rapid fall in blood pressure and practical cessation of heart action, which returns after a few seconds.

This dog was anesthetized with ether and then given an intravenous injection of sodium-barbital, 0.250 gram per kgm. of body weight.

Fig. 2. In the first part of this experiment, a saturated solution of bilirubin in N/10 sodium hydroxide, neutralized to litmus paper with N/10 hydrochloric acid, was injected intravenously. Second injection of 30 cc. of the same solution did not cause any change in the heart rate. A later injection of a saturated solution of bilirubin in sodium-carbonate 1 per cent, 25 cc., had no effect on heart or lungs.

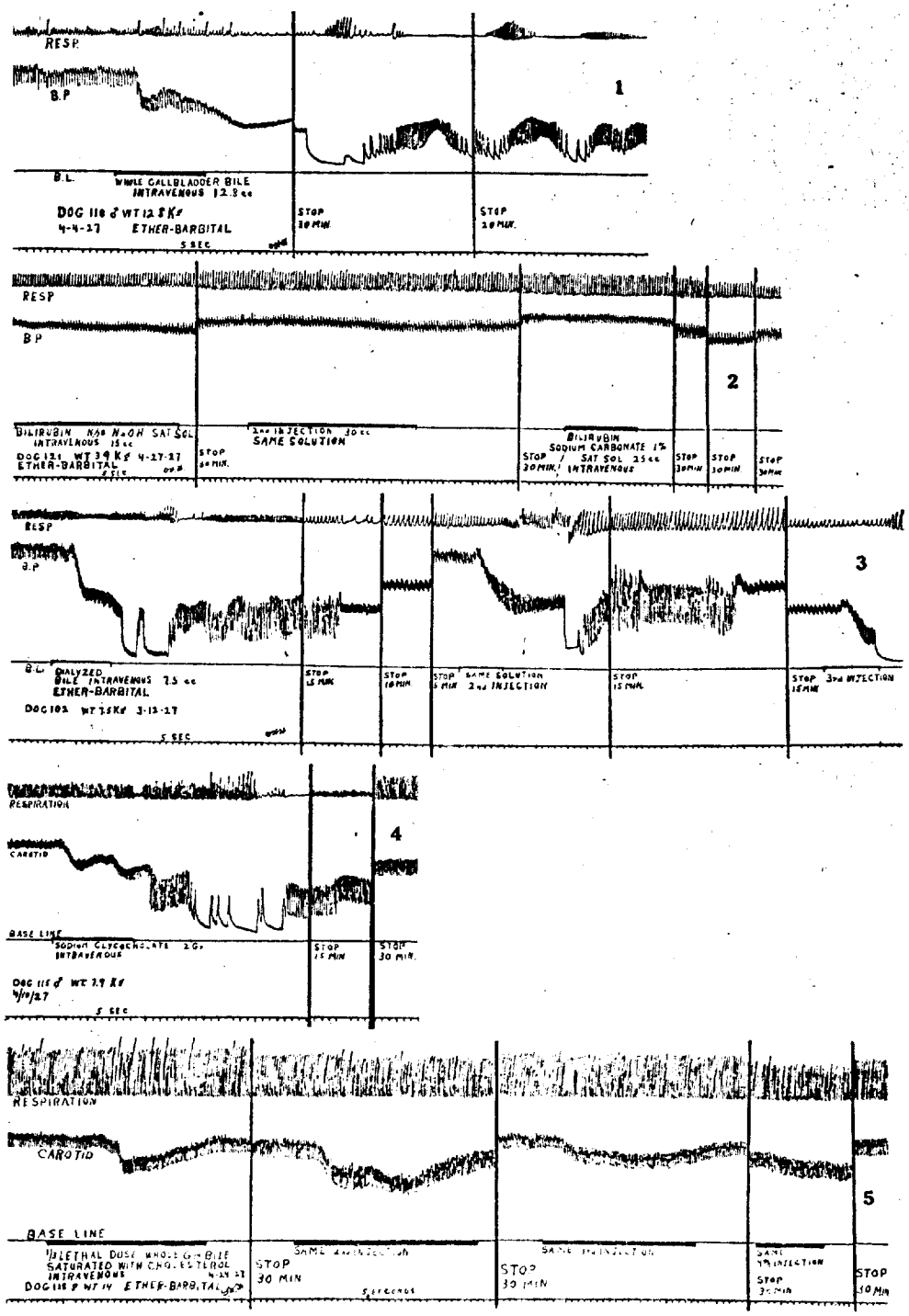
Fig. 3. Dialyzed bile, which was made up to the original concentration of dog gall-bladder bile, was injected intravenously. It produced the same results quantitatively as the whole gall-bladder bile.

Non-dialyzable portion of bile when injected did not cause any change in the heart rate, blood pressure or respiration.

An interesting instantaneous change in the amplitude of the heart beat can be seen in the tracing at two different places.

Fig. 4. Sodium glycocholate, calculated dosage similar to the total crystalloid content of bile was dissolved in distilled water. When injected intravenously it produced the same results quantitatively as whole gall-bladder bile.

Fig. 5. This tracing shows the protective action of cholesterol against bile. Four intravenous injections of whole gall-bladder bile saturated with cholesterol were given, which is much greater than the lethal dose of bile, and still the blood pressure 30 minutes after the last injection is practically normal. This was followed for two hours and there was no indication of cardiac failure. The same amount of bile injected without cholesterol would have killed the dog before the fourth injection.


RESP.
B.P
1
B.L.
WHOLE GALLBLADDER BILE
INTRAVENOUS 12.8 cc
DOG 116 ♂ WT 12.8 Kg
4-4-27
ETHER-BARBITAL
5 SEC
STOP 30 MIN
STOP 20 MIN.
RESP
BP
2
BILIRUBIN NaO NaOH SAT SOL
INTRAVENOUS 15 cc
DOG 121 WT 3.9 Kg 4-27-27
ETHER-BARBITAL
STOP 30 MIN.
2nd INJECTION 30 cc
SAME SOLUTION
BILIRUBIN
SODIUM CARBONATE 1%
SAT SOL 25 cc
STOP 30 MIN.
INTRAVENOUS
STOP 30 MIN
STOP 30 MIN
STOP 30 MIN
RESP
B.P
3
B.L.
DIALYZED
BILE INTRAVENOUS 7.5 cc
ETHER-BARBITAL
DOG 102 WT 7.5 Kg 3-13-27
5 SEC
STOP 15 MIN
STOP 10 MIN
STOP 15 MIN
SAME SOLUTION
2nd INJECTION
STOP 15 MIN
STOP 15 MIN
3rd INJECTION
RESPIRATION
CAROTID
4
BASE LINE
SODIUM GLYCOCHOLATE 2 Gr
INTRAVENOUS
DOG 115 ♂ WT 7.9 Kg
5 SEC
STOP 15 MIN
STOP 30 MIN.
RESPIRATION
CAROTID
5
BASE LINE
½ LETHAL DOSE WHOLE GALL BILE
SATURATED WITH CHOLESTEROL
INTRAVENOUS
DOG 118 ♂ WT 14 ETHER-BARBITAL
SAME 2nd INJECTION
STOP
30 MIN
5 seconds
SAME 3rd INJECTION
STOP
30 MIN
SAME 4th INJECTION
STOP
30 MIN
STOP
30 MIN

Figs. 1–5

Sterilized and frozen bile were each injected intraperitoneally in five dogs. The symptoms and autopsy findings were the same as those found in the use of fresh bile. The toxicity value remained unchanged.

The evaporated alcoholic extract of dried bile redissolved in water and made up to the original strength of the bile caused the death of a dog in three days when 5 cc. per kilogram were used subcutaneously. This amount of fresh, frozen or sterilized bile kills in less than twenty-four hours. Eight cubic centimeters of this extract per kilogram were required to kill four dogs in twenty-four hours when given intraperitoneally. Apparently some of the toxic material was either lost in the drying or in extracting with alcohol. The residue left from alcoholic extraction was partly dissolved in water and the rest held in suspension and injected intraperitoneally. It required 10 cc. per kilogram to cause death. Three dogs were injected, one with 5 cc., the second with 8 cc., and the third with 10 cc. The first two lived and the third died within 24 hours. This suspension would cause embolism if used intravenously.

Water extracts of dried bile caused typical symptoms and autopsy findings of bile peritonitis and quantitatively accounted for the toxic portion of bile. The residue from the water extractions was put in suspension and injected intraperitoneally only, without any toxic symptoms, even when 20 cc. per kilogram were used.

Subcutaneous injection of bile in ten dogs caused more variable effects than the intraperitoneal. Dogs receiving more than 4 cc. per kilogram died within twenty-four hours, and some of the dogs receiving as low as 2 cc. per kilogram died within the same time. The process of absorption in many instances seemed to be interfered with by excessive edema at the site of injection. Almost immediately following the injection of bile the dogs vomited and appeared thirsty, lapping water repeatedly, only to emit it almost as soon as taken. In a few hours the dog became groggy; respiration and heart slow and irregular; urine, if any, contained albumin; pleural and peritoneal cavities contained fluid; petechia of intestines, stomach, omentum, brain and lungs; marked congestion of liver, kidneys and spleen. Dogs receiving less than 2 cc. per kilogram had large necrotic areas at the sites of injection. These areas were very resistant to healing.

Intravenous injection of whole gall-bladder bile caused variable symptoms, depending on the amount injected and particularly on the rate of injection. A small amount injected rapidly would kill the dog while a much larger quantity given slowly caused only transient symptoms. Seven dogs were injected intravenously, five with varying quantities, and two with divided doses repeated every two hours. The dogs tolerated the bile better when given at intervals, but even when a total of 5 cc. per kilogram was given, the general condition of the dog gradually grew worse and an intervening infection caused fatal termination. Three of the five dogs

which lived longer than one week died with pneumonia; one dog lived eight days. Both dogs dying within twenty-four hours had hyperemic and edematous lungs. The first symptoms following injection were retching and vomiting. If the injection is rapid, there is bradycardia and irregularity of the heart. Three of the dogs had large thrombi formations in the veins extending from the points of injection. All dogs continued to pass large quantities of albumin in the urine; hematuria was present for a few hours. It was difficult to determine the effects of dosage as much depended on the rate. Dogs receiving 2 cc. and 2.5 cc. in single injection per kilogram died within twelve hours. Dogs receiving between 1 and 2 cc. per kilogram in single injection, or less than 4 cc. in divided doses, lived longer than one week, but all died within twenty-seven days. Even though the injection does not cause early death, there seems to be permanent impairment rendering the animal unusually susceptible to infections, especially pneumonia.

Chemical preparation of bilirubin. Attempts were made by various means to isolate bilirubin quantitatively from the gall-bladder bile of dog. This bile was selected because all our previous work had been done with whole gall-bladder bile of dogs. If bilirubin could be isolated from this bile, the process of determining the toxicity would be simplified. An apparently easy process of isolating pure bile pigment from bile was given by King and Stewart (3). They stated, referring to Gmelin, that when calcium lactate is added to whole bile a precipitate forms with bilirubin making an insoluble calcium bilirubinate. This is filtered and all the other constituents of bile pass through the filter. Then 10 per cent hydrochloric acid is added to the calcium bilirubinate, which removes the calcium radical, and by repeated washings with water, nothing remains but the pigment. The only problem remaining is to put the bilirubin into a suitable solution to inject. This appeared so simple that we tried it repeatedly with dog gall-bladder bile and with beef bile, with various dilutions of bile and reagents and under various temperature and light conditions, but I was never able to obtain pure bilirubin. Even with aid of Dr. F. C. Koch, and after considerable search of the literature, we found no reliable method by which bile pigment could be isolated quantitatively from bile. H. S. Deninger, from the Chemical Department, worked for a year, making many attempts by various methods to isolate the pigment from bile, but was unsuccessful. In all these processes we obtained a few crystals which microscopically appeared to be bilirubin, but a considerable portion of the material changed in color from day to day as we were working with it. Even though we did all the work in the dark, we were unable to obtain a stable and pure pigment.

Orndorff and Teeple (16) and Kuster (17) isolated chemically pure bilirubin from ox gallstones. The following method was used: About a pound

of reddish cattle gall stones was secured. These gall stones were practically all calcium-bilirubin stones, contained very little carbonate and practically no cholesterol. They were dried at 100° until they remained at

TABLE 1

Bilirubin and other bile pigments

All the solutions of bilirubin were saturated. The two greenish solutions were non-toxic. The death of mouse 8 was probably due to some irrelative cause. Whole bile of dog is toxic for mice

MOUSE	MATERIAL	SOLVENT	AMOUNT	METHOD OF INJECTION	EFFECTS
			cc.		
1	Bilirubin	NaOH 0.8	2	Intraperitoneal	No toxic effects
2	Bilirubin	NaOH 0.8	1	Subcutaneous	No toxic effects
3	No bilirubin	Sterile human serum	2	Intraperitoneal	No toxic effects
4	With bilirubin	Sterile human serum	2	Intraperitoneal	No toxic effects
5	Bilirubin	N/10 NaOH H_2O to neutrality to litmus	2	Intraperitoneal	No toxic effects
6	Bilirubin	N/10 NaOH H_2O to neutrality to litmus stand 24 hours greenish	2	Intraperitoneal	No toxic effects
7	Bilirubin	N/10 NaOH HCl ½ cc.	1	Intraperitoneal	No toxic effects
8	Bilirubin	N/10 NaOH H/10 HCl 1 cc.	1	Intraperitoneal	Dead fifth day, autopsy negative
9	Bilirubin	N/10 HCl to neutral to litmus brownish	2	Intraperitoneal	No toxic effects
10	Bilirubin	N/10 HCl to neutral to litmus	2	Intraperitoneal	No toxic effects
11	Same solution but green		2	Intraperitoneal	No toxic effects
12	Whole dog bile		1	Intraperitoneal	Dead twelve hours. Typical bile peritonitis

constant weight. About half the stones were used for the first preparation. They were powdered and sifted through a 30 mesh sieve: 1, extracted with extra pure ether in a Soxhlet apparatus for 30 hours, then dried in the open air, and later dried for 48 hours in the oven at 100°;

2, again placed in the Soxhlet and extracted with extra pure chloroform for 72 hours. The material was then dried in the open air, and again placed in the drying oven for 24 hours. 3. The powder was then extracted with boiling water repeatedly to remove whatever water soluble substances might still be present. 4. The residue was again dried and then digested for 24 hours with a large quantity of 1 per cent hydrochloric acid. The solution was allowed to stand until the insoluble portion settled to the bottom, and the supernatant liquid decanted. This was repeated a number of times until the supernatant liquid no longer gave a test for calcium. Then the insoluble portion was washed many times with double distilled water until the filtrate gave no reaction for chlorides. 5. The powder was again dried in the oven to constant weight and then extracted in the Soxhlet with pure ether for 12 hours, and again dried, then extracted, 6, with absolute alcohol until the alcoholic filtrate was no longer colored. This latter process was continued for 48 hours. 7. Again dried, replaced in the Soxhlet, and extracted with pure chloroform for 150 hours. This final chloroform extract was supposed to contain pure bilirubin, but on microscopic examination the crystals were not entirely uniform. Accordingly, the chloroform extracts were allowed to evaporate somewhat and were then precipitated with absolute alcohol, the supernatant liquid poured off, again thrown into solution with chloroform and precipitated with alcohol a number of times, until finally the crystals appeared entirely uniform under the microscope. An attempt was made to crystallize bilirubin with dimethyl-aniline with boiling point 192.2 to 192.6°. Also, quinine sulphate was used to precipitate bilirubin crystals, but they did not appear to be as uniform as those obtained by the absolute alcohol method.

The entire work was done in the dark except for a small Ruby light. A number of writers state that bilirubin is changed over into the other pigments by the action of light alone. The various extracting liquids were saved from the extraction of the gallstones, concentrated and prepared for injection.

The purity of bilirubin was tested by microscopic examination of the crystals. We compared my microscopic specimens with the photographs of the crystals which were prepared by Prof. S. H. Gage (16) of Cornell University, also Kuster (17). The crystals from the various methods of final crystallization are identical with those in the photograph by Professor Gage. Our bilirubin appears to be the same as that isolated by Orndorff and Teeple (16). The microscopic method of determining the purity of crystals is very accurate.

The question then arose as to whether or not the powder, which we shall call bilurubin, is the same as the bilirubin in the circulating blood in humans and in experimental animals with jaundice and also the same as the bilirubin present in the bile, or has the chemical process of isolation and

purification changed this unstable pigment into some non-toxic form. The method of detecting the purity of the pigment and the comparison of this particular pigment with that previously tested in blood serum and called bilirubin, was by means of the color analyzer of Keuffel and Essen (18). The analysis of wave lengths as Doctor Sheard (19) found for bilirubin in the blood stream of the dog and of the human, is the same as for our pure bilirubin thrown into solution with N/10 sodium hydroxide. This method of analysis seems to be extremely accurate and the detection of very minute quantities of bilirubin in solution is made with considerable ease.

The bilirubin gave the ordinary tests, which included the Bonamo, Gmelin, Hammarsten, Huppert-Cole, Nakayama, Rosenbach, Salkowski-Schiffer, Smith and van den Bergh. The bilirubin compared favorably with the quantitative method of calculation given by van den Bergh.

The bilirubin did not contain any biliverdin. There appeared to be no protein and only an infinitesimally small amount of iron. We feel sure that this pigment is the identical pigment that has been called bilirubin by so many writers, and the same pigment that is being tested for in the blood stream by the more critical analyses in experimental animals and in man. We wish particularly to point out that the pigment occurring in the blood stream in the human is not biliverdin but is bilirubin.

Solubility. We had considerable difficulty in getting bilirubin into proper solutions for injection intravenously into animals so that the menstruum itself would not cause the symptoms which might be attributed to bilirubin. Bilirubin was practically insoluble in physiological salt, Locke's, Ringer's and other commonly used salt solutions. It was very soluble in sodium carbonate, but when this solution was rendered neutral to litmus, a precipitate formed, consequently it was not deemed suitable for injection. Several investigators have used the sodium carbonate method without any neutralization and some report the effects of the bilirubin on the heart particularly, but when a similar amount of pure sodium carbonate solution was injected rapidly intravenously, we noted similar reactions to that of the bilirubin-sodium carbonate solution. Sodium hydroxide N/10 was a very good solvent, and this was brought almost to neutrality to litmus with N/10 hydrochloric acid, and no precipitate was formed. The same was found to occur with ammonium hydroxide and potassium hydroxide. Dog serum was used as a solvent for bilirubin but we found that only a small amount went into solution in fresh serum. On letting the sterile dog serum stand for 36 to 48 hours in the ice-box, the bilirubin went into solution very readily. Human cerebrospinal fluid was a poor solvent even though the solution was allowed to stand in the ice-box for days. The best solvent other than the carbonate and hydroxide was found to be sterile fresh human serum. As to the value of the different

solutions of bilirubin, much may be said in favor of the simpler solutions, as that of sodium hydroxide, but various workers have criticized this, stating that the sodium and potassium, calcium and magnesium ions render the bilirubin non-toxic. We have used both methods of injection, but prefer as a solvent the human serum or dog serum. Serum is the ordinary solvent for the bilirubin when in the blood stream. The normal human serum alone and also saturated with bilirubin when injected intraperitoneally in mice and pups, and intravenously in pups and dogs, and subcutaneously in mice, pups and dogs, gave no pathological reactions of any kind.

There were no symptoms of any kind attributable to bilirubin. There was no change in the rate or amplitude of the heart, no change in blood pressure and no change in respiration. There was no vomiting at any time and no diarrhea. No adhesions were formed in the peritoneal cavity. There was no edema or sloughing of the subcutaneous tissues when injected subcutaneously. There was no anuria. All the animals continued to eat and drink and live their normal course of life, the same as the control animals. The bile pigment was slowly absorbed and at least part of it eliminated in the urine.

It would appear from this work and theoretically that bilirubin is an excretory product, non-toxic, inactive, and not concerned with any of the pathological processes which occur in jaundice, except pigmentation, or in any of the poisonous actions of the bile.

We know of no way by which biliverdin can be obtained absolutely pure. A thorough search of the chemical literature did not reveal any method. On placing alkaline solutions of bilirubin in the air, they took on a beautiful green color. When these solutions were analyzed spectroscopically, we found there was a small amount of biliverdin but a very large portion of bilirubin. When these solutions were injected, they did not cause symptoms of any kind. Either the amount of pure biliverdin is too small or it is non-toxic.

When bilirubin solution was exposed to the air and sunlight for a few weeks, the solution took on a dirty brown appearance, and this apparently was what has been called bilihumin, but again, spectroscopical examination showed most of the pigment to be bilirubin. Injection of this mixture of pigments gave no toxic symptoms.

Bilirubin-A is the name applied to that part of bilirubin which is insoluble in alcohol and soluble in chloroform. The portion which is soluble in alcohol, called by some writers bilirubin-B, is referred to by Orndorff and Teeple (20) as biliverdin, biliprasin and bilifuscin. Spectroscopic examination of this showed it to be practically all bilirubin. Bilirubin-B when injected was non-toxic. The bilirubin-A fraction was injected as noted previously as plain bilirubin.

The various extracts of gall stones were saved, each evaporated to dryness, and in some instances redissolved in suitable solutions for injection. Water extracts of gall stones when used in sufficient quantity were toxic—this extract contained bile salts as the chemical tests were positive. One gram of the water extract of gall stones redissolved in water caused no effect when injected into the peritoneal cavity of a dog weighing 7 kgm. When five grams were injected into a dog weighing 6 kgm. the dog died in 24 hours with typical symptoms and autopsy findings of bile peritonitis. Six grams of water extract were obtained from one-half pound of gall stones. The chloroform extract (0.7 gram) was insoluble in water but dissolved in sodium hydroxide, and this was neutralized to litmus with hydrochloric acid. This extract caused vomiting immediately but no other toxic symptoms. Alcoholic and ether extracts of gall stones were insoluble in solutions that were suitable for injections. The relative toxicity of these extracts was slight as they were obtained from a large quantity of stones.

Dialyzed bile. On advice of Dr. A. B. Hastings, bile was dialyzed through highly pure colloidin membranes according to the method of Bechhold (20). These were made immediately before use, by dipping a large test tube into the collodion solution twice and letting the collodion dry enough so that the membrance could be removed intact. The membrane was placed for a few seconds in chloroform water, and washed several times in distilled water, then bile was placed within and the top thoroughly clamped. The dialyzing tubes were then placed in a jar of freshly distilled water, the water was saturated with carbon dioxide and covered with oil, and the jars kept in the ice-box at 36°. The carbon dioxide was allowed to bubble through the water every 8 hours. This kept the water at a constant pH and permitted the dialysis under controlled conditions. The water was changed frequently and the bile dialyzed continuously for five days. The dialysate was retained and also the non-dialyzable portion. The dialysate had the same color as the original bile. The non-dialyzable portion had a beautiful pea-green color, and the collodion membranes were a beautiful cyan-blue. Three-hundred cubic centimeters of whole gall-bladder bile of the dog were used. The dialysate contained 21.44 grams per 100 cc., and the non-dialyzable portion contained 2.4 grams per 100 cc.

The non-dialyzable portion was injected intraperitoneally and subcutaneously in mice, and subcutaneously, intraperitoneally and intravenously in dogs, in quantities similar to that used with whole bile, and in some instances triple the quantity. There were no symptoms of any kind attributable to the non-dialyzable portion of bile. The dialysate was found to have the same properties quantitatively as whole bile, when injected into mice and dogs. The symptoms produced by the injection of the dialysate were similar to those produced by whole bile. The toxic portion of bile is dialyzable, and the non-dialyzable portion of bile is non-toxic.

Impure salts of bile. Impure salts of sodium taurocholate (commercial) were used in the same quantity by weight as the solid portion of the dialysate. Injections were made intravenously, subcutaneously and intraperitoneally of sodium taurocholate and sodium glycocholate in varying quantities per kilogram of body weight, varying from 0.7 gram per kilogram of body weight to the same quantity as the dialyzable bile was found in gall-bladder bile. The various quantities of salts gave varying severity of symptoms in proportion to the quantity of the salt injected. These

TABLE 2

Pure bile salts and dialysate of bile. Tests were made to determine the toxicity of the various constituents of bile in small animals. The salts and dialysate caused toxic symptoms. All of these injections were intraperitoneal

MOUSE	MATERIAL	QUANTITY	SOLVENT	EFFECTS
1	Sod. Glycocholate	0.125 gr.	Dist. H_2O 0.5 cc.	Dead 30 minutes
2	Glycocholic Acid	Sat. Sol.	Dist. H_2O 2 cc.	Lived, no toxic effects
3	Sod. Taurocholate	0.125 gr.	Dist. H_2O 0.5 cc.	Dead 30 minutes
4	Sod. Glycocholate	0.031 gr.	Dist. H_2O 0.1 cc.	Dead 30 minutes
5	Sod. Glycocholate	0.015 gr.	Dist. H_2O 0.05 cc.	Dead 36 hours
6	Glycocholic Acid	0.04 gr.	N/10 NaOH 2 cc.	Dead 45 minutes
7	Dialysate	2 cc.	In H_2O	Dead 45 minutes
8	Undialyzable	2 cc.	In H_2O	Lived
9	Undialyzable	2 cc.	In H_2O	Lived
Pup				
1	Dialysate	2 cc.	In H_2O	Dead 5 hours
2	Undialyzable	2 cc.	In H_2O	Lived
Dog				
98	Undialyzable	5 cc. per kgm.	In H_2O	Lived
99	Dialysate	5 cc.	In H_2O	Died 24 hours
90	Na Taurocholate	0.09 gr.	H_2O 4 cc.	Lived. Diarrhea
91	Na Glycocholate	0.08 gr.	H_2O 4 cc.	Lived. Diarrhea
92	Na Taurocholate	0.13 gr.	H_2O 2 cc.	Edema, dead 3 days
93	Na Glycocholate	0.16 gr.	H_2O 2 cc.	Diarrhea; edema, dead 3 days
95	Na Taurocholate	0.2 gr.	H_2O 6.4 cc.	Vomiting; diarrhea, lived
97	Na Taurocholate	0.6 gr.	H_2O 20.0 cc.	Vomiting; diarrhea, lived

symptoms were the same as when a similar amount of bile was used. Quantities which were calculated to be equal to the total quantity of solids in the gall-bladder bile produced very much more marked and severe symptoms and death occurred in a much shorter time than when whole bile was used. It would appear from this that either a miscalculation was made in the salt quantity of whole bile, or that something in the bile acted as a protective agent when whole bile was injected. However, the absolute quantity of salt in the whole gall-bladder bile of dogs was not

determined with great accuracy. Calculations were made on the basis of the dialysate as sodium taurocholate which occurs practically alone in dog bile. It would appear that the dialyzed portion of bile contained at least a very great percentage of salt. The impure salts that were used were labeled at various percentages of purity by the manufacturers, ranging from 40 per cent to 80 per cent. Nothing is known as to their methods of preparation.

Pure salts of bile. Pure salts of bile were then obtained from Dr. Theodor Schuchardt, Gorlitz, Germany, and were used for the injections. When these salts were injected in the same quantity by weight as the impure salts, the toxic effects were the same, and the symptoms produced compared very favorably to those above mentioned.

Bile acids. The only pure bile acid that could be obtained was glycocholic acid, from Doctor Schuchardt. This was practically insoluble in water, salt solutions and blood serums of both man and dog. Any quantity that could be dissolved in any of these solutions was not sufficient by any method of injection to produce any toxic symptoms. Glycocholic acid was thrown into solution by sodium hydroxide. Strong sodium hydroxide was used and when neutralized with hydrochloric acid, an insoluble precipitate was formed. So a slightly alkaline solution of glycocholic acid in hydroxide was used for injections, and these solutions when calculated in terms of the pure salts gave quantitatively the same results as were obtained with the other salts.

Taurocholic acid: No pure taurocholic acid was obtainable,[1] so comparative tests with this material could not be made. Many attempts to prepare pure taurocholic acid were unsuccessful.

After it had been determined quantitatively that glycocholic and taurocholic salts contained the toxic portion of bile, attention was then directed to the different parts of these salts. It has already been proven by Schmidt (21) that taurin is non-toxic even when injected intravenously in the human in such large quantities as 10 grams. We injected glycocoll intravenously in dogs, and did not observe any toxic symptoms. Cholic acid was only very slightly soluble in distilled water or salt solution. When these solutions were injected only very slight effects were observed. Then cholic acid was dissolved in strong sodium hydroxide solution and brought nearly to neutral to litmus. When the solution was made neutral there was formed a dense precipitate immediately. Sodium cholate when injected gave the same effects quantitatively as sodium glycocholate and sodium

[1] Doctor Schuchardt replied May 24, 1927, as follows: "Leider mussen wir Ihnen mitteilen dass wir keine Angaben gefunden haben die uns eine Weg zeigen wurden Natrium taurocholic. etc. in absolut. chem. reiner Qualitat heraustellen . . . . Ferner bitten wir Sir, zur Kenntnis zu nehmen, dass wir Acid. taurocholic. nicht liefern konnen, so dass wir auch keine Offerte unterbreiten konnen."

taurocholate. The toxic portion of bile is in the cholate part of the bile salts.

Cholesterol. Cholesterol was injected in saturated alcoholic and saturated physiologic salt solutions, and gave no symptoms. These solutions were used both intravenously and intraperitoneally.

The action of bile on the cardio-vascular system. On reviewing the action that has been demonstrated on various tracings taken when intravenous injections of bile and the various constituents of bile were made in dogs, it would appear that there was a variety of actions. The preliminary slowing of the heart was modified by sectioning the vagi. Practically the complete slowing of the heart was eliminated by the use of atropin which paralyzed the vagus nerve endings, but when a similar quantity of the salts which caused the stopping of the heart was then injected after the use of atropine there was still a cessation of the action of the heart, with a return after 10 to 30 seconds. This alternation of beating and stopping continued for some time before death occurred.

We conclude that the heart action is modified by the bile salts, through various systems, first by acting on the vagus endings, but the principal action is that through the coronary circulation in which the bile salts act on the heart muscle.

Landois (14) reported that when small quantities of bile salts were injected the blood pressure was slightly elevated. This can be demonstrated if very minute quantities are injected, but the elevation was only very slight and was very transitory. When larger quantities of salts were injected, there was a definite and rapid fall in blood pressure. If a very rapid injection of the salts was made, the blood pressure dropped immediately to zero, and this rapid fall in blood pressure may be attributed to vagus action and to failure of the myocardium. The use of atropin does not prevent this action, although it comes on more slowly after its use. Peripheral dilatation of the arteries has probably not had time to take place.

We noted in practically all cases with ligation of the common duct after the dog lived for a number of weeks there was a marked depression of the blood pressure and the pulse was barely perceptible for some time before death of the dog. This depression may be explained in two ways: 1, the toxic action of bile salts (which is seriously questioned) and 2, the marked disturbance in nutrition, as all of these animals lost a very large proportion of weight. Of course, there is also a myocardial defect to be considered in malnutrition. This fall of blood pressure is similar to the hypotension that we so frequently find in late human jaundice.

The action of bile on the gastro-intestinal tract. This will be discussed in a separate paper to appear soon; and we refrain from further discussion except to state that when large amounts of bile were injected by any one of the three methods, vomiting and bloody diarrhea occurred.

The action of bile on the respiratory system. After injection of bile intraperitoneally, intravenously and subcutaneously, a very large proportion of the dogs that did not die within the first 36 hours died later of pulmonary involvement. Some of the dogs had massive pneumonia, others had small areas of pneumonia—some of them a few and others numerous areas; in a few of the dogs dying early there was hemorrhagic edema throughout small portions of the lung or throughout the entire lung. The immediate action of the absorption of bile on respiration is shown in the tracings. The amplitude of respiratory movement is diminished and the respiration rate is very much slower. When a large amount of bile has been injected, the respiration may cease for a minute or longer, to return with gradually increasing amplitude, then gradually decreasing, and finally a complete cessation for another 10 to 30 seconds. Typical Cheyne-Stokes type of respiration frequently lasted for hours. Sometimes this was associated with diminution in heart rate or cessation of the heart beat and at other times was independent of heart action. As a whole, the respiration rate decreased and the amplitude was diminished.

The action of bile on the urinary system. It was early noticed that in bile peritonitis there was a complete cessation of the passing of urine. We at first thought of this as being a reflex phenomenon due to stimulation of the nerves going to the kidneys. Accordingly, four dogs were used to test out this hypothesis and the nerves going to the kidneys were dissected out as completely as possible and sectioned. Bile was then injected into the peritoneum, and the anuria appeared as in previous experiments. The dogs died within 24 hours without secreting more than a few cc. of urine and that was highly blood tinged and contained a large amount of albumin and bile. It would appear, then, that after the effects of the nerves had been eliminated and noting that the blood pressure was very markedly lowered in bile peritonitis, that this is simply a failure of the kidneys to secrete following diminution in blood pressure. In order to test out this hypothesis, the ureters were catheterized, and it was noticed that the kidneys continued to secrete until the tracings showed a fall in blood pressure to approximately 60 mm. Hg following the injection of bile, when suddenly all secretion of the urine ceased and did not return so long as the blood pressure remained low. So failure of the kidneys to secrete is attributed to fall in blood pressure. In dogs that recovered from the initial injection of bile, the urine continued to show albumin for from a few days to several weeks, and in some of the dogs that lived for several months and had previously received a fairly toxic dose of bile, but not lethal, the urine showed albumin continuously. A part of the time the elements are formed and there occur red and white cells and renal epithelium in the urine, while later the urine shows a few casts or debris. In the dogs receiving a sublethal injection of bile there is an irritation of the

kidney tissue and this may be explained by the cytolytic action as suggested by Marawitz (22).

The action of bile on the nervous system. Various writers, especially Meltzer and Salant (23), have reported that small amounts of bile or bile salts cause a stimulation of the nervous system. We have noticed in a few of the dogs an early appearance of tonic or clonic convulsions. These occurred mainly in the animals which had not previously been given ether or barbital, but occurred to such an extent in one of the dogs which had received ether and barbital, as to render the dog valueless for further experiments. The dog continued with convulsions in spite of extremely large doses of barbital intravenously, and these convulsions only ceased after giving a large amount of ether, at which time the blood pressure fell considerably. When convulsions did occur it was early and before very much of the bile or bile salts had been absorbed, as later on the opposite effects were noted. There was no instance of death occurring immediately after or with convulsions.

Theory of the action of bile acids. Fats enter into the formation of the limiting membrane of all living cells. The bile salts diminish the surface tension and when blood cells are brought in contact with bile they rapidly disappear by disintegration. Theoretically we may conclude that the bile salts act on (dissolve) the limiting membrane of the cell, change the surface tension, and destroy the normal cellular metabolism. This reaction not only occurs with red blood cells, but with muscle cells and probably all the living cells with which bile salts come in contact. This action then is quantitative; when only a small amount of the salt comes in contact with the cell there is a slight change but not sufficient to prevent recovery, a reversible action.

CONCLUSIONS

1. Bile is toxic when injected intraperitoneally, subcutaneously and intravenously.
2. When bile is injected in sufficient quantity to cause death in twenty-four hours, the presence of bacteria in the bile does not modify its toxicity.
3. The toxicity of bile is not modified by boiling or freezing.
4. Bilirubin is non-toxic.
5. Bile dialysate has the same toxicity as whole bile.
6. The non-dialyzable portion of bile is non-toxic.
7. Pure bile acids are non-toxic, due to their insolubility.
8. The cholate portion of the salts of the bile acids, such as sodium glycocholate and sodium taurocholate, are toxic.

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Effect of Dietary Bile Acids on Plasma Cholesterol of the Rat

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Beher et al. ('60) have reported that dietary hyodeoxycholic acid reverses the hypercholesteremic effect of cholic acid in female mice and prevents cholesterol accumulation in liver and carcass. It was independently observed in this laboratory (Howe et al., '60) that lithocholic, chenodeoxycholic and hyodeoxycholic acids counteract the hypercholesterolemic effect of cholic acid in the male mouse. More recently it was shown that antibacterial agents completely eliminate the anti-cholic acid effect of lithocholic acid in the mouse but not that of hyodeoxycholic acid (Howe and Bosshardt, '61). In the rat, Portman and Bruno ('61) studied the effect of feeding a number of bile acids and derivatives among which were lithocholic, deoxycholic and apocholic acids. The first of these was found to be without effect on plasma cholesterol in this species, whereas the others were observed to possess definite plasma cholesterol-elevating properties although not to the same extent as cholic acid. On the other hand, Yamasaki et al. ('59) reported that deoxycholic and apocholic acids depress plasma cholesterol in the rat and that cholic, hyodeoxycholic and chenodeoxycholic acids all elevate it. In the experiments of Portman and Bruno, 1.45% of cholesterol was included in the diets, while in those of the Japanese investigators the diets were cholesterol-free.

Yamasaki and his co-workers ('59) also reported that the ingestion of 0.6 gm of deoxycholic acid daily by 6 healthy men for 5 or 8 days reduced their average plasma cholesterol level from 160 mg/100 ml to 110 mg/100 ml. This observation is in contrast to that of Mosbach et al. ('58) who, after prolonged daily oral administration of 0.5 gm of deoxycholic acid to humans, observed no effect on the concentration of serum lipids.

Reported herein are the results of additional experiments concerned with the effect of bile acids on the concentration of plasma cholesterol in the rat.

EXPERIMENTAL

Male rats (120 to 150 gm) of the Holtzman strain were randomly allocated into groups of 10 animals. Each group was housed communally and fed ad libitum. The basal diet consisted of 24% of casein,[1] 4% of salt mixture,[2] 10% of hydrogenated coconut oil,[3] 62% of glucose and a complete vitamin addendum.[4] All substitutions were made at the expense of glucose. After 12 days, the animals were anesthetized with pentobarbital sodium[5] given intraperitoneally and bled from the heart to obtain samples for total plasma cholesterol determinations which were carried out by the method of Abell et al. ('52).

In later experiments in attempts to confirm their results, a diet similar to that of Yamasaki et al. ('59) was used and some of the bile acids were given by intubation. The diet used in these experiments consisted of casein, 20%; Cellu Flour,[6] 10%; salt mixture,[7] 3%; dried brewer's yeast, 3%; peanut oil, 5%; sucrose, 59%; and the complete vitamin addendum used in the earlier experiments.

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[1] Labco, The Borden Company, New York.
[2] Wisconsin no. 4.
[3] Hydrol, Duchee's Famous Foods, Chicago.
[4] Micronutrients mg/100 gm: thiamine, 0.8; riboflavin, 1.6; pyridoxine, 0.8; niacinamide, 4.0; Ca pantothenate, 4.4; p-aminobenzoic acid, 4.0; inositol, 20.0, biotin, 0.02, folic acid, 0.2; vitamin B_{12} triturate (0.1% trituration with mannitol), 30.0; choline methionine tartrate, 60.0; vitamin A palmitate, 0.4, calciferol, 0.005; α-tocopherol, 4.2; menadione, 1.0; butylated hydroxyanisole, 20.0.
[5] Nembutal, Abbott Laboratories, North Chicago, Illinois.
[6] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[7] See footnote 2.

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RESULTS

The first series of experiments was designed to determine whether lithocholic, hyodeoxycholic or chenodeoxycholic acids would reverse the hypercholesterolemic effect of cholic acid in rats receiving a diet containing cholesterol and, if so, whether antibacterial agents might alter this effect. The results are shown in table 1. It is questionable whether lithocholic acid exerted any effect at all in this species in the absence of antibiotics ($P > 0.1$). However, at the higher level in the presence of dietary antibiotics there appeared to be a significant reduction in plasma cholesterol ($P < 0.05$). This is in sharp contrast with the results obtained with the mouse (Howe and Bosshardt, '60) in which the very definite anti-cholic acid effect of lithocholic acid was found to be cancelled by feeding antibiotics. Hyodeoxycholic acid acts similarly in both species, counteracting the hypercholesterolemic effect of cholic acid and remaining unaffected by dietary antibiotics ($P < 0.05$). Most unexpected was the observation that chenodeoxycholic acid instead of counteracting the effect of cholic acid as it does in the mouse, very definitely augmented its plasma cholesterol elevating property.

Two experiments were performed in attempts to confirm the results obtained by Yamasaki et al. ('59) with deoxycholic and hyodeoxycholic acids. In the first rats were fed commercial laboratory chow or a purified diet containing deoxycholic acid, or animals maintained on these diets were given deoxycholic acid by stomach tube. The results are shown in table 2. In the second, a diet similar to that of the Japanese investigators was used and either deoxycholic, hyodeoxycholic or cholic acids was administered by both methods. A compilation of the results is shown in table 3. These data confirm what has been reported many times, that bile acids in the absence of dietary cholesterol have no great effect upon the plasma cholesterol concentration of the rat. They do reveal, however, under the conditions of our experiments that deoxycholic acid tends to elevate it while hyodeoxycholic acid is without significant effect. In our hands the administration of 80 mg of the bile acids daily by gavage, the quantity used by Yamasaki and associates produced a high incidence of mortality.

[3] Purina Chow, The Ralston Purina Company, St. Louis.

TABLE 1

Effect of lithocholic, hyodeoxycholic and chenodeoxycholic acids on cholic acid-cholesterol induced hypercholesterolemia in the rat and the interaction of antibacterial agents

Dietary supplement[1]	Plasma cholesterol	12 [illegible] weeks
	mg/100 ml	[illegible]
None (60)	90.4 ± 1.8[2]	[illegible]
0.1% Cholic acid (60)	136.5 ± 4.8	[illegible]
0.1% Cholic acid, 0.2% lithocholic acid (19)	123.4 ± 5.2	[illegible]
0.1% Cholic acid, 0.4% lithocholic acid (20)	119.0 ± 4.8	[illegible]
0.1% Cholic acid, 0.2% hyodeoxycholic acid (20)	103.1 ± 4.0	[illegible]
0.1% Cholic acid, 0.4% hyodeoxycholic acid (20)	98.6 ± 3.5	[illegible]
0.1% Cholic acid, 0.2% chenodeoxycholic acid (30)	172.1 ± 19.3	[illegible]
0.1% Cholic acid, 0.2% oxytetracycline,[3] 2.0% succinyl sulfathiazole (19)	254.8 ± 10.3	[illegible]
0.1% Cholic acid, 0.2% oxytetracycline, 2.0% succinyl sulfathiazole + 0.2% lithocholic acid (19)	236.5 ± 22.2	[illegible]
0.1% Cholic acid, 0.2% oxytetracycline, 2.0% succinyl sulfathiazole + 0.4% lithocholic acid (20)	205.4 ± 14.3	[illegible]
0.1% Cholic acid, 0.2% oxytetracycline, 2.0% succinyl sulfathiazole + 0.2% hyodeoxycholic acid (20)	155.1 ± 6.1	[illegible]
0.1% Cholic acid, 0.2% oxytetracycline, 2.0% succinyl sulfathiazole + 0.4% hyodeoxycholic acid (20)	122.8 ± 5.1	[illegible]

[1] All diets contained 1% cholesterol.
[2] Standard error.
[3] Kindly supplied by Chas. Pfizer and Company, Inc., Brooklyn, New York.

TABLE 2

Effect of deoxycholic acid on plasma cholesterol concentration of rats fed commercial chow[1] or a purified diet containing 10% hydrogenated coconut oil

Supplement	Purified diet		Commercial chow	
	Plasma cholesterol	12-Day weight gain	Plasma cholesterol	12-Day weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*
None	55.5 ± 1.9	49	46.8 ± 1.1	64
10 mg Deoxycholic acid daily by intubation	66.4 ± 0.9	57	47.9 ± 0.8	51
0.05% Deoxycholic acid	60.5 ± 2.6	61	59.7 ± 2.4	68
0.1% Deoxycholic acid	73.8 ± 1.7	59	59.8 ± 1.0	70
0.1% Cholic acid	75.9 ± 2.2	54		
1% Cholesterol	90.0 ± 4.1	58		
1% Cholesterol + 0.1% cholic acid	111.8 ± 4.4	53		
1% Cholesterol + 0.1% deoxycholic acid	157.7 ± 9.6	56		
1% Cholesterol + 0.1% cholic acid + 0.1% deoxycholic acid	193.1 ± 18.5	53		

[1] Purina Chow, The Ralston Purina Company, St. Louis.

TABLE 3

Comparative effects of deoxycholic, hyodeoxycholic and cholic acids on the plasma cholesterol concentrations of rats

Supplement	Plasma cholesterol	12-Day weight gain
	mg/100 ml	*gm*
None	61.9 ± 1.7	50
80 mg Deoxycholic acid daily by intubation	69.9 ± 2.7	12
80 mg Hyodeoxycholic acid daily by intubation	58.9 ± 2.1	36
80 mg Cholic acid daily by intubation	65.8 ± 1.6	21
0.1% Deoxycholic acid	71.4 ± 2.6	52
0.1% Hyodeoxycholic acid	70.2 ± 1.9	54
0.1% Cholic acid	70.8 ± 1.8	56
0.4% Deoxycholic acid	75.2 ± 3.7	41
0.4% Hyodeoxycholic acid	64.7 ± 2.4	51
0.4% Cholic acid	71.2 ± 2.3	48

DISCUSSION

In species as closely related as the mouse and rat which have been shown to have very similar bile acid patterns (Danielsson and Kazeno, '59), it is unexpected that chenodeoxycholic acid exerts opposite effects. This finding confirms that of Yamasaki et al. ('59) that chenodeoxycholic acid exerts an hypercholesterolemic effect in the rat.

The relative inactivity of lithocholic acid in the rat is also unexpected, but since its anti-cholic acid effect in the mouse appears to be mediated through intestinal bacteria this difference might be explained on the basis of differences in bacterial flora in the two species. This hypothesis is especially attractive in light of the recent report by Norman and Sjovall ('60) that in the rat lithocholic acid is derived from chenodeoxycholic acid probably through the action of bacteria.

It is difficult to account for the contrasting effects of deoxycholic and hyodeoxycholic acids described here and reported by the Japanese workers. In the rat it has been shown that, while deoxycholic acid arises from cholic acid through bacterial action, it is readily reconverted to cholic acid in the liver. On this basis exogenous deoxycholic acid might be expected to elevate blood cholesterol in this species. On the other hand, differences in strain of rats, diet, or bacterial flora might influence the metabolic products obtained from this bile acid and consequently its effect on plasma cholesterol.

SUMMARY

It has perviously been shown in the mouse that dietary lithocholic, hyodeoxycholic and chenodeoxycholic acids counteract the hypercholesteremic effect of exogenous cholic acid. In contrast, in the rat it has now been observed that lithocholic acid has little or no anti-cholic acid effect and that chenodeoxycholic acid augments rather than counteracts the effect of dietary cholic acid. As in the mouse, hyodeoxycholic acid neutralizes the plasma cholesterol-elevating effect of cholic acid and in neither species is this effect eliminated by dietary antibacterial agents.

It has been reported that orally administered deoxycholic acid depressed plasma cholesterol in the rat and that hyodeoxycholic acid elevated it. Under the conditions of our experiments deoxycholic acid exerted only an elevating effect and hyodeoxycholic acid was without significant effect.

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Effect of Bile Acids on Plasma Cholesterol in the Mouse[1]

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It has been demonstrated that elevation of the cholate content of the plasma of the rat by biliary obstruction or by continuous intravenous injection of cholate into intact rats results in a prompt hypercholesterolemia (Byers et al., '50; Friedman and Byers, '51). It has been further reported that in pathological conditions in man such as nephrosis, myocardial infarct, xanthoma and hypothyroidism which are associated with high plasma cholesterol, elevated plasma cholate is also found (Friedman et al., '52). These latter results have not been confirmed by Wysocki and associates ('55) who reported instead that in hypercholesterolemic humans and monkeys chenodeoxycholic acid but not cholic acid is elevated.

The hypercholesterolemic effect of cholic acid was originally explained on the basis that it increased the absorption of exogenous and endogenous cholesterol. However, Friedman and Byers ('51) and Pihl ('55) have advanced evidence to show that exogenous bile acids often exert no effect on cholesterol absorption while causing a marked elevation of plasma cholesterol.

More recently it has been demonstrated in intact animals, that cholic acid reduces both rate of synthesis and mobilization of liver cholesterol (Beher and Baker, '58; Beher and Anthony, '58; Beher et al., '59). Further, Bergström and Danielsson ('58) have shown that it is the feedback of bile acid conjugates in the portal blood that controls the further oxidation of cholesterol to bile acids in the liver. A similar inhibition has also been shown to occur in an *in vitro* system (Whitehouse and Staple, '59).

There are at least two pathways of oxidation of cholesterol to bile acids in the animal organism (Bergström, '55; Siperstein and Chaikoff, '55). It has been demonstrated (Bergström, '55; Bergström and Lindstedt, '56; Lindstedt, '57) that no compound containing a carboxyl group in the side chain, with the exception of deoxycholic acid (Bergström et al., '53a),[2] is converted in the mammalian body to cholic acid; i.e., in the production of cholic acid the hydroxylation of the nucleus must occur before the oxidation of the side chain. On the other hand, lithocholic acid (Bergström et al., '53b) and chenodeoxycholic acid (Bergström and Sjoväll, '54; Matschiner et al., '57; Mahowald et al., '57) are each oxidized to more polar acids, none of which are identical with cholic acid. Some of these products have been identified as isomers of hyocholic acid, (3α,6α,7α) trihydroxycholanic acid (Hsia et al., '58a, b).[3]

In view of the many systems of checks and counterchecks found in the animal organism, it seemed logical to look for factors which increased or decreased the production of cholic acid or for factors which have an effect directly opposite to that of cholic acid. Recorded here are observations made in the course of an investigation of the effect of various bile acids on the plasma cholesterol concentration of the mouse.

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[1] Since the preparation of this manuscript hyodeoxycholic acid has been reported to reverse the effect of cholic acid in female mice and to prevent cholesterol accumulation in the liver and carcass (Beher, W. J., G. D. Baker and W. T. Anthony 1960 Effect of cholic and hyodeoxycholic acids on metabolism of exogenous cholesterol in mice. Proc. Soc. Exp. Biol. Med., *103*: 385).

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EXPERIMENTAL

The experimental procedure is similar to that reported earlier (Howe and Bosshardt, '60). Male weanling mice were housed individually, 8 per group, and allowed to feed ad libitum. The basal diet consisted of casein, glucose, salt mixture, cellulose, and a vitamin addendum was supplemented at the expense of glucose. In many of the experiments a hydrogenated coconut oil,[4] was used as a dietary supplement after it was found that this substance enhanced the hypercholesterolemic effect of the bile acids.

At the end of a 12-day period the mice were weighed and decapitated after light chloroform anesthesia in order to obtain a pooled blood sample from each group of 8 animals. Plasma total cholesterol was determined by the method of Abell et al., ('52).

RESULTS

Some of the data obtained using the bile acids[5] and bile acid derivatives in amounts equivalent to 0.1% of cholic acid with 1% of cholesterol and 10% of hydrogenated coconut oil are recorded in table 1. The structures of the bile acids involved in this study are shown in figure 1.

Taurocholic acid appears to be somewhat less hypercholesterolemic than unconjugated cholic acid. This agrees with the previously reported finding that taurine lowers plasma cholesterol in the Cebus monkey (Portman and Mann, '55) and in the hypercholesterolemic rat (Hermann, '59). We were able to confirm this observation in the mouse and shall report our results in a later communication. Of considerable interest also is the observation that acetylation of methyl cholate in the 3 and 7 positions renders it completely inactive. The most striking observation obtained from this study, however, was that lithocholic acid was not only inactive in elevating plasma cholesterol but actually appeared to reduce it below the control level. In view of variations previously reported (Howe and Bosshardt, '60) and indicated here, these differences can be considered significant.

In view of the observations shown in table 1, it was logical to test lithocholic

[4] Hydrol, Durkee Famous Foods, Chicago.

[5] The cholic acid used in these studies was obtained from Nutritional Biochemicals Corporation. Its infrared absorption spectrum deviated slightly from that of a highly purified sample in the Merck standard files. However, by reversed phase chromatography no difference could be noted. The glycocholic acid, methylcholate, ethylcholate, methyldeoxycholate, methyl-3, 7-diacetylcholate, and chenodeoxycholic acid were prepared in the Merck Research Laboratories; the deoxycholic acid, lithocholic acid and hyodeoxycholic acid were purchased from Nutritional Biochemicals Corporation and the taurocholic acid from Wilson Laboratories. The infrared spectrum of none of these samples deviated from expectation. Finally the infrared absorption spectrum of the dehydrocholic acid obtained from Mann Research Laboratories showed some OH absorption and must be considered somewhat impure.

TABLE 1

Effect of bile acids on plasma cholesterol in the mouse when incorporated into the diet at levels equivalent on a molecular basis to 0.1% of cholic acid

Compound	Number of tests 8 animals/test	Av. plasma cholesterol[1]	Av. weight gain, 12 days
		mg/100 ml	*gm*
None	10	146 ± 16.8[2]	10.8
Cholic acid	10	258 ± 28.6	8.8
Taurocholic acid	5	185 ± 11.1	9.5
Glycocholic acid	5	231 ± 17.9	9.5
Deoxycholic acid	3	237 ± 9.7	10.7
Lithocholic acid	2	106 ± 2.5	10.6
Dehydrocholic acid	2	263 ± 1.0	9.9
Methyl cholate	4	243 ± 17.5	8.8
Ethyl cholate	2	243 ± 5.0	10.0
Methyl deoxycholate	3	231 ± 19.4	9.5
Methyl 3,7-diacetylcholate	2	151 ± 3.0	8.8

[1] In this and subsequent tables each cholesterol determination was made on a pooled blood sample from the 8 animals in each group.

[2] Standard error.

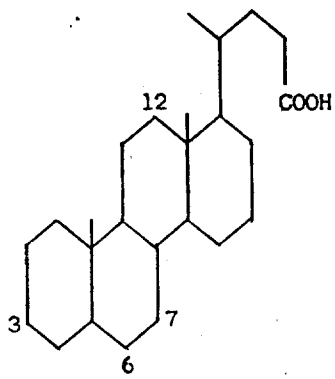

Fig. 1 Cholanic acid.
Lithocholic acid
—3α-hydroxycholanic acid
Deoxycholic acid
—3α, 12α-dihydroxycholic acid
Chenodeoxycholic acid
—3α, 7α-dihydroxycholanic acid
Hyodeoxycholic acid
—3α, 6α-dihydroxycholanic acid
Cholic acid
—3α, 7α, 12α-trihydroxycholanic acid
Hyocholic acid
—3α, 6α, 7α-thihydroxycholanic acid
β-Muricholic acid
—3α, 6β, 7β-trihydroxycholanic acid
Dehydrocholic acid
—3,7,12-triketocholanic acid

acid under a variety of conditions. It seemed likely that the most striking results might be obtained in animals receiving cholic acid and this system was used first with the results shown in table 2.

From these data lithocholic acid obviously counteracts the hypercholesterolemic effect of cholic acid. Accordingly a series of experiments was carried out to study the effect of lithocholic acid on other diets. The results are shown in table 3.

Analysis of variance of these data indicates that lithocholic acid especially at 0.2 to 0.4% of the diet ($P \gtrless 0.05$) depresses the plasma cholesterol concentration of mice maintained with adequate purified diets.

It seemed desirable to determine whether lithocholic acid is effective against other bile acids which were shown to elevate plasma cholesterol. The results of experiments designed to check this point are shown in table 4. The basal diet again was supplemented with 10% of hydogenated coconut oil and 1% of cholesterol. Lithocholic acid can be seen to be effective in every case.

By means of radiolabeling techniques, lithocholic acid has been shown to be metabolized in the bile fistula rat to three more polar compounds (Bergström et al., '53b). In the surgically-jaundiced rat one of the products of metabolism has been identified as β-muricholic acid, 3α,6β,7β-trihydroxycholanic acid.[6] In a like manner, chenodeoxycholic acid and hyodeoxycholic acid (Hsia et al., '58a) have been shown to have muricholic acid as a common metabolic product. It is within the realm of possibility that lithocholic acid may exert its anti-cholic acid effect indirectly through muricholic acid. This hypothesis is supported to some extent by the finding of the St. Louis group that β-muricholic acid was found in the bile of rats fed laboratory chow, but not in the bile of those receiving a purified diet (Matschiner et al., '57). In our observations rats receiving chow have

[6] See footnote 3.

TABLE 2

Effect of lithocholic acid on concentration of plasma cholesterol of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in the diet (12-day experiment)

Dietary supplement	Percentage of dietary lithocholic acid					
	0.0		0.1		0.5	
	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*
10% Hydrogenated coconut oil[1] (3)[2]	236	7.6	216	7.2	131	5.9
15% Mixed fat (1)	182	7.6			138	7.6
5% Linseed oil (1)	206	12.6	146	12.8	136	10.3

[1] Hydrol.
[2] Figures in parentheses indicate number of groups of 8 animals each used at each level of lithocholic acid.

TABLE 3

Plasma cholesterol and weight gain as influenced by lithocholic acid and various fats

Fat supplement	Percentage of lithocholic acid									
	0.0		0.05		0.1		0.2		0.4	
	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*
15% Mixed fat	139	13.9	117	12.9	113	12.8	113	13.0	100	9.1
5% Hydrogenated coconut oil[1]	107	10.8	129	10.7	132	9.1	103	11.2	99	9.1
5% Corn oil	114	12.4	120	12.6	114	11.5	95	12.5	80	6.3
None	100	10.5	107	10.6	89	10.0	77	9.7	95	6.7
5% Linseed oil	84	11.2	99	11.7	91	12.0	87	10.7	92	9.9
5% Cod liver oil	84	12.5	87	13.6	82	12.7	66	11.6	64	8.7

[1] Hydrol.

shown consistently lower concentrations of plasma cholesterol than those ingesting a purified diet. It might therefore be inferred that the presence of muricholic acid in the chow-fed rats may be responsible for their lower plasma cholesterol concentrations. At any rate, it seemed worthwhile to check the other precursors of β-muricholic acid, chenodeoxycholic and hyodeoxycholic acids for anti-cholic acid effect.

The results of the tests with chenodeoxycholic acid are shown in tables 5 and 6. From these data it is apparent that chenodeoxycholic acid has much the same effect as lithocholic acid in counteracting the hypercholesterolemic effect of cholic acid.

A comparison of the effect of hyodeoxycholic acid with lithocholic and chenodeoxycholic acids is shown in table 7. These observations show that hyodeoxycholic acid is the most effective of the three bile acids in counteracting the hypercholesterolemic effect of cholic acid.

Some of the dietary supplements, especially at the higher levels used, caused a definite growth inhibition. The results of experiments which show that this inhibition *per se* was not responsible for the depressed plasma cholesterol concentrations are recorded in an earlier publication (Howe and Bosshardt, '60). In addition, a group of weanling mice receiving the basal diet with cholesterol and cholic acid were restricted to 2 gm of food daily which limited their growth to approximately 2 gm in 12 days. The plasma cholesterol concentration of pooled sample from these animals was 307 mg/100 ml. Values obtained by assay of pooled samples from animals similarly restricted with comparable rates of growth receiving in addition 0.4% of lithocholic acid and 0.4% of hyodeoxycholic acid were 113 and 135 mg/100 ml, respectively.

DISCUSSION

In light of the observation that lithocholic and chenodeoxycholic acids, both of which appear to arise in the liver, counter the hypercholesterolemic effect of cholic acid it is tempting to assume that a balance of the various bile acids may be a body mechanism for the control of plasma cholesterol. A variety of factors might in-

TABLE 4

Effect of lithocholic acid on concentration of plasma cholesterol (mg/100 cm³) of mice receiving 0.1% of various bile acids added to a basal diet containing 10% of hydrogenated coconut oil and 1% of cholesterol (12-day experiment)

Dietary bile acid	Percentage of lithocholic acid		
	0.0	0.2	0.4
Cholic acid	315(7.7)[1]	144(8.0)	143(7.4)
Taurocholic acid	213(8.6)	139(9.4)	118(7.5)
Glycocholic acid	296(7.6)	159(7.8)	122(9.2)
Deoxycholic acid	255(7.1)	157(6.6)	120(7.0)
Methyl cholate	259(6.8)	170(7.5)	135(5.7)
Dehydrocholic acid	262(8.4)	138(7.0)	118(5.0)

[1] Figures in parentheses indicate weight gain in grams.

TABLE 5

Comparison of effect of lithocholic and chenodeoxycholic acids on plasma cholesterol concentration of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in 12-day test

Dietary supplement	Percentage of bile acid added			
	Lithocholic acid			Chenodeoxycholic acid
	0.0	0.1	0.5	0.1
None	234(9.6)[1]	169(10.1)	106(8.7)	153(9.6)
5% Mixed fat	195(12.4)	187(12.7)	142(6.6)	169(12.4)
5% Cod liver oil	158(11.0)	123(11.6)	116(7.2)	144(12.4)
5% Hydrogenated coconut oil[2,3]	250(10.1)	160(9.8)	123(5.4)	169(8.4)

[1] Figures in parentheses show average weight gain in grams.
[2] Hydrol.
[3] All figures are an average of values obtained with two groups of 8 mice each.

TABLE 6

Comparison of anti-cholic acid effect of lithocholic acid and chenodeoxycholic acid in 4-day test

Dietary supplement	Plasma cholesterol
	mg/100 ml
5% Hydrogenated coconut oil[1]	136
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol	177
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol + 0.1% chenodeoxycholic acid	147
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol +0.1% lithocholic acid	134
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol + 0.5% lithocholic acid	128

[1] Hydrol.

fluence this balance and in so doing alter the plasma cholesterol concentration. For example, Gordon et al., ('57a, b) have reported an increase in total bile acid excretion in man after the administration of sunflower seed oil with its high content of linoleic acid and this result has been confirmed by Haust and Beveridge, ('58) with linoleic acid alone, but with the further observation that this increase could be accounted for by an increase in the dihydroxycholanic acid fraction. Eriksson, ('57) has found that a thyroid-active material, which is known to influence plasma choles-

TABLE 7

Concentrations of plasma cholesterol (mg/100 ml) of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in combination with graded levels of selected bile acids in 12-day test

Dietary supplement	Percentage of bile acid in diet					
	0.0	0.025	0.05	0.1	0.2	0.4
	Exp. 1, 10% Hydrogenated coconut oil[1]					
Lithocholic acid	288(6.2)[2]	250(6.0)	224(8.7)	186(6.7)	177(7.1)	175(6.0)
Hyodeoxycholic acid	—	224(5.6)	192(8.2)	177(8.2)	145(7.1)	110(5.9)
Chenodeoxycholic acid	—	257(7.0)	214(7.2)	219(8.8)	191(5.9)	—
	Exp. 2, 0.5% Linoleic acid					
Chenodeoxycholic acid	246(10.3)	—	—	—	171(10.5)	—
Hyodeoxycholic acid	—	—	—	—	128(10.3)	—

[1] Hydrol.

[2] Figures in parentheses indicate weight gain in grams.

terol in some species, increases the ratio of chenodeoxycholic acid to cholic acid in the rat.

On the other hand, hyodeoxycholic as far as is known arises only through bacterial action on hyocholic acid in the intestinal tract of the pig (Bergström et al., '59). It is possible that lithocholic and chenodeoxycholic acids may exert their effects through bacterial transformation to unidentified products. Germ-free rats have been found to possess higher concentrations of blood cholesterol than normal controls despite the fact that Norman and Sjoväll ('58) could find no evidence that the total quantity of unabsorbable transformation products was increased in the latter.

The observation that taurocholic is less hypercholesterolemic than unconjugated cholic acid and that taurine decreases the hypercholesterolemic effect of cholic acid may open an attractive avenue of research. It is known that enzyme systems exist for conjugation and hydrolysis of cholic acids. Factors which influence these reactions may be of importance in regulating blood cholesterol. In a like manner, that acetylation of cholic acid neutralizes its activity may also be important. While no similar enzyme systems are known for this acetylation, that bile salts are required for the esterification of cholesterol with fatty acids has been established (Swell and Treadwell, '50). It has been suggested that this reaction is brought about by a primary esterification of the bile salt followed by transesterification. More than molar quantities of bile salt are known to inhibit cholesterol esterification (Swell et al., '53). Again if such a system existed, a study of the factors which influence it could be important

SUMMARY

From studies carried out in the weanling mouse, it was concluded that:

1. Taurocholic acid is less hypercholesterolemic than cholic acid.

2. Acetylation of methyl cholate in the three and 7 poistions eliminates its hypercholesterolemic action.

3. Lithocholic acid exerts a hypocholesterolemic effect and neutralizes the action of hypercholesterolemic bile acids.

4. Chenodeoxycholic acid has much the same action as lithocholic acid while hyodeoxycholic is more effective than lithocholic or chenodeoxycholic acid in neutralizing the hypercholesterolemic effect of cholic acid.

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Dietary Bile Acids and Lipid Metabolism II. The Ductular Cell Reaction Induced by Lithocholic Acid.* (28300)

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Proliferation of bile ductules occurs in a number of spontaneous and experimentally induced hepatic diseases in man and animals. The proliferation, generally associated with the infiltration of inflammatory cells, has been designated as the "ductular cell reaction"(1). In man, it is a frequent lesion in obstructive jaundice, post necrotic cirrhosis, acute and subacute viral hepatitis, and acute and subacute alcoholic injury(1,2). In experimental animals, it is an especially prominent finding in ethionine, butter yellow, thioacetamine, senechio, and dimethylnitrosamine intoxications and avitaminosis A(1,3). The reaction has also been observed in rabbits following long term ingestion of desiccated bile(4). The active agent of bile has been shown to be lithocholic acid(5,6). In studies to determine the influence of dietary bile acids on cholesterol metabolism in chicken, we observed a marked increase in size of the liver in chickens fed lithocholic acid. Microscopic examination revealed the increase in liver size to be the result of the ductular cell reaction.

Materials and methods. A total of 139 chickens from 2 experiments were fed a basal diet described in Table I. At the expense of glucose, bile acids and/or cholesterol were added to the basal diet at a level of 0.2% and 2%, respectively, as depicted in Table II. The chickens were fed the experimental diets for the time periods indicated in Table II, then all of the birds except 32 (8 each from groups 9, 10, 11 and 12) were sacrificed. The remaining chickens at this time were fed the basal diet without additives for an additional 3 weeks prior to sacrifice.

All of the chickens were necropsied immediately after sacrifice. The liver was weighed and thin slices of liver, gall bladder, heart, aorta, lung, spleen, small intestine and kidney were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 μ in thickness, and stained with Harris' hematoxylin and eosin. Duplicate tissue sections of liver were also stained by Masson's trichrome, Gomori's iron and Giemsa's stains, and subjected to the periodic acid-Schiff (PAS) reaction preceded by diastase digestion.

Results. Gross findings. The liver was the only organ in which gross alteration was observed. In the birds fed lithocholic acid alone, or in combination with cholic acid or cholesterol (Groups 3, 4, 7, 11), the liver was enlarged up to 3.5 times that of the control chickens (Table II). In birds fed all 3 of the above additives (Group 8) there was no significant increase in liver weight. The surface of the enlarged livers was slightly nodular.

Microscopic findings. No lesions were observed in the control birds or the chickens fed cholic acid, deoxycholic acid or cholesterol alone or in combination (Groups 1, 2, 5, 7, 9, 10, 12). In the chickens fed lithocholic acid alone, or in combination with cholic acid or cholesterol (Groups 3, 4, 7, 11), there was a marked periportal proliferation of bile ductules and interlobular bile ducts throughout the liver (Fig. 1, 2). The newly formed biliary tissue existed as patent

TABLE I. Composition of Basal Diet.

Component	g/100 g diet
Glucose	54.99
Assay protein C-1	20.40
Salt mix	5.31
Corn oil	5.00
Non-nutritive fiber	4.00
Glycine	.40
DL-methionine	.30
Vitamin mix	.40
Choline chloride	.20

* The results of these studies with regard to growth and plasma and liver lipids will be reported separately.

TABLE II. Additives to Basal Diet and Liver Weight.*

Group	No. chickens	Weeks on diet	Additive	Liver wt†	
1	11	8	None	2.74 ± .39	
2	9	8	Cholic acid	2.57 ± .23	
3	8	8	Lithocholic acid	6.45 ± 1.44	
4	6	8	Cholic acid + lithocholic acid	8.16 ± 1.76	
5	11	8	Cholesterol	3.03 ± .26	
6	11	8	Cholic acid + cholesterol	3.52 ± .28	
7	7	8	Lithocholic acid + cholesterol	11.08 ± 4.91	
8	12	8	Lithocholic acid + cholic acid + cholesterol	3.69 ± .33	
9	16	3	None	2.2 ± .2	2.6 ± .4‡
10	16	3	Cholic acid	2.1 ± .3	2.6 ± .4‡
11	16	3	Lithocholic acid	8.5 ± 2.0	3.2 ± .3‡
12	16	3	Deoxycholic acid	2.2 ± .3	2.6 ± .3‡

* Bile acids and cholesterol were added at a level of 0.2% and 2% of the diet respectively, at the expense of glucose.

† Mean liver weight expressed at % total body wt ± standard deviation.

‡ % liver weight after returning the birds to the basal diet for an additional 3 weeks; see Materials and Methods.

tubules and solid islands and cords of cells without obvious lumens (Fig. 3). These structures extended in finger-like projections from the enlarged portal tracts into the hepatic lobules. In some cases the proliferated bile ductules extended and joined with similar proliferated bile ductules originating from other hepatic triads. The proliferated bile ductules and cells were supported by mature collagenous connective tissue which was in greater abundance than that which surrounds bile ducts in the normal liver. Numerous polymorphonuclear leucocytes infiltrated and surrounded the proliferated bile ductules. Small collections of lymphocytes were occasionally seen, but similar collections were seen in the livers of the control chickens. An occasional mitotic figure was observed in the hyperplastic epithelial cells. Large bile ducts, and the gall bladders appeared normal. Bizzare hepatocytes were not observed, nor were mitotic figures seen in hepatocytes. However, focal hepatic necrosis was observed in some of the affected livers. No unusual nonglycogenic PAS positive staining materials were observed in the abnormal livers. Minimal to extensive deposits of iron positive material were present in the livers of birds from all groups. There did not appear to be an increase in stainable iron in the abnormal livers.

The extent of the ductular cell reaction was approximately the same whether lithocholic acid was fed for 3 or 8 weeks. However, the reaction was more extensive in chickens fed lithocholic acid in combination with cholic acid or cholesterol than in those receiving lithocholic acid alone. In the chickens fed the combination of lithocholic acid, cholic acid and cholesterol, the reaction was absent to minimal in 10 birds and in 2 birds of the degree as seen when lithocholic acid was fed alone.

In the 8 chickens fed lithocholic acid for 3 weeks and then returned to the basal diet for 3 weeks prior to sacrifice, the ductular cell reaction was minimal. The portal triads were more cellular than normal, and short projections of cells extended from them into the hepatic lobules. These cells resembled fibroblasts, but were interpreted as ductular cells (interstitial cells, oval cells).

Discussion. The ductular cell reaction as seen in man and experimental animals consists of the proliferation of bile ductules and epithelial cells not forming obvious tubules with lumina. These latter cells have been called interstitial cells(1), oval cells(7), and more preferably ductular cells(8,9,10). Electron microscopic observations support the concept that these cells are derived from ductular and ductal epithelial cells(2). In addition to the proliferation of ductular cells, accumulation of inflammatory cells is a feature of the ductular cell reaction(9). The reaction in the chicken, as described here,

has the same characteristics as that which occurs in other experimental animals and man. Although the ductular cell reaction is known to occur in several diseases and experimental intoxications, the pathogenesis is not entirely understood. It has been suggested by Popper and Schaffner(8,11) that the reaction may be the result of excretion of an irritating material into the ductules and periductular tissue which serves as a stimulant for ductular proliferation and incites the inflammatory cell reaction and periductular fibrosis. In the experiments reported here, the dietary lithocholic acid may represent this irritating material, or result in production of the irritant. The synergistic effect of cholic acid or cholesterol, neither of which produces the ductular cell reaction when fed alone or in combination, is not understood. It is also interesting that feeding the combination of lithocholic acid, cholic acid and cholesterol results in only a minimal reaction. There is some experimental evidence to suggest that the irritant may be the product of an antigen antibody complex in the periductular tissues(8,12). By immunohistochemical technics a non-glycogenic, periodic acid Schiff (PAS) positive antigen (mucopolysaccharide) has been shown to be present in ductule cells and bile in several types of liver diseases(8,12). This material may represent the irritant, or the antigen may elicit antibody formation and the irritant material may be the product of an immunologic reaction. It is possible that toxic chemicals (butter yellow, ethionine, etc.) damage hepatocytes or ductule cells with the production of the PAS positive antigen. However, in the chicken livers with the ductular cell reaction, abnormal deposits of non-glycogenic PAS positive material were not observed.

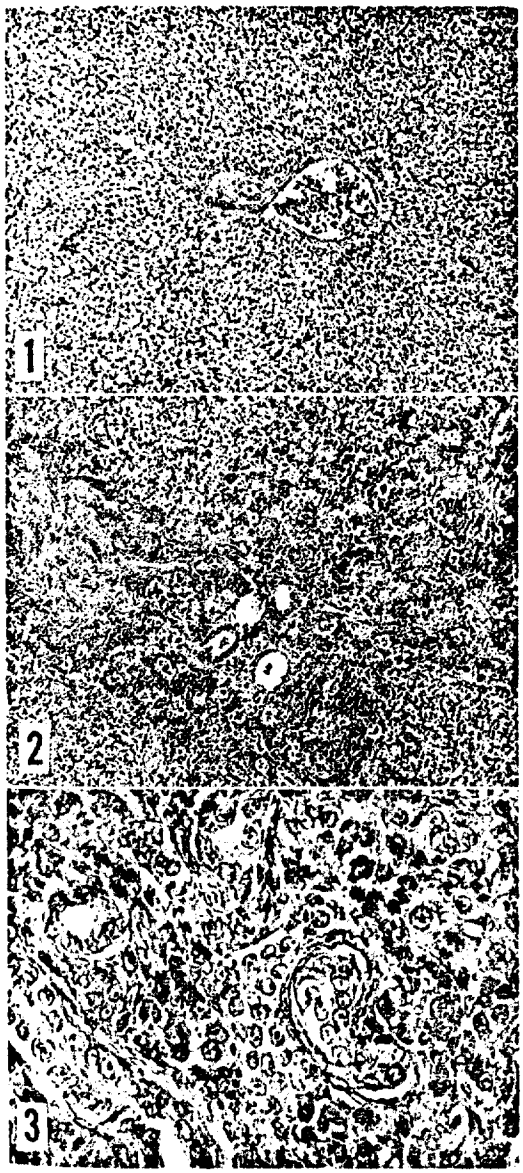

FIG. 1. A normal hepatic triad in liver of a chicken fed the basal diet without additives. Hematoxylin and eosin (H&E) stain. × 75.

FIG. 2. Proliferation of bile ductules in liver of a chicken fed the basal diet to which lithocholic acid (0.2%) was added. The dark staining cells are polymorphonuclear leukocytes. H&E stain. × 75.

FIG. 3. Proliferated ducts, ductules and ductular cells (oval cells) in liver of a chicken fed the basal diet to which lithocholic acid (0.2%) and cholic acid (0.2%) were added. H&E stain. × 375.

Whatever the mechanism involved in the study reported here, it is dependent on the dietary presence of lithocholic acid since removal of this bile acid from the diet results in regression of the proliferated bile ductules, the accompanying fibrosis and the inflammatory cells.

Summary. Dietary lithocholic acid alone and in combination with cholic acid or cholesterol has been shown to induce the ductular cell reaction in the livers of chickens.

Dietary cholic acid, deoxycholic acid, or cholesterol did not alter liver structure. Removal of lithocholic acid from the diet resulted in regression of the reaction.

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Factors Influencing Cholesterol Absorption[1]

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THE FIRST clear demonstration of some of the factors concerned in the absorption of cholesterol was made by Mueller (1, 2) who used the dog. It was found that cholesterol is readily absorbed from the intestine into the chyle and may be found in the thoracic duct. When given as the free sterol a portion is esterified and when given in the form of an ester a portion is hydrolyzed, so that cholesterol is absorbed with approximately the same proportion of free to esterified cholesterol as is found normally in the chyle. He also showed that the bile and pancreatic secretions were intimately connected with cholesterol absorption. A definite esterase appeared to be concerned with the change of cholesterol to cholesterol ester (3).

It has been suggested that neutral fat is necessary to provide fatty acid for cholesterol absorption. In fact, evidence has been presented to support the view that cholesterol absorption would take place only in the presence of fat, and on a fat free diet, the cholesterol fed could be recovered quantitatively from the feces (4, 5). Dubach and Hill (6), however, found that cholesterol was absorbed equally well by the rabbit whether or not neutral fat was added to the ordinary rabbit chow. More recently Bollman and Flock (7) studied intestinal lymph in rats and found no difference in cholesterol absorption with or without fat in the diet. To resolve this confusion was the main objective in this study.

METHODS

Throughout these experiments Holtzman-Rolfsmeyer strain young rats (70-95 gm. initial weight) were used. For cholesterol balance studies they were kept in individual cages and all, irrespective of sex, were given the same number of calories of rat food made up into a paste with water to prevent spilling. The weight of diet that would be completely consumed by all the animals was determined by gradually decreasing each day for a brief period the amount given until complete consumption resulted. As they grew a commensurate increase in the amount of food was likewise attempted. This was necessary in order to insure the intake of known amounts of cholesterol and fat in the diet. The composition of the basal diet used throughout these experiments is shown below.

COMPOSITION OF BASAL DIET (B.D.)

Casein (vitamin free)	23	Supplemented with 1 drop (70 U.S.P. units of vitamin A) of cod-liver oil every 4 days to each rat.
Corn syrup powder	72	
Salt mixture	5	
Yeast	7.5	
Fat content	0.05%	As a bulk, 0.5 gm. of nonnutrient cellulose was added to each daily ration.
Caloric value	354 (per 100 gm. of diet)	

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Feces were collected over specific fixed periods and dried to constant weight for the first series at 100°C. in the oven and for the rest of the experiments at 70°C. in a vacuum oven. Dried feces were finely pulverized and extracted in a Soxhlet apparatus with ether for 48 hours. Preliminary experiments to measure the time for complete extraction were made and 48 hours was found to be optimal.

TABLE 1A. DAILY LIPIDS EXCRETION PER GROUP (8 RATS/GROUP)

PERIOD	NO. OF TESTS		GROUP	II	III	IV	V
I 4 weeks	2	Daily diet/group, gm.	Basal diet	80	80	74.4	74.4
			Cholesterol	0	1.0	0	1.0
			Corn oil	0	0	2.0	2.0
		Fecal lipids, mg/8 rats/day	Total lipids	227	1021	236	1109
			U.M.[1]	109	814	120	841
			Sterols	69	778	90	800
		A.C.A.[2]			291		290
		% Absorption			29.1%		29.0%
II 2 weeks	3	Daily diet/group, gm.	Basal diet	96	96	85.6	85.6
			Cholesterol	0	0.5	0	0.5
			Corn Oil	0	0	4.0	4.0
		Fecal lipids, mg/8 rats/day	Total lipids	235	661	298	753
			U.M.	113	509	162	537
			Sterols	59	459	94	491
		A.C.A.			100		103
		% Absorption			20%		20.6%
III 2 weeks	4	Daily diet/group, gm.	Basal diet	96	96	80	80
			Cholesterol	0	0.25	0	0.25
			Corn oil	0	0	6.0	6.0
		Fecal lipids, mg/8 rats/day	Total lipids	253	527	328	504
			U.M.	115	337	171	293
			Sterols	56	261	110	220
		A.C.A.			45		140
		% Absorption			18%		56%

[1] Unsaponifiable material. [2] Apparent cholesterol absorption (mg.).

After weighing the ether soluble material as the total lipids, the lipids were saponified with 10 per cent alcoholic KOH and the nonsaponifiable materials were extracted with ether. Cholesterol was determined by means of the Liebermann Buchard reaction modified by Kingsley and Schaffert (8).

In order to test the validity of the use of the Liebermann-Buchard reaction for fecal sterol determination, a comparative study between the colorimetric method and the Windous gravimetric method, using digitonin, was made. The digitonin method measures total sterols including cholesterol, coprosterol and dihydrocholesterol whereas the colorimetric method measures mainly cholesterol, but coprosterol and dihydrocholesterol are known to give a feeble color. Table 2 shows that the colorimetric method can be satisfactorily used for the fecal sterols determination and also that

the fecal sterols excreted from rats on the diet used in this study are almost all cholesterol which is in good agreement with Rosenheim and Webster (9), as the diets used in this study lack the dietetic factor, phrenosin, which is responsible for the conversion of cholesterol into coprosterol.

When the fecal lipids were brought into solution with alcoholic KOH for saponification, solubility of the samples was observed to vary with the diet. Samples from the rats on the lipid free diet dissolved completely in alcoholic KOH whereas samples from rats on the diet containing corn oil or oleic acid, particularly the latter, were not very soluble in alcoholic KOH. Acetone was tried as a solvent for these samples which showed poor solubility and the presence of phospholipid was indicated. Therefore, phospholipid determinations were carried out on some of the samples using the Fiske and Subbarow method (10).

Observations

***Series I.* Role of Fat in the Absorption of Cholesterol.** Forty rats equally divided between the sexes and weighing approximately 75 gm. each were divided into five groups. They were individually caged and fed according to the scheme shown in

Table 1B. Effect of diet on serum cholesterol

Group	Diet	Serum cholesterol, mg. %
I	B.D. + thiouracil	108±19
II[1]	Basal diet	74±13
III[1]	B.D. + cholesterol	88±17
IV[1]	B.D. + corn oil	76±13
V[1]	B.D. + corn oil + cholesterol	92±15

[1] The rats were killed at the end of *period III;* see table 1*A*.

table 1*A*. Feces were collected for a 3-day period, the 3-day period being concurrent for all five groups. During *period I*, which lasted 4 weeks, feces were collected for two (two test periods) 3-day periods. During *period II*, which lasted 2 weeks, feces were collected for three (three test periods) 3-day periods. During *period III*, which lasted 2 weeks, feces were collected for four (four test periods) 3-day periods. The total fecal sterols were determined. Table 1*A* shows the average daily lipids, unsaponifiable material, sterol excretion and the average apparent cholesterol absorption. The apparent cholesterol absorption (A.C.A.) was calculated by subtracting the amounts of sterol excreted from the amount of cholesterol ingested and by adding to the remainder the estimated control sterol excretion.

It will be noted in table 1*A* that the animals in group II excreted daily 69, 59 and 56 mg. of sterols, and 109, 113 and 115 mg. of nonsaponifiable material. These values were relatively constant over the total experimental period of 8 weeks.

Sterol excretion during period I. (Cholesterol, 1 gm. + fat, 2.0 gm. daily; C:F = 1:2.) During *period I*, which lasted 4 weeks, the endogenous excretion of sterol for group II was 69 mg.

In group III, which received daily 1.0 gm. of cholesterol but no fat during *period I* the daily excretion of sterol was 778 mg. The amount of sterol absorbed was 1000 mg. (ingested) − 778 mg. (excreted) = 222 + 69 mg. (endogenous excretion) = 291 mg. The basic assumption in this calculation is that the endogenous excretion of sterol remains the same when cholesterol is added to the diet.

In group IV, which received daily 2.0 gm. of fat (corn oil), the daily sterol ex-

cretion was 90 mg., or 21 mg. more than in group II. This increase of 21 mg. is due to sterols in the corn oil.

In group V, which received daily 1.0 gm. of cholesterol and 2.0 gm. of fat the daily sterol excretion was 800 mg. The amount of sterol absorbed was: 1000 mg. (ingested) — 800 mg. (excreted) = 200 + 90 mg. (control excretion) = 290 mg.

It should be noted that the sterol excretion in group III and group V was practically identical; in both groups 29 per cent of the cholesterol was absorbed. Thus, when the daily intake of cholesterol was 1.0 gm. and of fat 2.0 gm. (C:F = 1:2), the addition of the fat had no effect on the percentage of the apparent absorption of cholesterol.

Sterol excretion during period II. (Cholesterol, 0.5 gm. + fat, 4.0 gm. daily; C:F = 1:8.) Inspection of the data in table 1*A* for *period II* will show that the absorption of cholesterol was 20 per cent and that reducing the daily cholesterol intake from 1.0 to 0.5 gm. and increasing the fat intake from 2.0 to 4.0 gm. did not significantly affect the percentage of the apparent cholesterol absorption.

Sterol excretion during period III. (Cholesterol, 0.25 gm. + fat, 6.0 gm.; C:F = 1:24.) Inspection of the data in table 1*A* for *period III* will show that reducing the cholesterol and increasing the fat intake decidedly increases the percentage of the

TABLE 1C. EFFECT OF THIOURACIL ON FECAL LIPID EXCRETION IN RATS FED FAT FREE DIET

	NO. OF TESTS[1]	AVERAGE EXCRETION IN 3 DAYS, MG. Total lipids	Sterols
Control	4	360	79
Thiouracil[2]	4	395	75

[1] Four rats were used as a group in a test. [2] Dose, 100 mg. per kg. body weight per day.

apparent cholesterol absorption. It should be indicated that the foregoing percentages for cholesterol absorption are generally somewhat higher than in the series of experiments to be reported later. This is ascribed to improvement in experimental technique in the later studies.

Serum cholesterol in the groups in series I. At the end of each experiment, the animals were killed and the serum cholesterol was determined. The data are shown in table 1*B* as the average with the standard deviation for each group. When the data for groups II and IV, which groups were given no cholesterol, were combined and the data for groups III and V, which groups were given cholesterol, were combined, the difference in the serum cholesterol value was significant. Thus, the addition of cholesterol to the diet resulted in a rise in serum cholesterol, but the addition of fat to the diet with cholesterol under the prevailing experimental conditions did not significantly increase the serum cholesterol.

Effect of thiouracil on fecal excretion of sterols and on serum cholesterol with no fat or cholesterol in the diet. The animals in group I received thiouracil (0.1 gm./kg. of body weight daily) with the same basal diet which the animals in group II received. The thiouracil was given during the last 2 weeks of the total feeding period of 8 weeks. The data on serum cholesterol and the fecal excretion of sterols are shown in tables 1*B* and 1*C*, respectively. The addition of thiouracil significantly increased the serum cholesterol but did not affect the excretion of sterols in the feces. Thus, the level of serum cholesterol can be increased without significantly decreasing the endogenous fecal excretion of sterols.

***Series II.* Why Does Fat When Present in the Diet in a Large Amount as Compared to Cholesterol Decrease the Fecal Excretion of Sterols?** As shown in *series I*,

fat facilitates cholesterol absorption when it is given in a proportionally larger amount than cholesterol (24:1 ratio). This raises the question as to which component of fat, glycerol or fatty acids, is the factor that facilitates cholesterol absorption. As mentioned in the introduction, fatty acid has been suggested as the factor, but its facilitatory action in cholesterol absorption has never been demonstrated. So this second series of experiments was designed to determine the relative facilitatory action of corn oil, oleic acid and glycerol in cholesterol absorption.

Fifty rats weighing initially approximately 100 gm. were individually caged (two rats in a partitioned cage) and fed according to the following scheme. Free fatty acid and glycerol were administered in amounts approximately equal to those present in the amount of corn oil given.

PERIOD	DIET/RAT/DAY
1	8 gm. basal diet (practically fat-free)
2	8 gm. B.D. + 1.2 cc. corn oil
3	8 gm. B.D. + 1.2 cc. corn oil + 100 mg. cholesterol
4	8 gm. B.D. + 1.1 cc. oleic acid + 100 mg. cholesterol
5	10 gm. B.D. + 0.2 cc. glycerol + 100 mg. cholesterol
6	11 gm. B.D. + 0.2 cc. glycerol
7	9 gm. B.D. + 1.1 cc. oleic acid
8	11 gm. B.D.
9	11 gm. B.D. + 50 mg. cholesterol
10	10 gm. B.D. + 1.2 cc. corn oil + 50 mg. cholesterol
11	10 gm. B.D. + 1.1 cc. oleic acid + 50 mg. cholesterol
12	10 gm. B.D. + 1.2 cc. corn oil

Up to *period 7*, in each group the rats were fed the same diet for 6 days and the feces were collected during the last 4 days. From *period 8* to *12* the rats were fed the same diet for 4 days and the feces were collected during the last 2-day period. Feces from two rats in a partitioned cage were grouped as 1 sample in all the experiments in this series and an attempt was made to collect 10 samples in each period from the same group throughout the experiment but due to unsatisfactory food consumption, which occurred from time to time, this was not always achieved. Only 6 out of 10 samples were collected from the same group up to *period 7*. From *period 8* to *12*, all 6 samples were collected from the same groups and the sterols determined.

The apparent cholesterol absorption (A.C.A.) was calculated by the following formulas.

A. C. A. (*3*) = 800 mg.[3] − sterols excreted (*3*) + sterols excreted (*2*)[4]
A. C. A. (*4*) = 800 mg. − sterols excreted (*4*) + sterols excreted (*7*)[4]
A. C. A. (*5*) = 800 mg. − sterols excreted (*5*) + sterols excreted (*6*)[4]
A. C. A. (*9*) = 200 mg. − sterols excreted (*9*) + sterols excreted (*8*)[4]
A. C. A. (*10*) = 200 mg. − sterols excreted (*10*) + sterols excreted (*12*)[4]
A. C. A. (*11*) = 200 mg. − sterols excreted (*11*) + ½ sterols excreted (*7*)[4]

Parentheses indicate the period.

The data are summarized in tables 3*A* and 3*B*. The averages with the standard deviation are derived from 20 rats and 10 samples for each of the first seven periods and for six samples of the last five periods. It should be noted that oleic acid facilitates cholesterol absorption better than corn oil and that glycerol is inert. This without

[3] Four-day period for two rats (100 mg. × 4 day × two rats).

[4] Mean values for those periods were used in the calculation. Therefore variance for sterols excreted and for A.C.A. in a same period should be the same.

doubt indicates that the fatty acids are the active portion of fat molecule concerned in cholesterol absorption. Analysis of variance shows that the differences between corn oil, oleic acid and glycerol in facilitating cholesterol absorption are significant.

Phosphorus content of fecal lipids in relation to neutral fat and fatty acid ingestion. Since during the absorption of neutral fat or fatty acid the phospholipid in the chyle increases, it was decided to make an exploratory analysis of the fecal lipids for phosphorus obtained during the first seven periods.

As shown in table 3*C* the phosphorus content of the fecal lipids is many times higher with oleic acid than with corn oil in the diet and a trace or no phosphorus was found in the fecal lipids when the basal diet without corn oil of fatty acid was fed. Addition of cholesterol to the basal diet did not stimulate phospholipid excretion but when cholesterol was added to the diets which contained corn oil or oleic acid a further increase in phospholipid excretion resulted. These data show that the phospholipid excretion is related to the lipids in the diet and indicate further study of the subject.

Further study of effect of oleic acid on the absorption of cholesterol as reflected by the level of serum cholesterol. It was considered desirable to obtain more data toward

TABLE 2. COMPARISON OF STEROL DETERMINATION METHODS

DIET		KINGSLEY SCHAFFORT	WINDAUS
Without cholesterol	No. of tests	14	14
	Average cholesterol, mg.	122	103
	Difference		−14.9±12%
With cholesterol	No. of tests	16	16
	Average cholesterol, mg.	475	464
	Difference		−2.2±6.1%

substantiating the foregoing observation that oleic acid facilitated the absorption of cholesterol more than corn oil by determining the serum cholesterol level.

With this in mind at the end of the preceding experiment all the female rats in *series II* were divided into four groups and fed four different diets shown in table 4 for 3 weeks, and then they were killed. Serum cholesterol was determined and the results are shown in table 4. Analysis of variance of the data revealed a statistically significant difference between certain groups. Differences between the oleic acid fed groups (FA and FB) and the corn oil fed groups (FC and FD) and between the oleic acid fed group (FA) and the oleic acid plus cholic acid fed group (FB) were significant, but the difference between the corn oil fed group (FC) and the corn oil plus cholic acid fed group (FD) was not significant. These results support the previous finding that oleic acid facilitates cholesterol absorption better than corn oil, and in addition they show that cholic acid greatly potentiates the action of oleic acid but not of corn oil as judged by the elevation of the level of serum cholesterol.

***Series III.* Relation of Fat and Fatty Acid in the Diet to Fecal Phospholipid Excretion.** For the observations cited previously it was postulated that phospholipid excretion in the feces may be proportional to the amount of free fatty acid liberated from neutral fat in the intestine. The fact that oleic acid facilitates cholesterol absorption better than the fatty acid equivalent amount of corn oil suggests that corn oil might not undergo complete hydrolysis before its absorption, because if corn oil is completely hydrolyzed, one would expect the same degree of facilitation. The study of the phosphorus excreted in the fecal lipids provided support for this view, since

corn oil stimulated phospholipids excretion much less than oleic acid does, which suggests that only a fraction of the corn oil is hydrolyzed before its absorption. Furthermore, the observations made in the tests in *series I* demonstrated that fat facilitates cholesterol absorption only when fat is added to the diet to a certain rather high level (F:C = 24:1). This implies that the amount of fatty acid split from a small amount of corn oil is relatively so minute as compared to the fatty acid given as such that it causes no detectable difference in the absorption of cholesterol. On the other hand, corn oil contains only 30 per cent oleic acid; therefore it is impossible to estimate

TABLE 3. CHOLESTEROL ABSORPTION

PERIOD	1	2	3	4	5	6	7	8	9	10	11	12
Diet	Basal diet (B.D.)	B.D. + corn oil	B.D., corn oil, cholesterol	B.D., oleic acid, cholesterol	B.D., glycerol, cholesterol	B.D. + glycerol	B.D. + oleic acid	B.D.	B.D. + cholesterol	B.D., corn oil, cholesterol	B.D., oleic acid, cholesterol	B.D. + corn oil
	A. Cholesterol Absorption, Periods 1-7							*B. Cholesterol Absorption, Periods 8-12*				
Total lipids	109±18	378±92	1134±169	1321±151	967±116	183±29	764±73	95±23	265±42	290±40	518±155	174±42
U.M.	68±13	167±12	746±67	541±78	806±76	108±18	151±21	44±5	218±31	193±32	160±22	95±17
Sterols, mg.	38±16	111±15	649±65	426±59	752±70	69±15	69±12	19±5	198±30	155±16	105±19	52±12
Apparent cholesterol absorption			262±65	443±59	117±70				21±30	97±16	129.5±19	
% Absorption			32.8	55.4	14.6				10.5	48.5	64.8	
	C. Phosphorus Content of Fecal Lipids											
No. of samples tested	3	4	4	4	4	4	4					
Av. total lipids, mg.	112	383	1195	1388	933	200	768					
Av. phosphorus, mg.	0	1.95	4.7	17.8	0	0	9.1					
Range	0–trace	3.9–0.9	3.3–5.8	21.2–13.6	0–Trace	0–Trace	7.8–101.					

Note: these values are all 4-day periods/2 rats

the degree of hydrolysis of corn oil from the phospholipid data because the fatty acids other than oleic acid in corn oil may have a different effect on fecal phospholipid excretion, which was subsequently found to be the case. It is also possible that the fatty acids are absorbed as they are split from the corn oil and hence do not reach a concentration at which they improve the absorption of cholesterol.

The main object of the experiments in *series III* was to compare the relative potency of corn oil and the exact mixture of its constituent fatty acids in stimulating phospholipid excretion in the feces. The basic assumption of this series of experiments was that the effect of a mixture of the free fatty acids derived from corn oil on the excretion of phospholipid might be an index of the extent of the hydrolysis which corn oil undergoes in the intestine.

Experiments were conducted in a manner similar to those in *series II*. The rats were accordingly fed as in the following scheme:

PERIOD	DIET/RAT/DAY
1	B.D. + 1.2 cc. corn oil + 50 mg. cholesterol
2	B.D. + 1.1 cc. oleic acid + 50 mg. cholesterol
3	B.D. + 50 mg. cholesterol
4	B.D. + 1.0 cc. glycerol + 50 mg. cholesterol
5	B.D. + 1 gm. palmitic acid + 50 mg. cholesterol
6	B.D. + 1.1 cc. of corn oil fatty acids[5] + 50 mg. cholesterol
7	B.D. + 1.1 cc. of corn oil fatty acids
8	B.D. + 1.1 cc. of corn oil fatty acids + 0.15 cc. glycerol
9	B.D. + 1.2 cc. corn oil
10	B.D. + 1.0 gm. palmitic acid

Composition of corn oil fatty acids (11)

Linoleic acid	56.2
Oleic acid	30.1
Palmitic acid	9.9
Stearic acid	2.9
Above C_{18} saturated	0.2
Myristic acid	0.2
Hexadecenoic acid	0.5
	100.0

Besides these fatty acids, it contains also 1.5 to 2 per cent of unsaponifiable material as corn oil does.

Table 5 shows a summary of the fecal sterol excretion and the estimated apparent cholesterol absorption. During the first two periods, diets were fed for 4 days and feces were collected during the last 2 days of the period. For the remainder of the periods, diets were fed for 6 days and feces were collected for the last 4 days of the period. The results show that palmitic acid appears to be less effective in facilitating cholesterol absorption than either corn oil, oleic acid or the corn oil fatty acids. The effect of corn oil fatty acids is similar to that of oleic acid (compare with *series II*, table 3*B*).

The total phosphorus in the fecal lipids was determined on each sample and the results are shown in table 6. The average phosphorus excretion during *period 7 is* about 14 times greater than that during *period 9*, which indicates on the average only 7 per cent of corn oil is hydrolyzed before its absorption. Because if corn oil is completely hydrolyzed before its absorption, one would expect the same amount of phosphorus with corn oil and the corn oil fatty acids (on an equimolar basis). This, of course, assumes that the concentration of the fatty acid is not an important factor. Palmitic acid also stimulated phospholipid excretion but far less than the oleic acid or corn oil fatty acids did.

***Series IV.* Role of Bile Salts in Absorption of Cholesterol.** This series of experiments was actually conducted before *series II*, but because of its deviation from the main line of study, it has been placed here. The experiment was conducted similarly to that described under *series I*. Thirty-two rats were divided into four groups and fed according to the following scheme.

GROUP	II	III	IV	V
Diet/group/day	Basal diet	B.D. 1 gm. of cholesterol	B.D. 9.6 cc. of corn oil	B.D. + 9.6 cc. corn oil + 1 gm. cholesterol

Forty-five days after starting the experiment, desiccated thyroid (U.S.P. Abbott) was fed in a dose of 100 mg/rat/day for 5 days and the body weight change was measured 5 days later, results of which will be shown in *series VI*.

[5] Mixture of fatty acids obtained after hydrolysis of corn oil.

Two weeks after discontinuing thyroid administration, desoxycholic acid was fed to the rats in a dose of 110 mg/rat for a week to determine whether bile salts facilitate cholesterol absorption or not. Due to the toxic effect of desoxycholic acid, fecal samples were obtained only from group V and the sterol excretions before and during the administration of desoxycholic acid were compared. As shown in table 7, there appears to be no difference in sterol excretion between the two periods, indicating that administration of bile salts to normal animals does not further augment cholesterol absorption when neutral fat is fed in the diet. This, however, does not mean that bile salts are not necessary for the absorption of cholesterol.

Table 8 shows a summary of the toxic effects of desoxycholic acid. A marked sex difference is noted. The greatest protective effect was obtained with the diet containing both corn oil and cholesterol. Due to the observation of a marked sex difference in serum cholesterol levels on desoxycholate administration as shown in table 9, the following series of experiments were undertaken.

TABLE 4. EFFECT OF DIETS ON SERUM CHOLESTEROL LEVEL

GROUP		FA	FB	FC	FD
Basal diet plus/rat daily:	Cholic acid	0	100–50 mg.	0	100–50 mg.
	Cholesterol	100 mg.	100 mg.	100 mg.	100 mg.
	Corn oil	0	0	1.2 cc.	1.2 cc.
	Oleic acid	1.1 cc.	1.1 cc.	0	0
No. of rats		7	8	7	6
Serum cholesterol, mg. %		187±75	426±141	101±20	128±38

Series V. **Effect of Desoxycholate on Serum Cholesterol Level.** Thirty-two rats, half male and half female, weighing 150 to 200 gm. were caged, four rats to a cage. To half of the group of rats desoxycholic acid (100 mg/rat/day) was administered. Rats were killed at the 5th, 14th, 24th and 27th day and the serum cholesterol was determined. The results are shown in table 10. There is apparently no sex difference in the serum cholesterol level. Rats fed desoxycholic acid showed higher mean serum cholesterol levels than rats without desoxycholic acid in the diet; however, the difference is not statistically significant. It was thought that a sex difference in serum cholesterol levels might be demonstrated if the rats were pretreated with desiccated thyroid as in *series IV*. Therefore, a group of rats were pretreated with desiccated thyroid (100 mg/rat/day) for 5 days and 2 weeks later desoxycholate was administered daily in a dose of 100 mg/rat. Animals were killed at the 5th, 6th and 7th day after starting desoxycholate administration. Table 11 shows the results, which again show no sex difference in the serum cholesterol level. The rats were not caged individually and for this reason there occurred great variations in the serum cholesterol levels and due to this great variation, the mean difference between the groups with and without desoxycholate in the diet was not statistically significant. However, this is in accord with the results in table 4, *series II*.

Series VI. **Effect of Diets on the Toxicity of Thyroid Powder Manifested as Body Weight Loss.** This was to elucidate the role of cholesterol in hyperthyroidism since in hyperthyroid patients the serum cholesterol level is lower than normal and also since Barker *et al.* (12) reported that cholesterol is the most potent anticalorigenic agent he has found in thyroidectomized rats maintained at normal B.M.R.'s

with thyroxine. The rats used in *series IV* were fed four different diets described for the animals in *series IV*. Forty-five days after starting the experiment, desiccated thyroid (U.S.P. Abbott) was fed in a dose of 100 mg/rat/day for 5 days and the body weight change was measured 5 days later. As shown in table 12, cholesterol does not prevent the loss of body weight, but corn oil partially prevented this both in male and female rats. A marked sex difference was also noted.

Discussion

As indicated in the title the elucidation of the factors influencing the intestinal absorption of cholesterol is the main objective of this study. To accomplish this may be a life long task. In the observations presented here only the role of fat in cholesterol absorption is partly demonstrated.

Table 5. Sterols excretion

Period	Diet/rat/day	No. of tests	Av. sterol excretion, mg.[1]	% absorption	Period	Diet/rat/day	No. of tests	Av. sterol excretion, mg.[1]
1	B.D. 1.2 cc. corn oil 50 mg. cholesterol	2	186	33.0	7	B.D. 1.1 cc. corn oil fatty acids	5	86
2	B.D. 1.1 cc. oleic acid 50 mg. cholesterol	2	140	47.5	8	B.D. 1.1 cc. corn oil fatty acids 0.15 cc. glycerol	4	71
3	B.D. 50 mg. cholesterol	2	410	6.5	9	B.D. 1.2 cc. corn oil	4	106
4	B.D. 50 mg. cholesterol 1.0 cc. glycerol	2	415		10	B.D. 1 gm. palmitic acid	4	64
5	B.D. 1 gm. palmitic acid 50 mg. cholesterol	6	377	22.0				
6	B.D. 1.1 cc. corn oil fatty acids 50 mg. cholesterol	4	244	60.5				

Note: During *period 1* and *2*, the feces were collected for 2-day periods and during the rest of the period, the feces were collected for 4-day periods.

[1] Values for average sterol excretion are all for two rats for 4-day period except *periods 1* and *2*.

Balance Method. As Cook and Thomson (13) had pointed out, the balance method for studying absorption has some advantages but at the same time suffers from certain disadvantages. If there is additional synthesis or destruction in the gut of the constituent fed, then the results may be equivocal. It is well known that the breakdown of cholesterol occurs in rats (14) and in mice (15). If this breakdown of cholesterol occurs in the intestine before its absorption and is further intensified by the addition of fatty acids or corn oil, one would expect a decrease in sterol excretion in the feces without any absorption. This, however, is only a possibility and at the moment there is no evidence to support it. Therefore, discussion will proceed with the assumption that the breakdown of cholesterol is negligible in the intestine and that this is not influenced by adding fatty acids, corn oil or glycerol to a basal diet. A recent report by Kritschevsky *et al.* (16) appears to support this assumption.

Role of Fat. Results obtained in *series I*, *II* and *III* well illustrate that fat does facilitate cholesterol absorption and that fatty acid, part of the neutral fat molecule, is the active factor. Whether cholesterol ester formation with fatty acids is the indispensable step for its absorption or whether the solubility of cholesterol in fatty

acids is the important factor for its absorption is not clear from the results obtained in this study. Peterson's work (17) appears to support the former view. He found that chicks receiving sitosterols or a stigmasterol mixture in their diet showed a lower serum cholesterol level than those which did not receive these plant sterols. He has not offered an explanation for this. However, on the basis of this study the following explanation can be given. Sitosterols or stigmasterols have long been known not to be absorbed from the intestine (18) and it is also well known that stigmasterol or sitosterols form an ester with fatty acids in the presence of pancreatic enzymes. Therefore, it is quite possible that sitosterols or stigmasterols compete with cholesterol for fatty acids in the intestinal lumen, thereby decreasing available fatty acids for cholesterol absorption. Final evidence for this would come from a study of the fecal sterol excretion during plant sterol feeding. In either case the exact mode by which cholesterol ester or fatty acids pass through the wall of the intestinal epithelium is a separate question to be answered, which will be discussed later.

TABLE 6. TOTAL LIPIDS EXCRETION AND PHOSPHORUS CONTENT IN FECAL LIPIDS

PERIOD	3	4	5	6	7	8	9	10
Diet	B.D. + cholesterol	B.D., cholesterol, glycerol	B.D., palmitic acid, cholesterol	B.D., corn-oil, fatty acids, cholesterol	B.D. + corn oil-fatty acids	B.D., corn-oil, fatty acids, glycerol	B.D. + corn oil	B.D. + palmitic acid
No. of tests	2	1	6	4	5	5	6	4
Av. total lipids excretion, mg.	565	511	1995	1284	1161	1156	375	1171
Av. phosphorus, mg.	Trace	Trace	2.55 ±1.0	14.1 ±1.89	14.1 ±2.88	12.7 ±3.9	1.06 ±0.59	2.57 ±0.86

These values are for 4-day period per two rats.

Is fat completely hydrolyzed before its absorption? This has long been a moot question. The lipolytic or solution hypothesis teaches that this fat is completely hydrolyzed to glycerol and fatty acids which are absorbed by the intestinal epithelium, resynthesized chiefly into neutral fat which passes as chyle to the lacteals and thence the thoracic duct into the venous system (19). The emulsion or particulate hypothesis teaches that fat and fatty acids are absorbed in the form of very small droplets into the lacteals and that lipase serves chiefly to release some fatty acid which, with a little alkali and bile salts emulsifies the major portion of the fat (20, 21). The evidences obtained in this work may be interpreted as supporting the latter view, i.e. the particulate hypothesis. Thus, if corn oil is completely hydrolyzed before its absorption, the degree of facilitation of cholesterol absorption, and the amount of phospholipid excretion would be the same with corn oil as with an equivalent amount of corn oil fatty acids in the diet, which however was not the case. Furthermore, as found in the experiments of *series I*, a small amount of fat did not facilitate cholesterol absorption, implying that the amount of fatty acids split off from a small amount of fat is too minute to cause a detectable difference. This interpretation would appear to be reasonable unless it is found, as indicated before, that the concentration of fatty acid in the intestine is an important factor, a very likely possibility.

Oleic acid or the corn oil fatty acids facilitate cholesterol absorption far better than palmitic acid does. This facilitatory action of fats and fatty acids appears to be

related to the stimulatory action of fats and fatty acids on phospholipid excretion in the feces. This is quite interesting in the light of the fact that cholesterol forms an ester in the blood mainly with unsaturated fatty acids (22) and that the ratio of unsaturated to saturated fatty acids in the tissue phospholipid molecule in the rat is 2 to 1 (23). Further studies with other saturated and unsaturated acids and with long and short chain fatty acids should reveal a more definite picture in this respect.

In regard to the phospholipid excretion in the feces almost no information is available in the literature. Bloor (24) in his monograph stated that there is not more than a trace of phospholipid in the feces. This was found to be the case when a fat free diet was given and the addition of cholesterol or glycerol did not increase the fecal phospholipid excretion as shown in *series II* and *III*. But when corn oil was added to the fat free diet, there appeared appreciable amounts of phospholipids in the feces and the addition of oleic acid or corn oil fatty acids increased phospholipid excretion further. However, palmitic acid, saturated fatty acid, showed only a mild stimulatory action on phospholipid excretion.

TABLE 7. EFFECT OF DESOXYCHOLATE ON STEROL EXCRETION

DIET/RAT DAILY	CONTROL: B.D., 1.2 CC. CORN OIL, 125 MG. CHOLESTEROL	DESOXYCHOLATE: B.D., 1.2 CC. CORN OIL, 125 MG. CHOLESTEROL, 110 MG. DESOXYCHOLATE
Test no. 1	1270	1140
2	1045	925
3	1010	1205
4	1000	1000
Av., mg.	1081	1068

These values are for 3-day period per four rats.

It was postulated that the ratio of phospholipid in the feces with corn oil (neutral fat) in the diet to that with corn oil fatty acids in the diet may be a measure of corn oil hydrolysis in the intestine. The validity of this hypothesis is not fully attested by the results obtained. If this hypothesis is found to be correct, it can be said that an average of only 7 per cent of the fatty acids are split off from the corn oil before its absorption, because corn oil has only one-fourteenth the potency of an equivalent amount of the corn oil fatty acids in stimulating phospholipid excretion. Recently using labeled glycerol and linoleic acids, Reiser *et al.* (25) demonstrated that approximately 24 to 45 per cent of the ingested glycerides was completely hydrolyzed during absorption and the remaining 55 to 75 per cent of the ingested glycerides was hydrolyzed to monoglyceride. The triglycerides they used were artificially made; therefore it is quite possible that the degree of hydrolysis of this artificial product may be different from those occurring naturally such as corn oil which was used in this study.

Role of Bile Salts. Schoenheimer (26) showed that the cholesterol content of rabbit serum is increased much more after feeding cholesterol with desoxycholic acid than without. His work has been considered to provide good evidence that bile salts facilitate cholesterol absorption. However, no evidence from balance studies has been presented on the role of bile salts in cholesterol absorption. Therefore, the experiment of *series IV* was undertaken and it was demonstrated that desoxycholate did not facilitate cholesterol absorption when corn oil was used in the diet (table 7). This is also well supported by the results in tables 4, 10 and 11 showing that bile salts (desoxycholate and cholate) did not significantly raise serum cholesterol level

above that of the control group. But when oleic acid was used in the diet, cholic acid induced a significant rise in serum cholesterol level over that of the control group (table 4), indicating that cholate facilitates cholesterol absorption in this condition. The explanation of this may be as follows: when corn oil is given in the diet, only a small fraction of fatty acids is split off from corn oil and forms a small amount of cholesterol ester in the intestine, which can be sufficiently taken care of by the bile salts physiologically secreted into the intestine. Therefore, addition of bile salts in this case would have no effect on cholesterol absorption. But when fatty acids are given with cholesterol, all the cholesterol given could be esterified with fatty acids. The amount of cholesterol ester thus formed is so large that the amount of bile salts normally secreted into the intestine may become insufficient to transport all of this cholesterol ester across the intestinal wall. This explanation also may hold for the

TABLE 8. EFFECT OF DIETS ON TOXICITY OF DESOXYCHOLATE

GROUPS		II	III	IV	V
Diet		B.D.	B.D. + cholesterol	B.D. + corn oil	B.D., corn oil, cholesterol
No. of rats	♂	5	4	5	5
	♀	3	3	3	3
No. of rats died	♂	4	3	1	0
	♀	0	0	0	0
Av. survival time of dead rats, days		3.5	5.3	5	
Av. body wt. change of the rats which survived, gm.	♂	−1	−7	−3	+5.4
	♀	+4.3	−2	0	+9
Av. serum cholesterol level of the rats which survived, mg. %	♂	80	50	70	70
	♀	92	235	95	198

following fact. In spite of adequate supply of fatty acids available for esterification of cholesterol given in *series II*, *periods 4* and *11*, and also in *series III*, *period 7*, a considerable amount of cholesterol escaped from being absorbed, which may be attributed to an insufficiency of bile salts. This, however, fails to explain the fact that in *period II* (*series II*) cholesterol in an amount less than that absorbed in *period 7* (*series II*) was given in the diet but still a considerable amount of cholesterol appeared in the feces. It is possible that a large portion of the cholesterol absorbed may be re-excreted in the feces.

If bile salts facilitate cholesterol absorption, how may this be achieved, is the next question to be answered. In 1916 Wieland and Sorge (27) found that among the bile acids desoxycholic acid is the best solvent for cholesterol and proposed the so-called 'choleic acid principle' to explain the absorption of fatty acids and cholesterol. But this simple solvent action of bile salts appears not to be responsible for the facilitation of cholesterol absorption, because addition of desoxycholate to a corn oil diet did not increase the absorption of cholesterol as shown in *series IV*, table 7. Verzar (28) stated that fatty acids form a water soluble compound with bile salts and are absorbed as such. This mechanism of fatty acid absorption may apply to the absorption of cholesterol ester or fatty acid droplets which contain cholesterol in solution

There is good indication that bile salts may form a chemical combination with cholesterol before absorption from the intestine. Hummel (29) showed that sitosterols lessened the toxic effect of bile salts in mice and Schoenheimer *et al.* (30) found that sitosterols decreased the accumulation of cholesterol in the liver when bile salts were fed, indicating that bile salts may combine with sitosterols and be excreted in the feces thereby preventing the absorption of bile salts from the intestine. If bile salts are all absorbed in animals fed sitosterols, one can hardly explain how sitosterols, which are known not to be absorbed from the intestine, can exert a beneficial effect on the toxicity of bile salts.

Although statistical analysis indicates that desoxycholate does not cause a significant increase in serum cholesterol level as shown in *series V*, there is a definite

TABLE 9. SERUM CHOLESTEROL LEVEL OF RATS FED DESOXYCHOLATE

DIET	GROUP	MALE, MG. %	FEMALE, MG. %
Without cholesterol	II and IV	72 ± 26.2(5)[1]	93 ± 17.7(6)
With cholesterol	III and V	67 ± 17.6(6)	213 ± 110(5)

[1] Figure in parentheses indicates number of rats tested.

TABLE 10. EFFECT OF DESOXYCHOLATE ON SERUM CHOLESTEROL

DIET/RAT	CONTROL: B.D., 1.2 CC. CORN OIL, 100 MG. CHOLESTEROL		DESOXYCHOLATE: B.D., 1.2 CC. CORN OIL, 100 MG. CHOLESTEROL, 100 MG. DESOXYCHOLATE	
	♂	♀	♂	♀
Day after starting the exper.				
5th	115	125	130	150
	130	145	115	150
14th	160	145	160	170
	115	145	100	125
24th	120	215	175	340
	210	100	240	165
27th	155	105	165	200
	130	135		550
Av., mg. %	142±32	139±35	155±47	231±145

tendency toward an increase. Recently Friedman and Byers (31) showed that intravenous injection of natural bile salts caused a significant rise in serum cholesterol level in fasting rats. (The article made no statement regarding hemolysis.) Recently (32) they again showed that the hypercholesteremia produced by biliary obstruction is associated with an accumulation of bile acid in the blood. If the hemolytic effect of bile salts, which they failed to eliminate as a contributory factor, is not wholly responsible for this rise, it may be due to either blocking the catabolism of cholesterol or to increasing the synthesis of cholesterol in the body. It is difficult to believe that cytotoxic substances such as natural bile salts would increase the synthetic activity of the cell. In this respect thiouracil resembles bile salts because thiouracil raised serum cholesterol of group I in *series I*, which received neither fat nor cholesterol in the diet.

In regard to the toxicity of desoxycholate, table 8 well illustrates the 'antitoxic' action of corn oil and of cholesterol. Cholesterol alone has only a slight, if any, preventive effect whereas cholesterol plus corn oil shows the greatest protective effect as shown in group V, table 8. Horrall (33) has described this action of cholesterol.

Earlier Tashiro and Oliver (34) reported that glycerol and cholesterol had no antagonistic action on bile salts and did not inhibit the ulcer forming power of the bile salts. However, Tsuruta (35) found that when stearic acid, crude phosphatides, lecithin, or cholesterol oleate was added to the bile before injecting into guinea pigs, this protected against the formation of peptic ulcer. Tsuruta also reported the sex difference in susceptibility to the toxicity of bile salts, which is in accord with the results shown in table 8. This sex difference may be closely related to the difference in lipid metabolism between the sexes. One could speculate that bile salts, when absorbed into the blood as free acids, exert a toxic effect but in combination with fatty acid the toxic effect becomes less and in combination with cholesterol ester it becomes least.

TABLE 11. EFFECT OF DESOXYCHOLATE ON SERUM CHOLESTEROL

DAY AFTER STARTING ADMINISTRATION OF DESOXYCHOLATE	CONTROL		DESOXYCHOLATE	
	♂	♀	♂	♀
5th	170	165	295	230
	190	160	240	355
			215	
6th	230	150	215	280
	180	150	220	195
			1100	
7th	175	170	240	290
	215	170	160	200
			240	
			240	
			210	
Av., mg. %	193±24	161±9	307±265[1]	258±62

[1] This large variation is due to a large deviation of 1100 from the mean.

Note on the Toxic Effect of Thyroid Powder. Barker (12) has recently reported that cholesterol is one of the most potent antithyroxine agents. Stimulated by this report, the *series VI* experiment was undertaken. The results (table 12) show that cholesterol has no effect in preventing the body weight loss induced by the administration of desiccated thyroid in contrast to the Barker's finding with injected cholesterol. Corn oil, however, prevented to a considerable extent the loss of body weight. Loumos (36), after studying the relation of dietary fats to the action of desiccated thyroid in rats, concluded that all fats do not bear the same relation to desiccated thyroid action, namely some fats such as cod liver oil, olive oil and lard antagonize the action of thyroid extract in various degrees and others such as cocoanut oil and Crisco do not. However, this author did not indicate the sex of the rats used in his study. A marked sex difference in response to the desiccated thyroid is well demonstrated.

SUMMARY

Fat in adequate quantities facilitated the absorption of cholesterol as determined by the difference between dietary and fecal sterol levels. The fatty acid portion of the neutral fat molecule was found to be the active factor which facilitates cholesterol absorption. The glycerol portion was without effect. Serum cholesterol levels were significantly higher in the groups of animals receiving cholesterol and oleic acid than

in those receiving cholesterol and corn oil. Cholic acid added to the diet significantly raised the serum cholesterol levels in rats receiving cholesterol and oleic acid. Cholic acid or desoxycholic acid added to the diet raised serum cholesterol levels only slightly in rats receiving cholesterol and corn oil, but the increase was not statistically significant. Addition of extra desoxycholic acid to the diet containing corn oil did not increase cholesterol absorption.

Excretion of phospholipid in the feces varied with the diet. When a fat free diet was fed, only a trace of phospholipid was found in the feces, and the addition of cholesterol or glycerol to a fat free diet did not alter the phospholipid excretion. But when corn oil was fed there appeared appreciable amounts of phospholipid in the feces and this was further increased by feeding various free fatty acids. Oleic acid was more potent than palmitic acid in stimulating phospholipid excretion. The addition to the diet of free fatty acids derived from corn oil increased the fecal phospholipid excretion approximately 14 times above the level seen when an equivalent amount of corn oil was fed. From this finding it was estimated that 7 per cent of the corn oil fatty acids was split off in the intestine prior to absorption.

TABLE 12. EFFECT OF DIETS ON TOXICITY OF DESICCATED THYROID

GROUP		II	III	IV	V
Diet		B.D.	B.D. + cholesterol	B.D. + corn oil	B.D., corn oil, cholesterol
Av. body wt. loss, gm.	♂	−22.4(5)[1]	−21.75(4)	−2.8(5)	−8.8(5)
	♀	−5(3)	−6.3(3)	−2.67(3)	−0.6(3)
Range of body wt. change	♂	−17 ↔ −30	−9 ↔ −32	+12 ↔ −8	−1 ↔ −12
	♀	3 ↔ −19	−3 ↔ −11	0 ↔ −4	+5 ↔ −4

Rats were fed on isocaloric basis.
[1] Figure in parentheses indicates the number of rats.

Thiouracil increased serum cholesterol significantly even when the diet contained neither fat nor cholesterol.

Cholesterol did not prevent the body weight loss induced by feeding thyroid powder. But corn oil was found to diminish the body weight loss considerably. There was noted a marked sex difference in response to thyroid powder.

Desoxycholic acid was found to be much more toxic to male than to female rats. Male rats could be protected by the following dietary additions in the order of their effectiveness: cholesterol, corn oil, and cholesterol plus corn oil.

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Letters to the Editors

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INFLUENCE OF BILE SALTS ON THE LYSIS OF ^{131}I-LABELLED PLASMA CLOTS IN HUMAN SUBCUTANEOUS TISSUE *IN VIVO*

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Bile salts, and particularly taurocholate, have previously been reported to release fibrinolytic activity in guinea-pig serum (Olesen 1965), but not in human plasma (Benedetti-Valentini & Luminari 1957).

The present investigation was an attempt to ascertain whether in the human organism it was possible to demonstrate an activation of fibrinolysis by bile salts *in vivo*.

As a measure of the fibrinolytic activity in the organism, the elimination of a subcutaneously opplied ^{131}I-labelled plasma clot was employed (Krøll 1966).

MATERIALS AND METHODS

A commercial preparation of sodium taurocholate (Georg T. Gurr, Ltd. London) was administered with meals in a dosage of 1.5 g per day to two normal aged male subjects (83 and 87 yrs.). In one of these the preparation had been given 3 weeks when the clot was applied, and medication continued during the whole study. In the other, medication was not initiated until 6 days after the application of the clot.

The fibrinogen preparation, ^{131}-labelling of the protein, preparation of the clotting mixture, application of the clot and the counting procedure were performed as previously described (Krøll 1966).

The control group consisted of 6 normal aged male subjects (74—84 yrs.).

RESULTS

The initial elimination rate (T/2) of the ^{131}I-labelled plasma clot was similar (approximately 6 days) in taurocholate medicated and control subjects. In the latter, however, the elimination curve remained monoexponential (A in Fig. 1) whereas in the former it apparently followed a biexponential course, the change in T/2 (to approximately 1.5 days) occurring about 4 days after the start of medication (B in Fig. 1), or with a

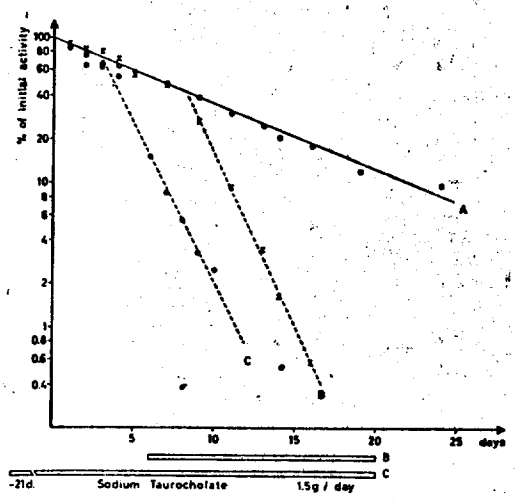

Fig. 1. The influence of sodium taurocholate on the disappearance rate of sbucutaneously applied ^{131}I-labelled plasma clots in 3 normal aged human subjects. *A* represent the control group, *B* and *C* taurocholate medicated as indicated.

similar lag period from sample application time in the subject on prolonged previous medication (C in Fig. 1).

DISCUSSION

A sustained increase in fibrinolytic activity, as noted in this study, has been observed also after the ingestion of other steroid substances (Fearnley 1965). How the effect on fibrinolysis is mediated is unknown, but bile salts have been previously demonstrated to accelerate tryptic activity (Christensen 1951), possibly by prolonging the action of trypsin (Elmslie 1965). A similar influence on plasmin activity might in part explain the significance of bile salts in fibrinolysis.

The concentration of bile salts required to induce fibrinolytic activity in serum (Olesen 1960) can not be expected to occur in the circulating blood (McIntyre & Wotton 1960) but might result locally from adsorption in fibrin deposits. That a time lag in fibrinolytic response apparently persists even after prolonged medication, also indicates a local rather than a systemic influence of bile salts. A similar mechanism has been suggested to explain the action of other thrombolytic agents (Fearnley 1965).

SUMMARY

Sodium taurocholate, in a dosage of 1.5 g per day, was found to cause a sustained increase in the fibrinolytic activity in two normal aged male human subjects. The elimination rate of a subcutaneously applied ^{131}I-labelled plasma clot was taken as a measure of the fibrinolytic activity *in vivo*.

That the fibrinolytic response apparently occurs with a time lag of about 4 days from the start of medication, and with a similar lag period from sample application in case of prolonged previous administration of the bile salt, indicates a local rather than a systemic influence of bile salts on the fibrinolytis process *in vivo*.

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THE COMPARATIVE STUDY OF THE LIVER OF RATS TREATED WITH CHOLIC AND LITHOCHOLIC ACID, ALONE OR ASSOCIATED WITH BUTTER-YELLOW

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1) In the last fifteen years or so, great progress has been made in the knowledge of the metabolites of cholesterol which are, for the most part, eliminated by the bile in the form of biliary acids (36). In the rat, the principal of these metabolites, cholic and chenodeoxycholic acid, which are the primary biliary acids, formed in the liver from cholesterol, are metabolized by the microorganisms of the intestine (5). In human bile as well, Wotton and Wiggins found that, in certain cases, chenodeoxycholic acid is the most abundant of the biliary acids, accompanied by lithocholic acid in a slight quantity. This latter acid also exists in the bile of the rat, as the product of the metabolism of cholesterol (35).

2) Biochemical investigations of this sort became active once again when Holsti (16-18) announced that he obtained cirrhosis of the liver in the rabbit by the daily gastric instillation of 0.15 g of totally dried bile (1956); then later, with different biliary acids (1958); and above all with the monohydroxycholanic acids, of which lithocholic acid was recognized as being the most cirrhogenic (1960). In animals of 2 kgs 500, 10 ml of a water suspension of the sodium salt of this last acid produces, in a matter of days, foci of degeneration of the hepatic cells, followed by the formation of a fibrous tissue; after three months, a diffuse cirrhosis, unpreceded by a massive fatty infiltration, sets in.

3) In 1957-58, a team of biochemists of Saint-Louis, largely utilizing the ^{14}C labeling, undertook a systematic study of the biliary acids of the rat (28). Some new trihydroxycholic metabolites of chenodeoxycholic acid were recognized; they are found in the feces of normal animals, in the bile of those with a cannulated biliary duct, and in the urine of those with a bound biliary duct (26). Of the different isomerisms obtained, the authors claim that cholic acid is the principal metabolite of deoxycholic acid, and that 3, 6, 7-trihydroxycholanic acid is the principal metabolite of chenodeoxycholic acid (27). Hyodeoxycholi acid, found in the bile of the pig, was also found in that of the rat (29). In this last animal, Beher et al. saw cholic acid, administered in the food, diminish the rat of synthesis of cholesterol and its mobilization in the liver; initially there is an elevation of the concentration of this steroid in the liver, which an inhibition of the incorporation of acetate in the cholesterol reverses (2). Inversely, hyodeoxycholic, lithocholic and deoxycho

acids diminish the concentration of cholesterol in the liver of the mouse (1).

However, Swedish biochemists, pursuing the work of Bergström, established that, in the rabbit, lithocholic acid is the one found in most abundant quantities in the feces and the bile, along with slight quantities of ursodeoxycholic acid and 7-keto-lithocholic acid (14). But, in the rat, though lithocholic acid represents more than 50% of the biliary acids in the feces, there are only traces of it to be found in the bile (31). The biliary acids of the bile are attacked in the caecum and the colon by microbial enzymes; there metabolites are partially reabsorbed into the entero-hepatic circulation, in the form of lithocholic and deoxycholic acid.

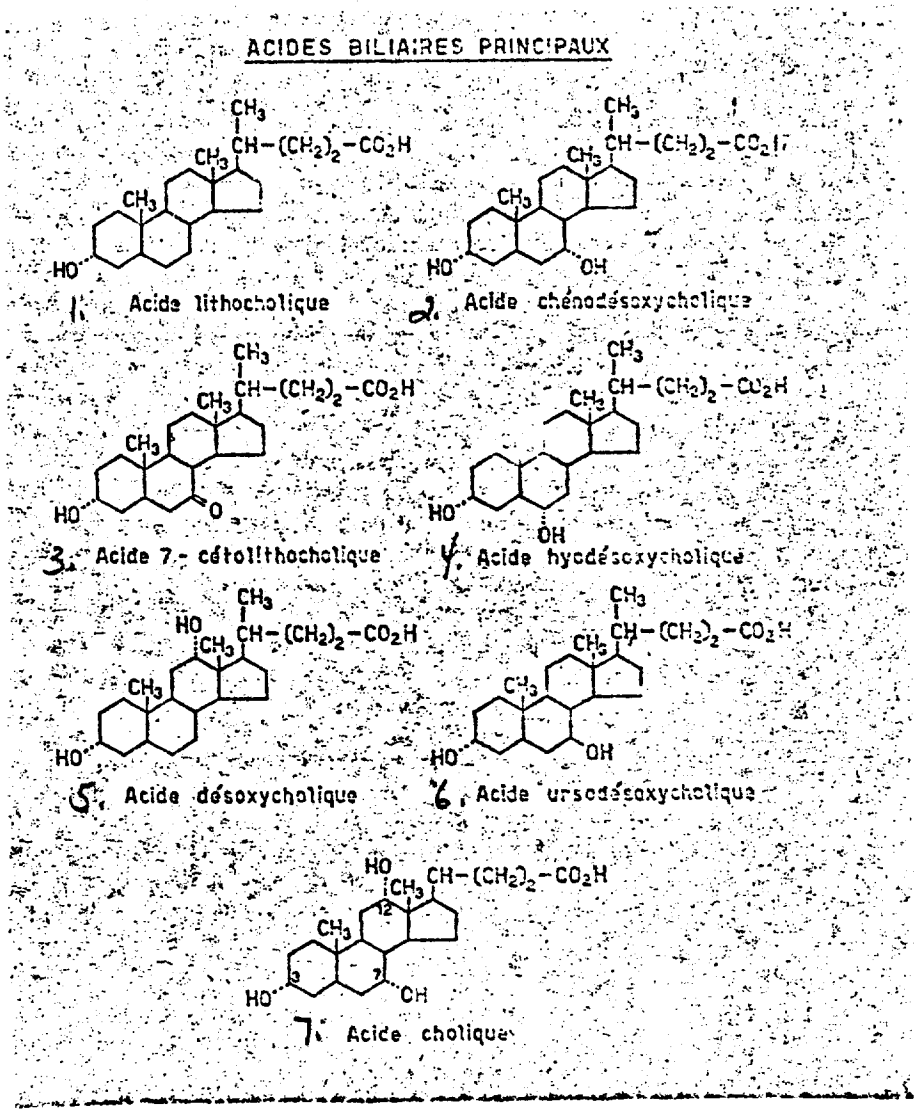
4) The metabolism of biliary acids in man is still being studied by Carey (7, 8), who began his investigation in 1955. Having confirmed the constant presence of chenodeoxycholic, cholic and deoxycholic acid in the blood serum, he admits that their quantities vary in normal individuals and icteric individuals (7). In the case of serious hepatic lesions, dihydroxycholanic acids increase, while trihydroxycholanic acids decrease (8). As early as 1957, Lindstedt (25), comparing the marked metabolites obtained after administration of cholic-^{14}C acid, whether to a normal subject or a subject with a biliary fistula, had concluded that deoxycholic and lithocholic acid are much less metabolized in man than in other species, probably because of the differences in the intestinal microbial flora. This was confirmed by other experimenters (15, 30): in normal man, only slight quantities of lithocholic and deoxycholic acid are metabolized in the intestine from chenodeoxycholic acid, and lithocholic acid is normally found only in very small quantities in the bile. But lithocholic acid even in these small quantites could be capable of provoking inflammation and fever (33).

5) However, in contrast to that which Beher et al. had demonstrated in the mouse, (3), and that which had been confirmed in the rat (34), the addition of cholic acid to the food of chicks does not increase the cholesterol of the blood serum, a hypercholesterolemic effect produced by the administration of lithocholic acid (10). It is not a question of the consequence of a metabolization of this acid by the microbes of the intestine, since, in germ-free chicks, the lithocholic acid which retards the growth of the animals, increases the plasmatic cholesterol and the volume of the liver. (11). This toxic action would probably be due to a disturbance of the lipidic metabolism in the liver, where the lithocholic acid decreases the biliary secretion and the conversion of cholesterol into biliary salts.

To this argument Hunt et al. add another (21). The histological examination of the liver of chicks receiving lithocholic acid revealed a proliferation of biliary canaliculi, which could be a primordium of the cirrhogenic process, described by Holsti in the rabbit. This proliferation under the effect of lithocholic acid was also obtained in the liver of a variety of monkey (19); then in the mouse, the guinea pig and the hamster (21). But while the rabbit and the hamster are the most sensitive, the rat is quite resistant; biliary excretion was only affirmed in three of these animals, out of six whose food contained 0.50% lithocholic

acid; the individual variations of susceptibility are notable.

PRINCIPAL BILIARY ACIDS



Key:
1.= lithocholic acid 2.= chenodeoxycholic acid
3.= ketolithocholic acid 4.= hyodeoxycholic acid
5.= deoxycholic acid 6.= ursodeoxycholic acid
7.= cholic acid

OUR EXPERIMENTS AND THEIR RESULTS

The microphotographs published in the article last mentioned (21) retain our attention. We recognized in them histological aspects of penetration, into the hepatic parenchyma, of the same oval cells whose origin and nature we had studied (23). There is no doubt that a toxic action of lithocholic acid occasioned a deficiency of hepatocytes, to which the process of regeneration represented by the proliferation of oval cells of biliary origin responded. Such a process constitutes an essential act of the formation of tumors produced in the liver by toxic chemical cancero-

have as a co-carcinogen; the experiments which are going to be reported here were undertaken immediately for the purpose of verifying this hypothesis. However, as it was going to be a questic of experiments of long duration, it was necessary to use a daily dose quite inferior to that used by the former authors, so that the majority of our animals would survive as long as possible.

1) Simultaneous administration of para-dimethylaminoazobenzene (DAB), or butter-yellow, and a biliary acid: cholic acid (CA), or lithocholic acid (LA).*

Conditions of the experiment. The semi-synthetic food, poor in proteins and riboflavin, which all the animals received exclusively, was that which we have been using for more than 15 years in our experiments of cancerization of the liver of the rat by DAB; its formula has recently been recalled (24). To this mixture were added 0.6 g/kg of DAB and 0.5 g/kg of one or the other of the two biliary acids in question. All the animals were Wistar males. The Ca lot was comprised of 9 rats of an average weight of 370 g; the LA lot of 10 rats of an average weight of 390 g.

At equal intervals, one animal of each of the two lots was sacrificed, after 26, 52, 89, 147, 189, 243 and 278 days of treatment. But on the other hand, two rats died spontaneously in each of the two lots: in 203 and 258 days in the CA lot, and 189 and 265 in the LA lot. Besides this, in the latter lot, the tenth animal was sacrificed the same day as the ninth, that is to say, on the 278th.

Comparative result. It was the inverse of that which had been presumed. The daily administration of lithocholic acid did not only not encourage the delays of appearance and succession of the stages which trace the toxic effect of DAB but, even more than cholic acid, it retarded them slightly.

First of all let us recall that, in the first two months of intoxication with DAB alone, the individual alteration of the hepatocytes causes their more or less differing death (stage I); bu the proliferation of small oval cells whose biliary origin is well established (22) is a principal assurance of the regeneration of the hepatocytes (stage II); these hepatocytes reconstitute, during the third month, a hepatic parenchyma in the form of nodules (stage III). Each of these nodules will present, more or less rapidly, signs of degeneration of its constituent elements, until a second group of biliary formations will encircle them (stage IV). In general, it is starting in the fourth month of intoxication that this stage is macroscopically evident, under an initially granular aspect, then a nodular one of one or several lobes of the liver, and finally of the entire surface. This appearance is the reflecti of characteristic changes in the microscopic structure. The foci of biliary neoformation evolve either in the form of rows of oval cells which divide and cirrhose the parenchyme, or of microcysts which grow and ramify into cholangiectasias; small disseminated adenomas form. They are the usual point of departure of carcinomas.

* The cholic and lithocholic acid were pure samples, in which the absence of notable quantities of impurities had been carefully verified by chromatography in thin layers.

The most precocious of these, observed in our experiments with DAB alone, was seen on the 110th day of the treatment (stage V). The invasion of the entire liver by large multiple tumors (stage VI) was encountered, at the earliest, beginning on the 150th day.

In the rats receiving cholic or lithocholic acid mixed with DAB in their deficient diet, the loss of weight was parallel in both lots, altough, also in both lots, it was accompanied by a slight increase of the panniculus adiposus.

The first macroscopic changes which, appearing on the surface of the liver, signalled the beginning of stage IV, were confirmed in 89 days in the case of the rat of lot CA (thus in normal delays) and in 147 days in the case of the rat of lot LA (thus very late). A first case of stage VI brought about the death of an animal of lot LA on the 189th day, and on the 203d day in lot CA.

Upon microscopic examination, the fatty surcharge which appeared in the centrolobular regions of the parenchyma under the influence of the deficiency of the diet, was found to be similar in the two lots, with variations which are either individual, or of one or another lobe in the same animal. It seems that the biliary acids administered rendered the hepatocytes more resistant to the toxic aggression of the DAB. The cytolyses, the inequalities of volume of the nuclei, and above all the beginning of the proliferation of the oval cells were clearly retarded and reduced; this protective action even seemed to be a little more marked in the case of lithocholic acid. Until the installation of the nodules of regeneration, the glycogen had remained abundant in all the animals and in all the lobules.

Apart from a few rare cases in one or the other of the two lots, where slight alterations of the spermatic line were remarked, the testicles remained actively functional, even in the rats which survived longest. In the same way, the adrenal glands retained a normal weight; we only remarked in their case a marked vacuolization of the cells of the internal part of the fascicula, a probable index of a lipidic surcharge.

In the face of this result, we judged it appropriate to undertake a new experiment, destined to allow us to compare the effect of the prolonged administration of cholic acid alone on the one hand, and of lithocholic acid alone on the other.

2) Daily prolonged administration through digestive route of cholic and lithocholic acid.

All the rats used were Wistar males. A CA lot was composed of 10 animals of an average weight of 380 g, which were submitted to the usual deficient diet, with 0.5 g/kg cholic acid added, and killed after 32, 95, 153, 211, 264, 285, 319, 333, 349 and 362 days. An LA lot included 6 animals of an average weight of 330 g; they received cholic acid only under the same conditions; they were sacrifieed after 28, 49, 103, 161, 272 and 372 days.

No mortality occurred among the 16 rats in the course of an experiment which lasted more than a year. A slight loss of weight was the immediate consequence of subjection to the diet. Consisting of a few dozens of grams toward the end of the first month, it generally increased a bit in the CA lot, until it passed 100 g. On the other hand, it had a tendency to diminish and

disappear in the LA lot.

In general, the liver of the animals presented a slight progressive increase in weight (reaching on the average 2 g, thus 20%), which was parallel in the two lots. Upon microscopic examination, the organ appeared normal. Histologically, it was not possible to find a characteristic difference between the resulting cuttings of the livers of the CA or the LA lot. In both the one and the other, steatosis is more marked or more slight; small clusters of lympho-hystocytaric cells are more frequent or rare in several portal canals; the hypertrophy, more or less marked, of the endothelial cells of the sinus is frequent, but in no case could we confirm the presence of iron in these elements. In the CA series, no traces of biliary proliferation were found. In the first two animals of the LA series, thus on the 28th and 49th days of treatment with lithocholic acid, an attentive examination revealed a tendency of some small perilobular biliary canals to emit rare oval cells; however, this also was not found in any of the other animals of this series.

The spermatogenesis was normal in most of the animals, reduce in the testicles of the last of the CA series, nearly completely disappeared in the last of the LA series; age could be a principal factor in this change. In the adrenals, of normal weight, there is found, in both lots, a slight increase of vacuolization of the cells of the internal zone of the fascicula along with (but above all in the reticular layer) a surcharge in chromolipoids, colorable by the periodic acid of Schiff, in the glandular cells and those of the endothelium.

Our attention was drawn by a leucocytosis (30,000) in a rat which had been receiving lithocholic acid for three months; but we found two analogous cases in the series receiving only cholic acid. This finding led us to carry out a histological examination of all the spleens of the four lots of the two experiments reported here; they were weighed, fixed, mounted, but not cut. The comparison of the average weights of these organs caused the appearanc of a difference according to the lots:

1) Lithocholic acid alone: individual weight nearly always normal; average weight 0g, 890;

2) Cholic acid alone: slight hypertrophy, but not progressive in most of the animals; average weight 1 g, 100;

3) Lithocholic acid + DAB:non-progressive hypertrophy in most of the animals; average weight 1 g, 390;

4) Cholic acid + DAB: marked hypertrophy in all the animals (from 2 g, 200 to 500 in three of them); average weight 1 g, 900.

The hypersplenia in the animals treated with DAB is explained by the influence of this toxic, which always brings about a certain degree of hemochromatosis, with surcharge of iron in the spleen. But it is remarkable that in each of the four comparative categories of animals, those having received cholic acid (CA+DAB, or CA alone) revealed a degree of hypertrophy of the spleen which was absent or less in those having received lithocholic acid (LA+DA or LA alone),

A histological study confirmed this impression: in the two lots -- cholic acid and lithocholic acid alone -- there exists a diffuse infiltration, by numerous polynuclear adults, of more or less congested spleens, sometimes with small hemorrhages. The funicle of the spleen expands in the course of the first months be-

cause of the formation of erythromyeloid foci. Simultaneously, the Malphigian spleen corpuscles atrophy, and their germinative centers disappear. Beyond the fifth month, myelopoiesis regresses strongly or disappears entirely; the reticular perifollicular cells and cordonals become charged with hemosiderine, and sclerosis sets in.

All these changes are generally more pronounced in the spleens of the animals having received cholic acid, than in those treated with lithocholic acid; the infiltration by the polynuclears corresponding with hyperleucocytosis of the blood, thus seems to have had its cause in a strong granulopoietic reaction in the bone marrow.

DISCUSSION AND CONCLUSIONS

1) There is something paradoxical in the fact that the placement on a hypoproteinic diet with lithocholic acid added, of rats in which one thus hoped to accelerate the effect of a hepatocancerogenic, seems to have been one of the causes of the inhibition of the phenomenon of biliary proliferation which several investigators had ascertained after the administration of lithocholic acid. Since this neoproduction of oval cells was not produced under the influence of this biliary acid administered daily for months to rats not receiving DAB either, one may suppose that the deficiency of the alimentary diet might have played a part, greater perhaps than that of the relatively weak dose of lithocholic acid. To be sure, previous authors have shown that the rat is much less sensitive than other animals to the cirrhogenic effect of lithocholic acid; in reality, our rats showed themselves to be totally unresponsive to this effect.

Moreover, from different points of view, cholic acid ordinarily exercised an inflammatory effect which was a little more marked than that of lithocholic acid (greater increase in weight of the liver and the spleen; lymphohistiocytic foci are a little more numerous in the hepatic parenchyma; myelinization and limphoid atrophy more pronounced in the spleen).

2) These experiments were already terminated when, in 1965, there appeared the work of Palmer (32), who related that he obtained, after 8 to 16 weeks, choledocholithiasis in young Sprague-Dawley rats which received lithocholic acid in their food (at a dose of 1% of a hypoproteinic diet: 8% of proteins). The author signals the strong dilatation of the biliary ducts in which calculi have formed; but, in the description he gives of the histological cuttings of the liver, he makes no mention of the existence of a proliferation of oval cells.

In our rats, which received both cholic and lithocholic acid, the biliary canals are neither multiplied nor distended, only slightly patulous in the histological cutting (fixation in the liquid of Bouin, usual methods of mounting in paraffin and staining). This difference from the results of Palmer (32) can be explained by the much inferior dose of lithocholic acid which we used in a different line of rats.

3) From the diverse works which have been reported here, one

can draw the conclusion that lithocholic acid exercises, in certain species of animals (notably in rabbits and chicks), a toxicity greater than that of other biliary acids, including cholic acid. However, this was not true in the case of the rat, in the conditions of our experiments. These differences, as well as several contradictions apparent in the results of the various investigators, are explained if one takes into consideration the two factors which condition the action of the biliary acids in the liver:

a) the passage of these bodies in the general circulation, which is only effected in very small proportion, whatever the quantities of these acids present in the bile may be. This passage depends on the alimentary diet, and above all on the integrity of the hepatic function. It is know, for example, that if lithocholic acid is a minor but normal constitutent of the human bile (5% in certain cases, according to Wotton and Wiggins (3) its concentration in the blood serum is subject to great variations. This concentration is greater in icteric individuals than in the normal subjects: this suggests the possibility of an etiological role played by lithocholic acid in the cirrhosis of the liver (9).

b) the interconversion and metabolization of the various biliary acids in the liver. In this organ there exists (probably in the microsomes) enzymatic systems susceptible to hydroxylizing the deoxycholic acids in position 7, to transform them into trihydroxycholanic acids (4). It has been shown, on the other hand, that cholic acid, once passed into the blood, persists there so lon in proportion as the liver is more injured. Thus an essential difference, in cirrhotics and in normal subjects, consists in the abnormally prolonged persistence (14 days instead of 6 hours) of a cholic-^{14}C acid after intravenous injection (6). This participation of cholic acid in the phenomena of chronic intoxication of the liver observed in man, find a favorable argument in the manifestations of a certain toxic action of this substance in the rats of our experiments. A more extensive study of the physiopathological properties of the various biliary acids (alone or in conjunction with toxants which one knows favor the transport) deser to be done, even when it is a question of an acid which normally exists in large quantities in the bile, such as cholic acid and deoxycholic acid, the proportions of which are respectively 34 to 39% and 25 to 29% in the human bile (18). This study should not be neglected, even in the case of biliary acids recognized as being without effect on the liver of certain species of animals, such as cholic, deoxycholic and chenodeoxycholic acid, which have recent been recognized as harmless in the chick.(12).

SUMMARY

Several investigators have found that lithocholic acid exercises a cirrhogenic effect on the liver of various animal species. One could thus suppose that this biliary acid, administer to rats receiving butter-yellow at the same time, would act as a hepatic-cocancerogenic.

Examined comparatively with cholic acid, lithocholic acid did not act any more than the cholic acid to accelerate the oncogen power of butter-yellow. Moreover, administered orally each for for some months to rats, it even demonstrated a toxicity slightly

weaker than that of cholic acid.

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Dietary Bile Acids and Lipid Metabolism. I. Influence on Lipids and Liver Size of Chicks. (28668)

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Several workers have demonstrated an influence of dietary bile acids on plasma and hepatic cholesterol levels of mice and rats (2,6). The results of these studies have shown dietary cholic acid to be hypercholesterolemic. Lithocholic acid, on the other hand, lowered blood and liver cholesterol levels of mice and rats.

Little work has been reported on the influence of dietary bile acids in the chick. Hegsted *et al*(7) demonstrated a cholesterol elevating effect of cholic acid in cholesterol-fed chicks. More recently, Edwards(8) reported that cholic and hyodeoxycholic acids did not influence serum cholesterol levels of chicks fed a cholesterol-free diet, but that lithocholic acid, in contrast to its effect in mice and rats, was hypercholesterolemic. These results suggest that chicks fed a cholesterol-free or supplemented diet respond differently to cholic acid supplementation.

The studies herein reported were undertaken to determine the influence of dietary cholic and lithocholic acids on lipid metabolism in growing chicks fed a cholesterol-free or supplemented diet.

Experimental. Male Hy-line chicks were used in all experiments. Chicks were distributed to the various experimental groups on the basis of body weight in such a manner as to have almost identical mean weights for all groups in each study. The length of the experimental periods is indicated in the results section.

The composition of the diet employed is shown in Table I. Bile acids and cholesterol were added to the diet at a level of 0.2 and 2.0 g/100 g of diet, respectively. All additions to the basal diet were made at the expense of glucose.

The chicks were housed in heated cages having raised wire floors. Food and water were supplied *ad libitum*. Body weight and food consumption were determined at weekly intervals.

Blood was drawn by cardiac puncture with a heparinized syringe at weekly intervals in Exp. 3 and at termination of each experiment. At termination of Exp. 1 and 2, the animals were sacrificed and livers were excised, freed of excess blood and weighed. Livers were then frozen and stored at —20°C until analyzed.

Plasma and liver lipids were determined as previously described(10). The data were analyzed statistically by "t" test.

Results. The results are presented in Tables II, III and IV for Exp. 1, 2 and 3, respectively.

Experiment 1 (Table II). The chicks used in this experiment were prefed a stock diet for one week and the experimental diets for 3 weeks.

Chicks fed lithocholic acid and cholesterol gained less weight than did chicks fed the basal diet ($P < 0.001$). Dietary lithocholic acid also increased plasma cholesterol and lipid phosphorus ($P < 0.001$) and increased liver size ($P < 0.001$), both in terms of weight and as a percentage of body weight. Lithocholic acid also decreased liver fat of chicks fed the cholesterol-supplemented diet ($P < 0.005$). Liver cholesterol levels of chicks fed a cholesterol-free diet were increased ($P < 0.01$), while those of chicks

TABLE I. Composition of Basal Diet.

Ingredients	Amount, g/100 g diet
Assay protein C-1*	29.40
Glycine	.40†
DL-methionine	.30†
Vitamin mixture‡	.40
Salt mixture‡	5.31
Choline Cl	.20
Non-nutritive fiber	4.00
Corn oil	5.00
Glucose	to 100

* Archer-Daniels Corp., Midland, Mich.
† In Exp 1 all diets contained 0.20% glycine and 0.35% DL-methionine.
‡ For composition see Leveille *et al.*(9).

TABLE II. Body Weight Gain, Liver Weight and Plasma and Liver Lipids of Chicks Fed Bile Acids With and Without Cholesterol.

Experiment 1*

Bile acid†	Body wt gain, g‡	Plasma: Cholesterol, mg/100 ml	Plasma: Lipid P, mg/100 ml	Liver§: Weight, % body wt	Liver§: Fat, %	Liver§: Cholesterol, mg/g	Liver§: Lipid P, mg/g
			No added cholesterol				
None	191 ± 24‖	140 ± 12	13.51 ± 1.55	2.8 ± .6	5.3 ± .3	4.4 ± .2	1.32 ± .25
Cholic	189 ± 15	157 ± 21	13.10 ± 1.22	2.5 ± .3	5.4 ± .5	4.0 ± .3	1.18 ± .08
Lithocholic	184 ± 22	459 ±117	32.12 ± 6.99	5.7 ± .3	5.1 ± .5	5.0 ± .4	1.11 ± .08
Cholic + lithocholic	182 ± 28	228 ± 93	16.86 ± 2.59	3.4 ± .4	3.4 ± .8	3.8 ± .7	.72 ± .14
			2% added cholesterol				
None	221 ± 18	344 ± 84	10.12 ± 1.44	2.9 ± .2	8.2 ± 1.6	29.2 ± 9.7	.97 ± .06
Cholic	199 ± 15	697 ±180	13.02 ± 2.40	3.0 ± .2	8.0 ± 1.2	28.3 ± 7.0	.94 ± .10
Lithocholic	155 ± 36	743 ±196	31.09 ± 9.56	6.5 ±2.1	4.8 ± .4	8.1 ± 1.4	.85 ± .06
Cholic + lithocholic	194 ± 17	495 ±219	10.92 ± 1.93	2.6 ± .3	6.2 ± .8	19.6 ± 6.2	.91 ± .10

* The chicks in this study were fed the experimental diets from 7th to 28th day of age.
† All bile acids added at a level of 0.2% of diet.
‡ Weight gain for 21-day experimental period.
§ All liver values expressed on a wet weight basis.
‖ Mean for 8 animals ± standard deviation.

fed cholesterol were decreased ($P < 0.001$) by lithocholic acid. The effects of lithocholic acid were partially or completely reversed by simultaneous feeding of cholic acid. The weight gain of chicks fed lithocholic acid in the cholesterol-supplemented diet was significantly improved when cholic acid was also fed ($P < 0.025$). The elevated plasma cholesterol and lipid phosphorus levels of lithocholic acid-fed chicks were reduced by cholic acid supplementation, particularly in chicks fed cholesterol. The increased liver size of lithocholic acid-fed chicks was also reduced by cholic acid supplementation.

Cholic acid did not significantly alter any of the parameters evaluated in chicks fed a cholesterol-free diet. In chicks fed cholesterol, however, cholic acid significantly increased plasma cholesterol ($P < 0.001$) and lipid phosphorus ($P < 0.025$).

No appreciable changes were observed in liver lipid phosphorus values as a result of the treatments employed.

*Experiment 2, (Table III).** Exp. 2 was designed to test the reproducibility of the results obtained with cholic and lithocholic acids in Exp. 1. Four-week old chicks which had been fed a stock diet were used and were fed the experimental diets for 4 weeks.

The effects of lithocholic acid supplementation are in good agreement with those obtained in Exp. 1. Lithocholic acid depressed weight gain of cholesterol-fed chicks ($P < 0.01$), increased plasma cholesterol and lipid phosphorus levels ($P < 0.001$), increased liver size ($P < 0.001$) and decreased liver fat ($P < 0.005$). Liver cholesterol was increased in chicks fed the cholesterol-free diet ($P < 0.001$) and decreased in cholesterol-fed chicks ($P < 0.001$).

As in Exp. 1, cholic acid did not significantly alter weight gain, liver size or liver lipids but significantly increased plasma cholesterol and lipid phosphorus of cholesterol-fed chicks ($P < 0.05$). In contrast to the results of Exp. 1, chicks fed cholic acid without a cholesterol supplement had significantly higher plasma cholesterol and lipid phosphorus levels ($P < 0.005$). Liver lipid phosphorus values remained unchanged.

Experiment 3 (Table IV). Exp. 3 was designed to determine whether the increased plasma cholesterol level noted in older chicks fed a cholic acid-supplemented, cholesterol-free diet (Exp. 2) might be related to age. In Exp. 3, chicks were prefed a stock diet for the first week and received the experimental diets for 8 weeks (7th to 63rd day

* Some of the data from Experiment 2 have been reported in a preliminary note, Leveille *et al.*(1).

TABLE III. Body Weight Gain, Liver Weight and Plasma and Liver Lipids of Chicks Fed Bile Acids With and Without Cholesterol.

Experiment 2*

Bile acid†	Body wt gain, g‡	Plasma: Cholesterol, mg/100 ml	Plasma: Lipid P, mg/100 ml	Liver§: Weight, % body wt	Liver§: Fat, %	Liver§: Cholesterol, mg/g	Liver§: Lipid P, mg/g
			No added cholesterol				
None	338 ± 74‖	153 ± 16	13.13 ± 1.53	2.63 ± .22	5.6 ± .6	4.1 ± .3	.85 ± .03
Cholic	294 ± 44	211 ± 29	18.11 ± 1.95	2.87 ± .22	5.8 ± 1.4	4.5 ± .5	.96 ± .10
Lithocholic	291 ± 32	458 ± 99	33.72 ± 6.56	5.87 ± 1.08	4.0 ± .9	5.3 ± .3	.96 ± .08
Cholic + lithocholic	320 ± 29	264 ± 35	20.10 ± 1.89	3.92 ± .38	5.2 ± .9	4.8 ± .8	.97 ± .16
			2% added cholesterol				
None	330 ± 40	450 ± 96	11.06 ± 1.46	2.83 ± .05	6.9 ± 1.2	12.1 ± 1.1	.98 ± .10
Cholic	332 ± 47	622 ± 142	13.60 ± 1.43	2.93 ± .33	6.4 ± 1.1	13.6 ± 2.7	.89 ± .09
Lithocholic	258 ± 35	739 ± 170	29.91 ± 4.29	5.37 ± .59	4.9 ± .4	8.2 ± .9	.87 ± .08
Cholic + lithocholic	261 ± 26	353 ± 81	11.41 ± 1.55	2.95 ± .14	5.7 ± .7	11.1 ± 2.1	.86 ± .03

* The chicks used in this study were fed the experimental diets from 28th to 56th day of age.
† All bile acids added at a level of 0.2% of diet.
‡ Weight gain for 28-day experimental period.
§ Liver values expressed on a wet weight basis.
‖ Mean for 6 animals ± standard deviation.

TABLE IV. Cholesterol and Lipid Phosphorus Levels of Plasma from Chicks Fed Diets With and Without Added Cholic Acid.

Experiment 3

Wk of exp*	Cholesterol, mg/100 ml: Control	Cholesterol, mg/100 ml: Cholic acid fed†	Lipid phosphorus, mg/100 ml: Control	Lipid phosphorus, mg/100 ml: Cholic acid fed†	No. of determinations: Control	No. of determinations: Cholic acid fed†
1	172 ± 29‡	210 ± 27	12.4 ± 1.7	15.4 ± 2.1	6	6
2	142 ± 18	148 ± 19	15.0 ± 3.5	14.5 ± 3.5	6	6
3	134 ± 22	149 ± 25	10.5 ± 1.2	12.4 ± 2.2	6	4
4	167 ± 21	166 ± 32	11.8 ± 2.2	12.3 ± 2.3	11	9
5	162 ± 22	156 ± 14	9.3 ± 1.0	9.2 ± .7	11	9
6	113 ± 9	118 ± 11	11.3 ± 1.4	11.1 ± 1.0	10	8
7	183 ± 12	186 ± 20	15.0 ± 1.4	14.6 ± 1.7	11	9
8	139 ± 14	147 ± 19	11.2 ± .9	12.3 ± 1.7	11	8

* Chicks were prefed a stock diet for 1 week, therefore value for week 1 represents sample taken on 14th day of age.
† Cholic acid fed at a level of 0.2% of diet.
‡ Mean ± standard deviation for number of determinations shown.

of age). Blood samples were obtained at weekly intervals for cholesterol and lipid phosphorus determinations. Cholic acid supplementation did not significantly alter plasma lipids at any time during the 8-week experimental period (Table IV).

Discussion. The data presented demonstrate that dietary lithocholic acid decreases body weight gain, increases liver size, plasma cholesterol and lipid phosphorus levels and decreases liver fat in the presence or absence of dietary cholesterol. Dietary lithocholic acid increased the liver cholesterol level of chicks fed a cholesterol-free diet and depressed liver cholesterol in cholesterol-fed chicks.

The increased liver size and plasma lipid levels found in lithocholic acid-fed chicks were partially reversed when cholic acid was fed simultaneously in a cholesterol-free diet. In chicks fed a cholesterol-supplemented diet cholic acid afforded almost complete protection from the effects of lithocholic acid. The protective effect of cholic acid was observed after feeding the experimental diets for 3 or 4 weeks and similar results have been reported concerning plasma cholesterol levels (5).

The mechanism(s) by which dietary lith-

cholic acid exerts the effects reported is not clear. Dietary lithocholic acid has been reported to induce liver cirrhosis in the rabbit (11) and Iguana iguana(12) and parenterally administered lithocholic acid has been shown to be pyrogenic in man(13). The increase in liver size observed appears to be the result of a biliary hyperplasia(1); however, the etiology of the lesion remains obscure.

The hypercholesterolemic effect of cholic acid when fed in conjunction with cholesterol and its lack of effect in a cholesterol-free diet, observed in these studies, is in accord with the reports of Hegsted *et al*(7) and Edwards (8). The indication from Experiment 2 that cholic acid might be hypercholesterolemic in older chicks fed a cholesterol-free diet was not borne out by the data obtained in an 8-week study (Exp. 3). Dietary cholic acid has been reported to increase liver cholesterol levels in mice(3) and rats(6); however, this bile acid did not significantly alter liver cholesterol levels in these studies with chicks.

Summary. Dietary lithocholic acid elevated plasma cholesterol and lipid phosphorus levels, and increased liver size of growing chicks. In cholesterol-fed chicks, lithocholic acid depressed liver lipid and cholesterol levels. The effects of lithocholic acid were partially reversed by cholic acid in chicks fed a cholesterol-free diet and almost completely reversed in chicks fed cholesterol. The plasma cholesterol level of cholesterol-fed chicks was increased by cholic acid feeding.

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EFFECT OF DIETARY LITHOCHOLIC ACID ON LIVER SIZE OF THE CHICK

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Lithocholic acid has been shown to counteract the hypercholesterolemic effect of cholic acid in the mouse and rat (Howe *et al.*, 1960; Portman and Bruno, 1961). Recently, Edwards (1961) has presented data demonstrating a lack of effect of dietary cholic acid on serum cholesterol levels of growing chicks. In this report (Edwards, 1961), dietary lithocholic acid was shown to have a hypercholesterolemic effect in the chick in contrast to its cholesterol depressing action in the rat and mouse (Howe *et al.*, 1960; Portman and Bruno, 1961).

The influence of dietary bile acids on cholesterol and lipid metabolism is presently under investigation in this laboratory. During the course of these studies, dietary lithocholic acid was found to influence liver size markedly.

Groups of six chicks were fed the experimental diets for 4 weeks (4 through 8 weeks of age). Prior to receiving the experimental diets, the chicks were fed a commercial diet. The composition of the basal diet was as follows in gm./100 gm. of diet: Assay protein C-1, 29.40; glycine, 0.40; DL-methionine, 0.30; salt mix (Leveille *et al.*, 1962), 5.31; vitamin mix (Leveille *et al.*, 1962), 0.40; choline Cl, 0.20, non-nutritive fiber, 4.00; corn oil, 5.00; and glucose to 100. Cholic and lithocholic acids (Nutritional Biochemicals

Corp.) were added at a level of 0.2% of the diet at the expense of glucose. At the termination of the study, the animals were sacrificed and the livers excised. Moisture was determined by drying the livers at 60°C. under vacuum. Liver fat was determined gravimetrically after chloroform: methanol (2:1) extraction.

The results obtained are presented in Table 1. Neither cholic nor lithocholic acid affected growth significantly, although the groups fed bile acids did have lower weight gains than did the control group. As can be seen, lithocholic acid caused a two-fold increase in liver weight (Table 1). The difference in liver size is not explained by differences in fat or moisture content as shown; in fact, the chicks fed lithocholic acid had a lower percentage of liver fat than did either of the other two groups. Preliminary results of other studies demonstrate that this increase in liver size resulting from the ingestion of lithocholic acid is easily reproducible.

Histological examination of the enlarged liver of representative chicks fed lithocholic acid show the enlargement of the liver to be due to a marked periportal biliary hyperplasia throughout the liver. The newly formed biliary tissue existed as patent tubules, and islands and cords of cells without obvious lumina. These structures, which were supported by a minimal amount of collagenous connective tissue, extended in fingerlike projections from the enlarged portal tracts into the hepatic lobules, resulting in subdivision of the lobules. Sections of other tissues (brain, heart, lung, spleen, kidney and gastrointestinal tract) were normal.

Although further observations are needed, the results of histological examination of these livers indicate rather conclusively that the enlargement is a result of the biliary hyperplasia noted. The etiology of the biliary hyperplasia cannot as yet be determined. This condition can occur as a result of a hepatic damage, chronic intoxications (ethionine), administration of carcinogenic substances and biliary obstruction. The latter acts either mechanically or through the absorption of biliary substances which act via the blood stream.

It is conceivable that the difference in liver size noted for chicks fed cholic (3α, 7α, 12α-trihydroxycholanic acid), or lithocholic (3α-hydroxycholanic acid) resides in the 7α or 12α-hydroxyl group, or both. Investigations are in progress to study this possibility and also to attempt to determine the etiology of the biliary hyperplasia.

TABLE 1.—*Effect of dietary bile acids on body weight gain and on liver weight, moisture and fat of the growing chick*

Treatment	Body weight gain[1]	Liver		
		Weight	Moisture[2]	Fat[2]
	gm.	gm.	%	%
Control	338±74[3]	15.8±2.4	69.7±0.6	5.57±0.63
0.2% Cholic	294±44	15.2±1.4	70.3±0.5	5.77±1.43
0.2% Lithocholic	291±32	31.0±6.8	72.2±1.3	3.98±0.87

[1] Weight gain from 4 through 8 weeks of age.
[2] Moisture and fat expressed as % of wet liver weight.
[3] Mean ± standard deviation for six chicks.

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Dietary Bile Acids and Lipid Metabolism. VI. Protective Effect of Cholic Acid in Lithocholic Acid Fed Chicks. (29169)

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Dietary lithocholic acid has been shown to depress body weight gain and increase plasma lipids and liver size of chicks(1-4). The increased liver size of lithocholic acid-fed chicks has been shown to be the result of a bile ductule proliferation(5).

The deleterious effects of lithocholic acid can be prevented, at least for a 3- or 4-week period, by simultaneously feeding cholic acid (4). Data presented here demonstrate that cholic acid protects chicks against the effects of dietary lithocholic acid for periods of up to 12 weeks.

Experimental. Male Hy-line White Leghorn chicks were fed a stock diet for 2 weeks. The chicks were then assigned to the experimental groups in such a way as to have almost identical average initial weights for all groups (128-130 g).

The chicks were reared in heated cages having raised wire floors. Food and water were supplied *ad libitum*. Body weight and food consumption were determined at weekly intervals. The basal diet employed was identical to that described previously(4). Lithocholic and cholic acids when fed were added at a level of 0.2% of the diet at the expense of glucose. Twenty-eight chicks were started in each of the 6 experimental groups as shown in Table I. Seven chicks from each group were sacrificed after having received the diets for 3, 6, 9 and 12 weeks.

At time of sacrifice, blood was obtained by cardiac puncture using a heparinized syringe. The liver was excised, blotted to remove excess blood and weighed; a slice of liver was fixed in neutral buffered formalin and the remainder was frozen and stored at —20°C for chemical analysis. Plasma and liver lipids

TABLE I. Experimental Design.

Exp group	Dietary variables
1	Control
2	.2% Lithocholic acid
3	*Idem* + .2% cholic acid
4	2% Cholesterol
5	.2% Lithocholic acid + 2% cholesterol
6	*Idem* + .2% cholic acid

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TABLE II. Body Weight, Liver Size, Plasma and Liver Lipids of Chicks Fed Lithocholic or Lithocholic and Cholic Acid-Supplemented Diets Containing or Free of Cholesterol for 3, 6, 9 or 12 Weeks.

Treatment group*		Body wt, g	Plasma: Cholesterol, mg/100 ml	Plasma: Lipid phosphorus, mg/100 ml	Liver†: Size, % body wt	Liver†: Fat, %	Liver†: Cholesterol, mg/g	Liver†: Lipid phosphorus, mg/g
3 wk	1	394 ± 35‡	127 ± 31	11.7 ± 2.6	2.2 ± .3	4.0 ± .4	3.1 ± .2	.91 ±.11
	2	314 ± 55	421 ±131	32.9 ± 6.4	6.9 ±2.4	4.7 ±1.1	4.2 ± .5	1.04 ±.09
	3	380 ± 21	244 ± 50	24.5 ± 3.5	4.6 ± .7	4.4 ± .5	3.5 ± .6	.98 ±.10
	4	426 ± 38	296 ± 79	12.9 ± 1.2	3.0 ± .3	5.8 ± .9	15.7 ±3.7	.87 ±.13
	5	360 ± 25	502 ± 90	26.7 ± 3.6	5.1 ± .7	4.4 ± .4	7.9 ±1.3	.90 ±.06
	6	369 ± 19	379 ±140	14.4 ± 2.8	3.5 ± .4	5.3 ± .6	12.9 ±4.3	.98 ±.08
6 wk	1	687 ± 47	109 ± 12	9.3 ± 1.1	2.2 ± .2	4.4 ± .5	3.7 ± .3	.95 ±.08
	2	601 ± 95	341 ± 87	23.0 ± 7.7	6.8 ±2.1	4.1 ± .4	4.6 ± .5	.93 ±.14
	3	688 ± 81	271 ± 44	19.7 ± 1.0	4.4 ± .8	4.0 ± .3	4.4 ± .3	.95 ±.12
	4	747 ± 39	460 ±140	15.8 ± 2.8	2.7 ± .2	6.6 ± .8	19.2 ±4.1	1.09 ±.13
	5	650 ± 93	658 ±211	28.3 ± 7.2	6.2 ±2.5	5.1 ± .7	9.1 ±2.8	.99 ±.10
	6	670 ± 71	333 ±122	14.6 ± 1.8	2.6 ± .3	6.5 ±1.2	15.1 ±5.9	1.03 ±.10
9 wk	1	1052 ± 65	118 ± 24	6.9 ± .8	1.8 ± .1	4.2 ± .3	3.6 ± .3	1.07 ±.03
	2	828 ±142	556 ±172	27.7 ± 7.0	9.0 ±2.4	3.9 ± .6	4.8 ± .8	.98 ±.09
	3	997 ± 59	316 ± 47	16.6 ± 4.5	4.6 ±1.2	3.9 ± .2	4.3 ± .2	.98 ±.08
	4	993 ± 76	453 ±156	9.0 ± 2.4	2.3 ± .3	5.5 ± .6	13.5 ±1.9	.85 ±.09
	5	934 ± 77	785 ±315	19.9 ± 9.5	6.3 ±2.5	4.1 ± .6	8.0 ±1.5	.90 ±.11
	6	992 ± 68	437 ±172	9.2 ± 1.9	2.5 ± .3	6.7 ± .5	16.6 ±1.9	1.08 ±.23
12 wk	1	1439 ±106	123 ± 16	11.2 ± 1.5	2.0 ± .1	5.2 ± .5	4.1 ± .3	1.05 ±.07
	2	1015 ±201	467 ± 62	23.2 ± 5.4	8.7 ±2.7	4.0 ± .3	5.2 ± .4	.90 ±.07
	3	1318 ±166	299 ± 39	18.9 ± 3.6	5.8 ±1.5	4.3 ± .6	5.3 ± .3	.91 ±.15
	4	1414 ±138	356 ±131	11.6 ± 2.8	2.4 ± .2	7.1 ±1.4	21.7 ±6.4	1.08 ±.11
	5	1177 ±168	944 ±395	24.9 ± 8.0	6.8 ±2.7	4.7 ±1.0	8.2 ±1.8	.87 ±.10
	6	1390 ± 89	464 ±150	16.2 ± 4.4	2.3 ± .2	7.3 ± .9	20.6 ±4.8	1.05 ±.08

* Groups 1-3 received a cholesterol-free diet; Groups 4-6 were fed the same diet supplemented with 2% cholesterol; in addition, Groups 2 and 5 received a supplement of .2% lithocholic acid; Groups 3 and 6 received supplements of .2% each of lithocholic and cholic acids.

† All liver values expressed on a wet weight basis.

‡ Mean for 7 chicks ± standard deviation.

were determined as previously described(6). The formalin-fixed tissue was prepared for microscopic examination as previously described(5). Results were statistically evaluated by means of the "t" test.

Results. Results are presented in Table II. Body weight was significantly lower in chicks fed lithocholic acid. However, the simultaneous addition of cholic acid overcame the body weight-depressing effect of lithocholic acid. Dietary cholesterol did not influence body weight. Plasma cholesterol and lipid phosphorus levels were significantly increased by lithocholic acid feeding. The lipemia induced by lithocholic acid feeding was less marked when cholic acid was fed simultaneously to chicks receiving a cholesterol-free diet and was absent in cholesterol-fed chicks receiving a dietary supplement of both bile acids. Dietary cholesterol, as would have been expected, increased plasma cholesterol levels significantly. Chicks fed a cholesterol-free diet had a significantly greater liver size when fed lithocholic acid; cholic acid partially overcame this increase. As shown in Table II, cholic acid completely prevented the increase in liver size resulting from lithocholic acid supplementation of a cholesterol-containing diet.

Liver fat was decreased slightly by lithocholic acid in chicks fed a cholesterol-free diet; however, this difference was significant only at the 12th week. Dietary cholesterol significantly increased liver fat and lithocholic acid decreased liver fat significantly in the cholesterol-fed chick. Addition of cholic acid to the lithocholic acid-cholesterol-supplemented diet resulted in liver lipid values intermediate to and significantly different from those observed in chicks fed cholesterol or cholesterol and lithocholic acid. Liver cholesterol levels were significantly increased in chicks fed lithocholic acid. Addition of cholic acid to the lithocholic acid-supplemented diet

resulted in slightly lower values than those observed in chicks fed lithocholic acid alone, but significantly higher than those from chicks fed the unsupplemented control diet. In the cholesterol-fed chick, lithocholic acid decreased liver cholesterol levels, while chicks fed both cholic and lithocholic acids had liver cholesterol levels which were not significantly different from those of chicks fed the control cholesterol-supplemented diet. Liver lipid phosphorus levels were not significantly altered by the dietary treatments used.

Microscopic examination of the livers revealed a marked ductular proliferation, as previously described, resulting from lithocholic acid ingestion(5). The degree of proliferation induced by lithocholic acid ingestion did not appear to be influenced by cholic acid in chicks fed a cholesterol-free diet. In the cholesterol-fed chick, however, cholic acid markedly influenced the degree of ductular proliferation. All chicks fed lithocholic acid in combination with cholesterol showed a very marked proliferation of bile ductules. Only a very modest involvement was seen in the livers of chicks fed diets containing the combined supplement of cholesterol, cholic and lithocholic acids.

Discussion. The data presented demonstrate that cholic acid somehow protects chicks from the deleterious effects of dietary lithocholic acid. In chicks fed cholesterol, cholic acid affords virtually complete protection against both the biochemical and morphological changes induced by dietary lithocholic acid. However, in chicks fed a cholesterol-free diet, only partial protection is afforded against the biochemical changes noted and no apparent protection is given against the morphological changes seen in the liver as a result of lithocholic acid feeding.

Bile ductule proliferation is induced by a number of different agents both carcinogenic and noncarcinogenic(7) and by biliary obstruction or ligation(8). Recent studies have added lithocholic acid to the list of compounds capable of inducing hyperplasia of bile ductules(2,3,4,5). As pointed out by Songster *et al*(9), the morphological appearance of the biliary proliferation seen in these various intoxications is sufficiently similar to suggest a common mechanism. Songster *et al* (9) suggest the intriguing hypothesis that bile duct hyperplasia may result from an alteration of bile acid metabolism. The present study demonstrating that biliary hyperplasia or the "ductular cell reaction"(7) can be induced by ingestion of a bile acid and that ingestion of a second bile acid may prevent or at least reduce the severity of the hyperplasia, would strengthen such a hypothesis. The possibility remains that cholic acid may act, not on a metabolic pathway, but simply by interfering with the absorption of lithocholic acid.

Summary. Dietary lithocholic acid increased plasma lipid levels, decreased liver fat, increased liver size and induced a marked biliary hyperplasia in chicks fed either a cholesterol-free or supplmented diet. Lithocholic acid increased liver cholesterol levels in chicks fed a cholesterol-free diet and decreased liver cholesterol in chicks fed a cholesterol-supplemented diet. Cholic acid supplementation partially prevented the changes in liver size and plasma liver lipid levels resulting from ingestion of a lithocholic acid-supplemented cholesterol-free diet. In cholesterol-fed chicks, cholic acid afforded almost complete protection against the effects of dietary lithocholic acid.

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On the Excretion of Bile Acid Derivatives in Feces of Rats fed Cholic Acid-24-^{14}C and Chenodesoxycholic Acid-24-^{14}C.

Bile Acids and Steroids 19.

By

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It has been demonstrated that bile acids are the main metabolic products excreted in the bile of rats fed cholesterol-4-^{14}C (BYERS and BIGGS 1952, BERGSTRÖM 1952, SIPERSTEIN, JAYKO, CHAIKOFF and DAUBEN 1952). The bile acids were identified as taurocholic acid and taurochenodesoxycholic acid (BERGSTRÖM and SJÖVALL 1954). These acids account for more than 95 per cent of the activity found in the saponifiable fraction of the bile. When the acidic steroids present in the feces of rats fed labelled cholesterol were investigated, it was found, however, that only a minor part of the total radioactivity was present as conjugated bile acids. The rest was divided between several groups of substances, which could be separated by chromatography (BERGSTRÖM and NORMAN 1953). The splitting of the peptide bond and the further modification of the bile acids which occur during the intestinal passage, are mainly the result of the action of microorganisms (NORMAN 1955).

The aim of the present investigation was to study whether the products found on chromatograms of the fecal acidic products obtained after feeding cholesterol-4-^{14}C can be found on chromatograms after feeding cholic and chenodesoxycholic acid-24-^{14}C.

The action of microorganisms on bile acids *in vitro* as well as *in vivo* has been studied by several authors (see SOLOMIA 1937, 1948). In 1942, SCHMIDT and HUGHES found that cholic acid is rapidly metabolized by the content of the coecum of guinea-pigs. They measured the disappearance of cholic acid injected into the isolated coecum; after 48 hours of incubation only about twenty per cent of the acid remained unchanged. SCHMIDT, HUGHES, GREEN and COOPER (1942) showed that cholic acid added to cultures of a strain of *Alkaligenes faecalis* isolated from human feces, was converted into different ketocholanic acids among which they isolated 3,7,12-triketocholanic acid. MORIMOTO and SHIMIZU (1951) found that 3,12-dihydroxy-7-ketocholanic acid is formed when cholic acid is incubated with a culture of *Bact. coli comm.* In the last few years extensive work has been done on microbiological hydroxylation of steroids. For a review, see PETERSON (1953).

Experimental.

The labelled bile acids used in these investigations were prepared by BERGSTRÖM, ROTTENBERG and VOLTZ (1953). 1—3 mg was brought into solution as sodium salt in 1—2 ml of 0.9 per cent sodium chloride and injected intraperitoneally into white male rats of the institute stock (weight about 150 g). The rats were kept in metabolism cages and the feces and urine collected daily into 80 per cent ethanol for two weeks. The animals had free access to their normal diet consisting mainly of oats and bread.

The feces from each 24-hour period were crushed and boiled twice with 80 per cent ethanol for three hours. The filtrate was transferred to a volumetric flask and an aliquot evaporated to dryness on an aluminium planchet for determination of radioactivity. A few micrograms of a bile acid with high specific activity was added and the total activity determined. In this way a correction for self-absorption was obtained. This was usually not more than 5—10 per cent of the true activity.

The remaining ethanol extract was evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of water acidified to pH 1 with hydrochloric acid and extracted three times with two volumes of butanol. The combined butanol extracts were washed with water to remove excess hydrochloric acid and then evaporated *in vacuo*. The remaining substance was dissolved in 70 per cent ethanol and subjected to a three stage counter-current distribution to remove lipid material that was not bile acids (cf. NORMAN 1955).

The residue from the combined aqueous phases was then fractionated with the reversed-phase partition chromatographic technique described

for bile acids by BERGSTRÖM and SJÖVALL (1951), SJÖVALL (1953) and NORMAN (1953). The fecal extract containing the bile acids was first chromatographed with solvent system C suitable for separation of conjugated bile acids.

Phase system C. 150 ml of methanol, 150 ml of distilled water, 15 ml of isooctanol and 15 ml of chloroform were mixed in a separatory funnel. 4 ml of the lower phase was used per 4.5 g of hydrophobic Supercel.

The material that remained in the stationary phase was eluted with chloroform and rechromatographed with phase system A.

Phase system A. 180 ml of methanol, 120 ml of distilled water, 45 ml of chloroform and 5 ml of heptane were mixed in a separatory funnel. 4 ml of the lower phase was used per 4.5 g of hydrophobic Supercel.

The fractions were collected by a time-operated fraction collector. Each fraction was titrated with 0.02 N sodium hydroxide in methanol. The solvent was then evaporated off in an oven at 100 °C. The material was redissolved in a small amount of 50 per cent ethanol and evaporated to dryness on copper planchets for determination of radioactivity.

Results.

Figures 1 and 2 show the excretion of activity for each 24-hour period in per cent of administered cholic and chenodesoxycholic acid. These figures also show the cumulative amount of activity excreted.

Figures 3 and 6 show the chromatograms obtained with phase systems C and A of the metabolites of cholic acid in the feces from the second to the fourth day. Figures 4 and 7 show the chromatograms with the same systems of the metabolites of chenodesoxycholic acid in the feces from the second day. For comparison we also reproduce two chromatograms of the acidic products in the feces on the seventh day after feeding cholesterol-4-^{14}C (BERGSTRÖM and NORMAN 1953). The solid lines connect titration values and the open circles connected by broken lines show the c.p.m. of each sample.

Depending on the amount of substance, the size of the column had to be varied. It has been found that the amount of solution necessary to elute a certain band is directly proportional to the size of the column. To facilitate the comparison between different curves the figures on the horizontal axis show the number of ml effluent which would be needed on a standard column made up of 4.5 g of Supercel. To obtain the actual amount of eluting solution used, one has to multiply with the factor given in brackets for each curve.

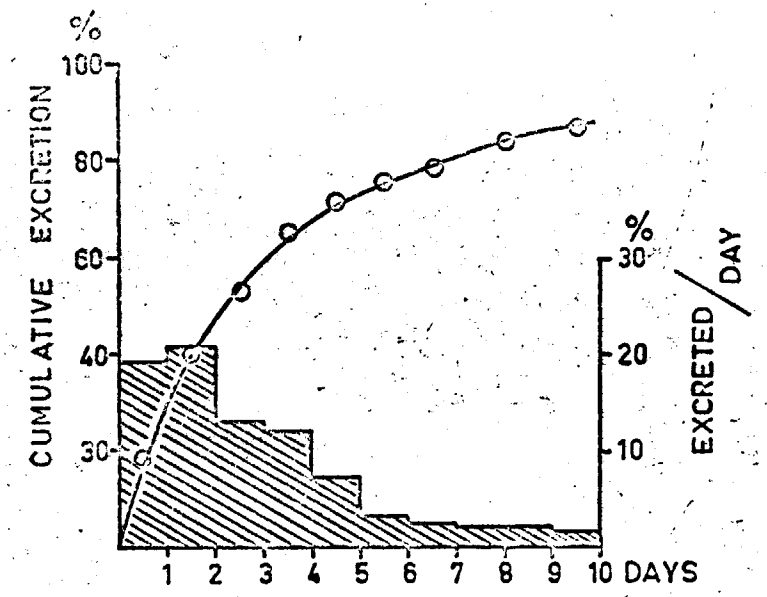

Fig. 1. Isotope excreted in the feces following intraperitoneal administration of cholic acid-24-^{14}C. Shaded areas represent the activity excreted each day in per cent of dose administered; open circles indicate the total amount excreted.

butanol extract of feces is of little significance as the feces contain many acidic products other than bile acids. The greater part of these compounds leave the column with or just after the front with solvent system C. Almost regularly, a distinct titration peak is seen at 130—160 ml in phase system A (this peak is helpful for orientation on the chromatogram). The total amount of these acidic compounds varies with the amount of feces in a 24-hour period.

Discussion.

Amount excreted: It is evident from figures 1 and 2 that carbon atom 24 of the bile acid is completely excreted in the feces. It has previously been shown by BERGSTRÖM, SJÖVALL and VOLTZ (1953), BERGSTRÖM, ROTTENBERG and SJÖVALL (1953) that only an insignificant amount of the activity appears in the expired carbon dioxide after administration of [24—^{14}C] labelled bile

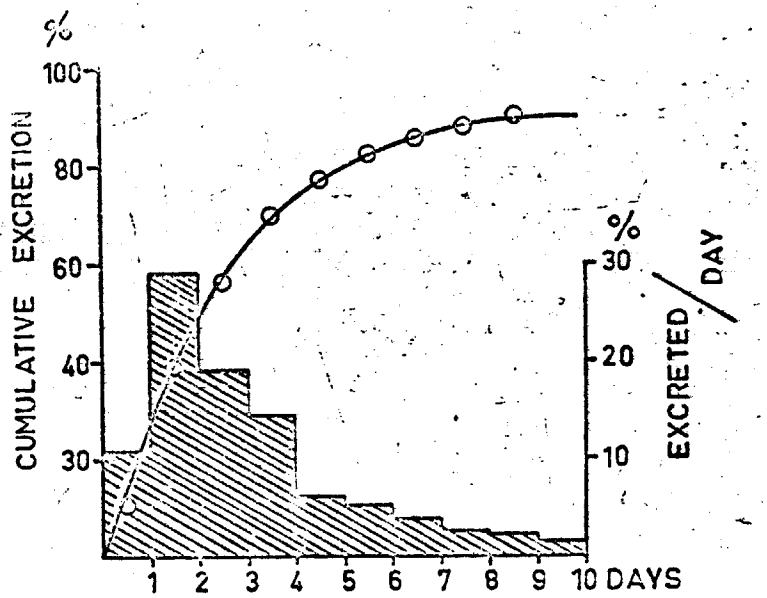

Fig. 2. Isotope excreted in the feces following intraperitoneal administration of chenodesoxycholic acid-24-^{14}C. For explanation, see fig. 1.

measurable activity in the urine. For a further discussion of the rate of elimination, see the paper by LINDSTEDT and NORMAN (1955).

Hydrolysis of the peptide bond: Cholic acid and chenodesoxycholic acid are excreted in the bile, completely conjugated — 1—2 per cent with glycine and the rest with taurine. On the chromatograms with solvent system C (figures 3 and 4), the taurine conjugates all appear in the front, glycocholic acid at 30—50 ml and glycochenodesoxycholic acid at 100—130 ml. It is seen that only a small amount of the acids remain in the conjugated form. A marked splitting of the peptide bonds thus takes place during the intestinal passage. Recent work by NORMAN (1955) has shown that the intestinal microorganisms are mainly responsible for the hydrolysis as practically no splitting of the peptide bonds takes place in rats treated with antibacterial agents. The hydrolysis appears to take place only in the lower part of the intestinal tract as SJÖVALL (1955) did not find any free acids in the content of the small intestine.

The proportion between free and conjugated forms present in

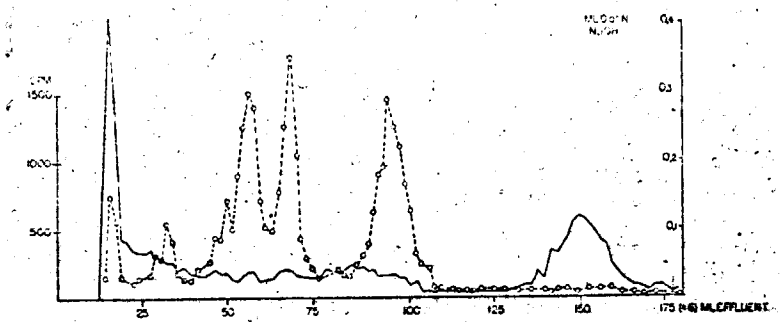

Fig. 3. Chromatographic separation of fecal acids from second to fourth day after administration of cholic acid-24-^{14}C. Phase system C, see page 3. Column: 27 g of hydrophobic Supercel.

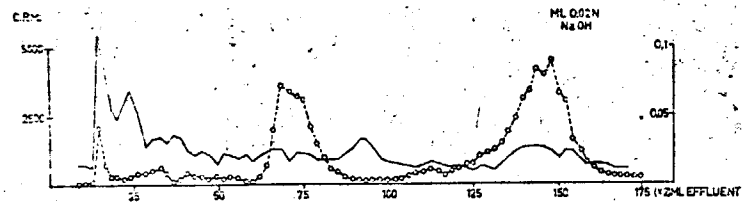

Fig. 4. Chromatographic separation of fecal acids from second day following administration of chenodesoxycholic acid-24-^{14}C. Phase system C, see page 3. Column: 9 g of hydrophobic Supercel.

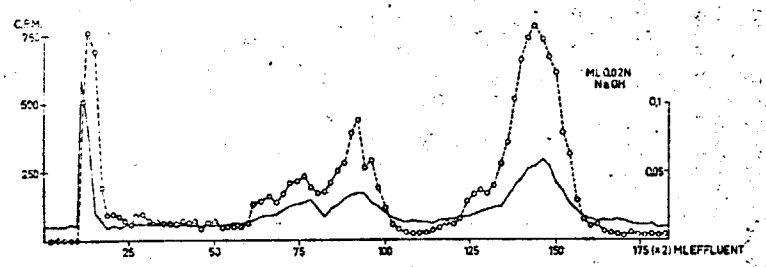

Fig. 5. Chromatographic separation of fecal acids from seventh day following administration of cholesterol-4-^{14}C. Phase system C, see page 3. Column: 9 g of hydrophobic Supercel.

the feces was observed to vary individually in the rat, and in one animal as much as one third of the total activity appeared in the "taurine" band.

Metabolites of cholic acid: It has been shown that cholic acid is not transformed into any new metabolites by the liver (BERGSTRÖM, ROTTENBERG and SJÖVALL 1953). The products separated on the chromatograms shown in figures 3 and 6, result, therefore,

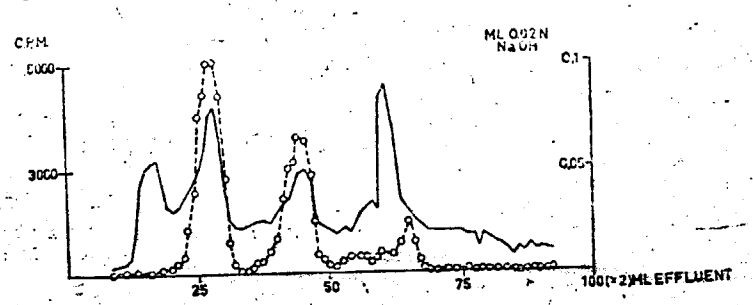

Fig. 6. Cholic acid-24-^{14}C i.p. Chromatographic separation of less hydrophilic acids remaining in stationary phase, fig. 3. Phase system A, see page 3. Column: 9 g of hydrophobic Supercel.

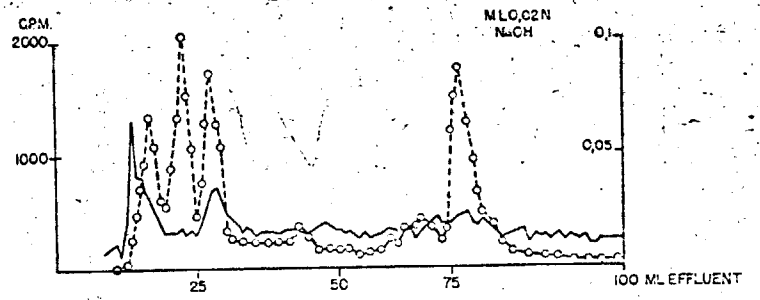

Fig. 7. Chenodesoxycholic acid-24-^{14}C i.p. Chromatographic separation of less hydrophilic acids remaining in stationary phase, fig. 4. Phase system A, see page 3. Column: 4.5 g of hydrophobic Supercel.

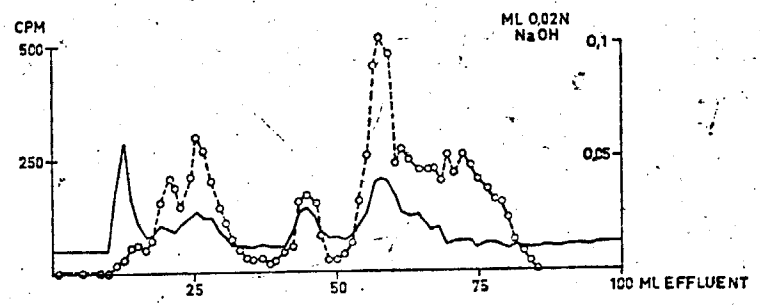

Fig. 8. Cholesterol-4-^{14}C i.p. Chromatographic separation of less hydrophilic acids remaining in stationary phase, fig. 5. Phase system A, see page 3. Column: 4.5 g of hydrophobic Supercel.

from the action of enzymes present in the microorganisms. At the position of free cholic acid (80—110 ml), a distinct peak is seen. This band was hydrolyzed and rerun on the same solvent

system with unlabelled cholic acid. All the labelled material moved with the cholic acid. It is noteworthy that this peak accounts for only 10 to 20 per cent of the total activity. At 50—75 ml, at least two peaks of a more hydrophilic character than cholic acid are regularly seen. More than 50 per cent of the total activity remained in the stationary phase in the run shown in figure 3. The chromatogram of this material with system A is seen in fig. 6 and shows three distinct peaks. The one at 30 ml corresponds to the position of a dihydroxycholanic acid.

When chromatograms of material from feces collected on different days are compared the relative sizes of the bands are found to remain fairly constant during the period studied.

Metabolites of chenodesoxycholic acid: When labelled chenodesoxycholic acid is given to rats with a bile fistula, most of the acid is excreted as taurochenodesoxycholic acid, but about 25 per cent is transformed into two more hydrophilic compounds, none of which is identical with cholic acid after hydrolysis (BERGSTRÖM and SJÖVALL 1954). A chromatogram of the metabolites in feces shows that the major part of the activity is distributed within two bands at 60—70 and 110—140 ml. After alkaline hydrolysis the bands appear at the same positions, indicating that they do not contain conjugated material. As these bands appear at the same positions as those found on chromatograms of hydrolysed material from fistula bile, it is not possible to decide to what extent they contain metabolites formed by the action of microorganisms.

When chromatograms were run on material collected on different days, one animal showed a decrease of the first band with time and a corresponding increase of the second band.

About 30 per cent remained in the stationary phase in the run shown in figure 4. The chromatogram of this material with system A is seen in figure 7. It presents a complicated mixture of different compounds; only a small band appears at the position of chenodesoxycholic acid.

Comparison of the chromatographic patterns obtained after feeding cholic acid, chenodesoxycholic acid and cholesterol.

Chromatograms with solvent system C: The most striking feature is the peak around 150 ml, which is present after feeding cholesterol and chenodesoxycholic acid but regularly absent after feeding cholic acid. At the present time it is not possible to decide whether this peak is homogeneous and contains identical substances in the two cases. The complex at 60—110 ml

seen on the "cholesterol" chromatograms corresponds to peaks in the same position on the "cholic and chenodesoxycholic" chromatograms.

Chromatograms with solvent system A: The complex around 25 ml on the cholesterol chromatogram corresponds to several peaks on the cholic and chenodesoxycholic chromatograms. In the interval 50—75 ml, bands are seen in all chromatograms. The most distinctive feature is the activity peak at 40 ml, accompanied by a titration peak which is seen on the cholesterol and cholic acid chromatograms but which is absent from the chenodesoxycholic chromatogram.

All the bands on the chromatograms of fecal acidic products obtained after feeding cholesterol, can thus be found on the chromatograms after feeding cholic and chenodesoxycholic acid. There are, however, large quantitative variations in different rats of our colony.

Summary.

Cholic acid-24-^{14}C and chenodesoxycholic acid-24-^{14}C were given intraperitoneally to rats and the excretory products in the feces were studied:

1. The given amount is practically completely recovered in feces within 10 days.
2. The acidic products in the feces have been separated by partition chromatography. Only minor amounts were found in the conjugated form. The complicated patterns indicate extensive changes on the bile acid molecule by microbial enzyme systems.
3. The different bands observed on the chromatograms of fecal material after administering labelled cholic and chenodesoxycholic acid together show the same bands that are observed after administration of ring labelled cholesterol.

The technical assistance of Miss K. Hyllén is gratefully acknowledged.

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The Turnover of Cholic Acid in Man.*

Bile Acids and Steroids 51.

By

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Problems related to the formation and excretion of bile acids in man have been studied by several authors (for reviews of earlier work see SOBOTKA 1937, JOSEPHSON 1941, TAYEAU 1949). Experimental difficulties such as the lack of reliable methods for the determination of the individual bile acids, have, however, made detailed quantitative studies impossible.

The estimates of the daily production of bile acids that have been published are usually based on determinations of the excretion of bile acids in bile fistulas in surgical cases or on the amount of the bile acids removed through a duodenal tube. By both these techniques, however, the normal enterohepatic circulation is broken. In animal experiments it has been repeatedly shown that the withdrawal of bile salts stimulates the liver to an increased synthesis (THOMPSON and VARS 1954, ERIKSSON 1956). We have tried to use a technique that would involve a minimum of disturbance of the normal physiological state. A tracer dose of ^{14}C-labelled cholic acid has been given and the specific activity of the circulating cholic acid determined in small samples removed through a duodenal tube at different times.

* A preliminary report of this work was read before the Norwegian Biochemical Society, June 1956.

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Experimental.

Cholic acid-24-^{14}C had been prepared according to BERGSTRÖM, ROTTENBERG and VOLKT (1953). Two different preparations with specific activities of 2.9 μC/mg and 1.005 μC/mg respectively were used. Stock solutions were prepared by dissolving the acid in acetone. One ml of the stock solution was added to about 2 g of inactive cholic acid in ethyl acetate. The cholic acid was crystallized (4.05×10^{-4} μC/mg and 8.75 μC/mg resp.) and used as a standard in the determination of radioactivity.

Administration of labelled acid. 2—4 ml of the stock solutions corresponding to 2.85—4.28 μC (2—5 mg) of cholic acid were evaporated to dryness and redissolved in a few drops of ethanol and then neutralized with 0.1 N sodium hydroxide. The solution was then diluted with water to 50 ml and given by mouth to voluntary male medical students (20—25 years old). The glass and the mouth were then rinsed with several portions of water. The subjects stayed on their ordinary diet and were told to avoid excess fatty food and alcohol.

Collection of bile samples. 20 hours after the administration of labelled acid the subjects swallowed a duodenal tube made from 2.1 mm polyvinyl tubing (Pharmaseal) (BLANKENHORN, HIRSCH and AHRENS Jr. 1955) loaded with 2 ml of mercury in a rubber balloon. After 3—5 hours the tube had usually passed through the pylorus as evidenced by the neutral reaction and green colour of the drainage. The subjects were then injected intravenously with 1.5—3 mg of a pancreozymine-cholecystokinine preparation (obtained from Professor E. JORPES, Stockholm) dissolved in water. In most cases a free flow of thick bile was then obtained from the tube within a few minutes. About 10 ml of this juice was collected and any excess given back through the tube.

Isolation of cholic acid. The bile was diluted with an equal volume of ethanol and the precipitate filtered off. The solution was evaporated to dryness on a water-bath and saponified with 25 ml of 2 N sodium hydroxide in a sealed glass-tube for 12—18 hours at 120° C. The free bile acids were then extracted with ether and cholic acid isolated by column chromatography on hydrophobic kieselguhr with the use of *isooctanol*/chloroform 1:1 as stationary phase and methanol/water 1:1 as moving phase [phase system C of NORMAN (1952)]. The fractions were titrated with 0.02 N sodium hydroxide. Those containing cholic acid were combined and extracted with ether after acidification. The cholic acid was crystallized from ethyl acetate. The yield varied between 5 and 50 mg depending on the concentration and amount of bile collected.

Determination of specific activity. The cholic acid was combusted and the specific activity of the carbon dioxide determined according to CLARCOCK (1954). The efficiency of the counting procedure was obtained from the combustion of a ^{14}C-polyacrylate of known strength (C. E. P. 1. The Radiochemical Centre, Amersham, England).

Results.

Evaluation of necessary assumptions for the determination of turnover rate.

1. *Bile acid pool.* The bile acids that predominantly occur in human bile are cholic, chenodeoxycholic and deoxycholic acids. These acids occur conjugated with glycine or taurine. The proportion between the acids varies between different individuals (WOOTTON 1953, ISAKSSON 1953—54, SJÖVALL 1957). The bile acids are practically completely confined to an anatomically restricted space consisting of the liver, bile ducts, gall-bladder, intestine and portal circulation. Under normal conditions the concentration of bile salts in the systemic circulation is negligible small (IRWIN, JOHNSTON and KOPALA 1944) and the bile acids are exclusively excreted in the feces (LINDSTEDT and NORMAN 1955). In healthy individuals this bile acid pool must be assumed to be in a steady state, *i. e.* the excreted bile acids are replaced by synthesis in the liver.

2. *Mixing within the pool.* The bile salts take part in an enterohepatic circulation as originally proposed by SCHIFF (1870). The time taken for a bile acid molecule to complete one enterohepatic circuit is not known. In animal experiments it has been shown, however, that an amount of labelled bile acid fed by mouth is rapidly absorbed and excreted in a bile fistula to the extent of 80—90 per cent within 2 hours (SJÖVALL and ÅKESSON 1955). It is also known that, when a meal reaches the duodenum, a large amount of bile acids is made available through the contraction of the gall-bladder (BORGSTRÖM, DAHLQVIST, LUNDH and SJÖVALL 1957). These facts indicate that a tracer dose of labelled cholic acid is rapidly mixed with the pool.

3. *Conjugation.* The labelled cholic acid was administered as its sodium salt. From preliminary work in humans and from many animal studies we know that the conjugation is complete when the acid has passed one time through the liver (NORMAN 1954).

4. *Interconversion of the bile acids into each other.* The interconversion of the different bile acids into each other in humans has not been much studied. LINDSTEDT and ZETTERSTRÖM (1957) fed cholic acid to a child and examined the bile soon afterwards. At this time no dihydroxycholanic acids were found. BERGSTRÖM and ZETTERSTRÖM (1957) in a similar experi-

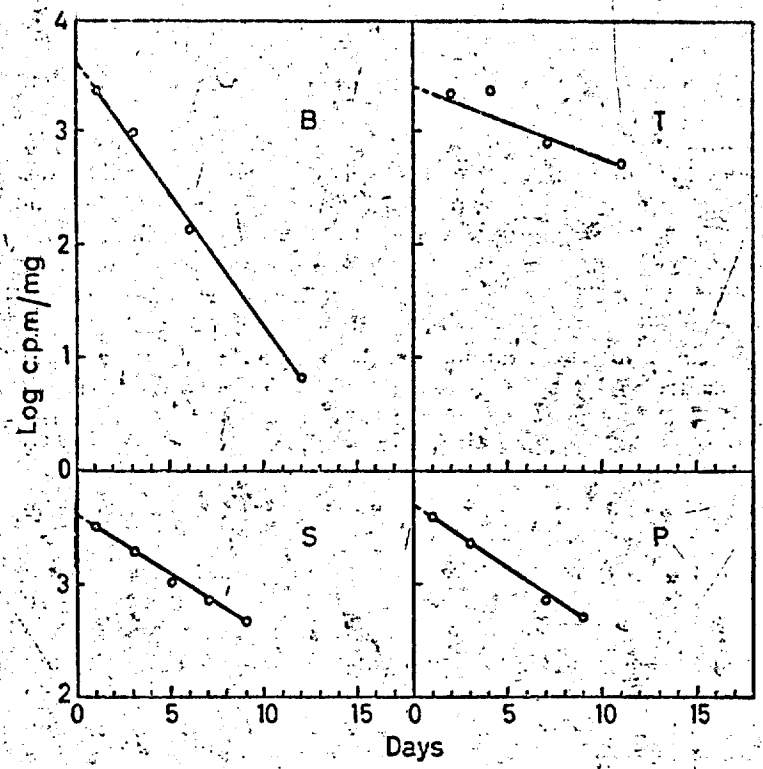

Fig. 1. Semilogarithmic plot of the specific activity of cholic acid in the pool.

ment found no evidence of formation of cholic acid from deoxycholic acid such as has been demonstrated in the rat by BERGSTRÖM, ROTTENBERG and SJÖVALL (1953). From now available evidence it, therefore, appears that the isotope does not re-enter the cholic acid pool once it has been removed from it.

Considering the facts discussed above it was reasonable to assume that the decay of specific activity of the cholic acid in the pool would follow first order kinetics. In this case

$$I_t = I_o e^{-kt}$$

where

I_t = specific activity of cholic acid in the pool at time t.
I_o = specific activity of cholic acid in the pool at zero time.
t = time in days.
k = "Elimination constant".

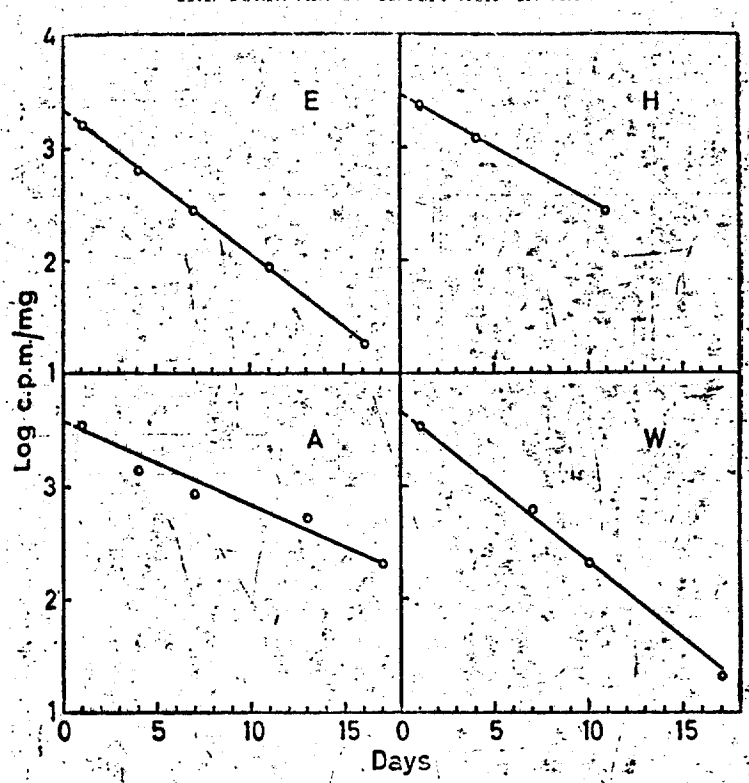

Fig. 2. Semilogarithmic plot of the specific activity of cholic acid in the pool.

and

$$t_{1/2} = \frac{\log 2}{k}$$

where

$t_{1/2}$ = half-life of cholic acid in days.

A plot of log I_t *versus* time would then give a straight line. As the first sample was taken one day after the administration of the tracer dose the value of I_0 was obtained by extrapolation to zero time. From the value of I_0 the amount of cholic acid in the miscible pool was calculated.

In fig. 1 and 2 a plot of log I_t versus time is shown. The linear fall is obvious. The values obtained from the graphs are tabulated in table 1.

Table 1.

Pool size and production of bile acids in man.

Subject	Half-life of cholic acid. Days	Cholic acid pool. g	Chenodeoxycholic acid pool. g	Deoxycholic acid pool. g	Total bile acid pool. g	Daily production of cholic acid. g
B.	1.2	0.50	0.79	0.51	1.88	0.33
T.	3.9	1.93	1.2	0.62	3.75	0.35
S.	3	1.29	1.70	0.92	3.91	0.30
P.	2.5	0.92	1.88	1.10	3.90	0.26
E.	2.4	2.29	1.88	0.78	4.97	0.69
H.	3.2	1.66	—	—	—	0.36
A.	4.2	1.27	1.25	0.80	3.32	0.27
W.	2.3	1.15	1.48	0.68	3.31	0.35
MEAN	2.8	1.38	1.45	0.77	3.58	0.36

With the use of a paper chromatographic technique (SJÖVALL 1956) SJÖVALL (1957) has determined the relative proportions between the three main bile acids in the subjects studied. When determinations were carried out at different times during the experiment only slight variations were encountered, and a mean value was therefore used. As the absolute amount of cholic acid in the pool had been determined by the isotope experiment, it was possible to use the quotients between the different bile acids to calculate the size of the total bile acid pool. The figures thus obtained are found in the table.

Discussion.

Choice of method. It has been repeatedly shown that the bile acids are extensively attacked by intestinal microorganisms (BERGSTRÖM and NORMAN 1953, LINDSTEDT and NORMAN 1955, EKDAHL and SJÖVALL 1955, GUSTAFSSON, BERGSTRÖM, LINDSTEDT and NORMAN 1957, LINDSTEDT 1957). No chemical methods are available that permit the determination of all bile acid derivatives in the feces. LINDSTEDT and NORMAN (1956) fed labelled bile acids to rats and estimated the half-life of the bile acids from the total excretion of isotope in the feces. This technique does not, however, give a figure for the size of the bile acid pool and would furthermore present technical difficulties in human experiments owing to the restricted amount of isotope that can be used.

As mentioned in the introduction it is not possible to reach a correct figure for the daily production of bile acids by measuring the excretion in a bile fistula or the recovery in duodenal drainage. In the present investigation only a few per cent of the total bile acids were withdrawn at intervals of 2—4 days, and this small amount would hardly upset the normal equilibrium. Cholecystokinine — a normal physiological stimulant — was used to bring about contraction of the gall-bladder at the sampling. The subjects had a meal a short time after the collection of the bile sample.

Half-life and pool size. The half-life of cholic acid varies between 1.2 and 4.2 days with a mean of 2.8 days. LINDSTEDT and NORMAN (1956) found the half-life of cholic acid in rats to be 2.3 days (1.2—3.1). In this animal chenodeoxycholic acid, litocholic and cholanic acids showed a half-life of the same order of magnitude. It must be pointed out, however, that the value obtained for cholic acid in this investigation cannot *a priori* be used for calculations of the turnover of chenodeoxy and deoxycholic acids in man.

When this work was started we believed that the three main bile acids in man were formed directly from cholesterol in the liver and that no interconversions between the acids took place. Recently, however, evidence has accumulated showing that deoxycholic acid is a "secondary" bile acid arising at least partially from the action of intestinal microorganisms on cholic acid (LINDSTEDT and SJÖVALL 1957, SJÖVALL 1957, LINDSTEDT 1957). If cholic acid is an obligatory precursor of deoxycholic acid the turnover of the cholic acid pool would be more rapid than that of the deoxycholic one in subjects where the bile contains more deoxycholic than cholic acid. In order to get a complete picture of the complex system it would be necessary to determine the turnover of the different bile acids in the same subject.

The size of the cholic acid pool showed large variations (0.58—2.29) giving a mean value of 1.39 g. It should be noted, however, that usually in the subjects with a small pool the half-life of the cholic acid was short. When the two other main bile acids were taken into account a value for the total bile acid pool of 3.58 g (1.88—4.97 g) was found. BERGSTRÖM and ERIKSSON (1957) found a value of 4.2—6.3 mg/100 g body weight for the bile acid pool in the rat, which is a figure of

the same order of magnitude as that found in the present work.

Relation to cholesterol metabolism. It is an established fact that the bile acids represent end-products of the cholesterol metabolism and there is no evidence against the assumption that cholesterol is an obligatory precursor of the bile acid. Our knowledge of the route by which the bile acids formed from cholesterol in man is, however, incomplete (ROSENFELD, HELLMAN and GALLAGHER 1955, SIPERSTEIN and MURRAY 1955).

From the data obtained in the present work we have calculated the daily formation of cholic acid to be 0.36 g (0.21—0.69). This corresponds to a daily degradation of 0.31 g of cholesterol. To this figure should be added the amount that is degraded to chenodeoxycholic acid which is probably of the same order of magnitude.

Summary.

Cholic acid-24-^{14}C has been given to male medical students. The specific activity of the circulating cholic acid has been followed. The half-life was 2.8 days and the size of the pool 3.58 g. From values of the quantitative composition of the bile the chenodeoxycholic and deoxycholic acid pools have been calculated.

I am much indebted to Professor E. JORPES and Dr. V. MUTT, Stockholm, for a generous supply of cholecystokinin.

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Bile Acids and Steroids

LXXXIII. ON THE INTERCONVERSION OF CHOLIC AND DEOXYCHOLIC ACID IN THE RAT

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Cholic acid and chenodeoxycholic acid are the main bile acids in rat bile, occurring in the approximate proportion of 8:2 (1). The occurrence of minor amounts of metabolites of cholic and chenodeoxycholic acid, formed by the action of intestinal microorganisms and liver enzymes, has recently been reported (2, 3).

The transformation of labeled cholic acid during the enterohepatic circulation has been studied by Norman and Sjövall (2) who isolated labeled deoxycholic acid and 7-ketodeoxycholic acid from the feces. These two acids were evidently formed by the action of intestinal microorganisms on cholic acid. Furthermore, labeled 7-ketodeoxycholic acid was found to give rise to deoxycholic acid in the intestinal tract.

The deoxycholic acid formed in the intestine is absorbed and hydroxylated at the 7α-position to cholic acid (4). This hydroxylation reaction has so far not been found in other species and explains the relative absence (1 to 2 per cent) of deoxycholic acid from rat bile.

The mechanism of the elimination of the 7α-hydroxyl group has been studied in the rabbit (5). In this animal 7-ketodeoxycholic acid was only of minor importance as an intermediate, since the 7β-hydrogen was almost completely retained in the deoxycholic acid, formed from cholic acid-7β-H^3, 24-C^{14}.

The present work was carried out in order to study the reaction mechanisms in the sequence

$$\text{Cholic acid} \xrightarrow{\text{intestine}} \text{deoxycholic acid} \xrightarrow{\text{liver}} \text{cholic acid}$$

and to obtain some information of the relative speed of these reactions.

EXPERIMENTAL

Cholic Acid-7β-H^3, 24-C^{14}—The doubly labeled cholic acid was prepared as described in the preceding paper (5), and a stock solution was made containing approximately 0.5 μc. of 7β-H^3 and 0.3 μc. of 24-C^{14} per mg. of cholic acid.

Animal Experiments—White male rats, 200 to 250 gm., of the institute stock were used. The diet consisted mainly of bread and oats. Cannulation of the bile duct was performed as described earlier (6).

In some experiments the action of the intestinal microorganisms on cholic acid was studied, and the effect of the liver enzymes on the metabolites formed had to be excluded. The acid as sodium salt in water solution was therefore injected with a fine needle into the cecum of the rat, immediately after a bile fistula had been made. The feces were collected in ethanol.

The germ-free rats used were reared under germ-free conditions according to the technique of Gustafsson (7). The sodium salt of cholic acid-7β-H^3, 24-C^{14} in water solution was autoclaved at 120° for 20 minutes and given orally to the animals. A bile fistula was made after 7 days and the bile collected during the following 2 hours was used.

Isolation and Chromatographic Separation—The bile and feces were collected, hydrolyzed, extracted, and chromatographed as described earlier (8, 9). The following phase systems were used:

System	Moving phase	Stationary phase
C	150 ml. of methanol	15 ml. of isooctanol
	150 ml. of distilled water	15 ml. of chloroform
F	165 ml. of methanol	45 ml. of chloroform
	135 ml. of distilled water	5 ml. of heptane

Four ml. of the stationary phase were used per 4.5 gm. of hydrophobic Super-Cel. All the chromatograms were run at a constant temperature of +23°.

Isotope Determination—Tritium and C^{14} activity in the doubly labeled acids administered and in the isolated bile acids were determined by gas phase counting as described previously (5).

RESULTS

Loss of 7β-H^3 in Cholic Acid-7β-H^3, 24-C^{14} during Enterohepatic Circulation—Cholic acid-7β-H^3, 24-C^{14}, 1 mg., was injected intraperitoneally into rats, and after different time intervals (0 to 7 days) a bile fistula was made. The bile was collected for 2 hours and the cholic acid isolated by chromatography with phase system C. Determinations of the ratio of isotopes in the administered and isolated cholic acids were made, and the amount of tritium retained was calculated. From the results shown in Table I it is obvious that a progressive loss of the tritium label occurs with time as the acid takes part in the enterohepatic circulation.

In order to prove that there is no loss of the 7β-H^3 label in cholic acid during the liver passage, the doubly labeled acid was administered to two germ-free rats and allowed to take part in the enterohepatic circulation for 7 days, at the end of which time the tritium retention in the normal rat was less than 5 per cent. As seen in Table III, the ratio of tritium to C^{14} remains constant in the cholic acid in the germ-free rats.

Retention of 7β-H^3 in Intestinal Formation of Deoxycholic Acid from Cholic Acid-7β-H^3, 24-C^{14}—In order to determine the reten-

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tion of the 7β-H^3 in the step, cholic acid → deoxycholic acid, the rehydroxylation of deoxycholic acid in the liver had to be excluded. This was accomplished by injecting the doubly labeled cholic acid into the cecum of bile fistula rats. The rats were killed after one and a half days and deoxycholic acid was isolated from the feces by chromatography with phase system F.

TABLE I

Determination of H^3 and C^{14} in administered cholic acid and in cholic acid isolated from rats after different times of enterohepatic circulation

Cholic acid	Duration of enterohepatic circulation	C^{14} c.p.m./mg.	C^{14} Mean	H^3 c.p.m./mg.	H^3 Mean	H^3/C^{14}	H^3 retained Per cent	H^3 retained Mean (per cent)
	days							
Administered		424		460				
		419	422	471	466	1.11	100	
Isolated from rat with	0	298		302				
preformed bile fistula		288	293	316	309	1.05	95	
Isolated from rats with	1	280		221				
intact enterohepatic		290	285	233	227	0.80	72	
circulation		314		251				
		318	316	283	269	0.85	77	75
	2	386		79				
		384	385	86	83	0.22	20	
		516		155				
		517	517	157	156	0.30	27	
		437		185				
		439	438	175	180	0.41	37	28
	4	590		103				
		595	593	112	108	0.18	16	
		564		259				
		569	567	271	265	0.47	42	
		506		185				
		510	508	198	192	0.38	34	31
	7	440		26				
		436	438	27	27	0.06	5.4	
		523		27				
		531	527	29	28	0.05	4.5	5
Isolated from germ-free rats with intact enterohepatic circulation	7	260		282				
		263	262	284	283	1.08	98	
		256		280				
		255	256	281	281	1.10	99	99

TABLE II

Determination of H^3 and C^{14} in deoxycholic acid in feces

	C^{14} c.p.m./mg.	C^{14} Mean	H^3 c.p.m./mg.	H^3 Mean	H^3/C^{14}	H^3 retained Per cent	H^3 retained Mean (per cent)
Administered cholic acid					1.10	100	
Deoxycholic acid isolated from feces	297		294				
Rat XVII	304	301	294	294	0.98	89	
Rat XVIII	236		221				
	237	237	216	219	0.92	84	87

TABLE III

Determination of H^3 and C^{14} in cholic acid after administration of deoxycholic acid-7β-H^3-24-C^{14}

	C^{14} c.p.m./mg.	C^{14} Mean	H^3 c.p.m./mg.	H^3 Mean	H^3/C^{14}	H^3 retained Per cent	H^3 retained Mean (per cent)
Administered deoxycholic acid, Rat XVII					0.98	100	
Cholic acid isolated from bile							
Rat XIX	315		17				
	320	318	13	15	0.05	4.8	
Rat XX	286		9				
	285	286	11	10	0.04	3.6	4.2

The band of radioactive deoxycholic acid was diluted with inactive deoxycholic acid and crystallized from acetic acid water and ethanol-water. The specific activity of successive samples remained constant.

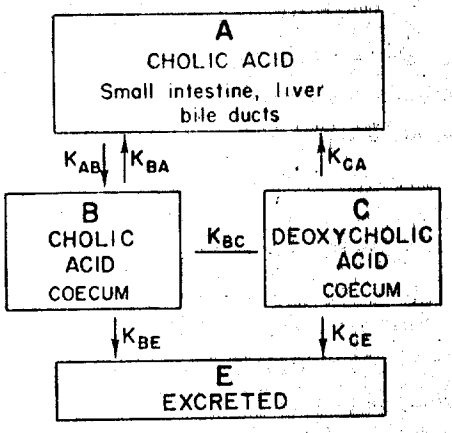
The results from the determinations of H^3 and C^{14} in this compound (Table II) showed that about 85 per cent of the original tritium label is retained.

Loss of Tritium Label in Deoxycholic Acid-7-H^3, 24-C^{14} during 7α-Hydroxylation to Cholic Acid—The deoxycholic acid which had been isolated from the feces after injection of doubly labeled cholic acid into the cecum was administered to two rats with a functioning bile fistula. About 50 per cent of the administered deoxycholic acid was 7α-hydroxylated to cholic acid, which was isolated by chromatography with phase system C and crystallized from ethyl acetate after dilution with inactive cholic acid. Determinations of the H^3 and C^{14} content in this acid (Table III) showed that almost all of the tritium label was lost during the hydroxylation in the liver.

DISCUSSION

Reaction Mechanism—The experiments in which deoxycholic acid was isolated from feces and readministered clearly showed that only a small loss of the 7β-hydrogen occurs in the conversion of cholic acid into deoxycholic acid and suggest that a shift from the 7β- to the 7α-position takes place. These results therefore confirm the findings obtained in the preceding paper (5), in which the reaction has been discussed.

Rate of Formation of Deoxycholic Acid—The fact that cholic acid and deoxycholic acid are converted into each other should obviously be taken into account in studies of bile acid turnover in the rat. Such studies have been carried out with the use of cholic acid-24-C^{14} at a time when it was assumed that the isotope did not re-enter the cholic acid pool (10). We have now tried to devise a model that is more suitable for discussions of the quantitative aspects of the cholic-deoxycholic acid metabolism in the rat and which could make it possible to estimate the amount of deoxycholic acid formed per day.



With the use of this model the following general set of equations[1] may be worked out. For tritium:

$$dA/dt = -K_{AB}\cdot A + K_{BA}\cdot B \quad (1)$$

$$dB/dt = K_{AB}\cdot A - (K_{BA} + K_{BC} + K_{BE})B \quad (2)$$

$$dC/dt = K_{BC}\cdot B - (K_{CA} + K_{CE})C \quad (3)$$

For C^{14}:

$$dA/dt = -K_{AB}\cdot A + K_{BA}\cdot B + K_{CA}\cdot C \quad (20)$$

$$dB/dt = K_{AB}\cdot A - (K_{BA} + K_{BC} + K_{BE})\cdot B \quad (21)$$

$$dC/dt = K_{BC}\cdot B - (K_{CA} + K_{CE})\cdot C \quad (22)$$

From studies with labeled cholic acid (2) it is known that the conversion to bacterial metabolites starts in the large intestine, *i.e.* the cecum, so it seems justified to consider the cholic acid as being divided between two compartments. The distribution of the bile acids in these compartments shows large individual variations owing to differences in nutritional status of the animals and to the nature of the intestinal microorganisms. In eight animals Norman and Sjövall (2) found 20 to 52 per cent of the total radioactivity in the large intestine after feeding of labeled cholic acid. In the calculations below it is assumed that 30 per cent of the total bile acids is located in the large intestine.

The proportion between cholic acid and deoxycholic acid in the large intestine also shows large variations. From chromatograms of the fecal bile acids collected in this study we have estimated the cholic and deoxycholic acid pools in the large intestine to be of about equal size, and in this simplified system no corrections have been made for the other bile acids in the large intestine, consisting of 7-ketodeoxycholic acid, 3α,7β,12α-trihydroxycholanic acid, 12-ketolithocholic acid, and very small amounts of unidentified microbial metabolites. The deoxycholic acid formation occurs to a small extent with an intermediate formation of 7-ketodeoxycholic acid, a fact that has no influence on the validity of these calculations. According to preliminary results obtained on germ-free rats by Norman and Sjövall,[2] small amounts of cholic acid and 3α,7β,12α-trihydroxycholanic acid may be formed from 7-ketodeoxycholic acid during the enterohepatic circulation, whereas no reduction of the 7-keto acid can be observed after one passage through the liver in a bile fistula rat. However, this reconversion of 7-ketodeoxycholic acid into cholic acid is too small to affect these calculations significantly.

[1] The equations are numbered according to their appearance in the Appendix at the end of the article.

[2] A. Norman and J. Sjövall, to be published.

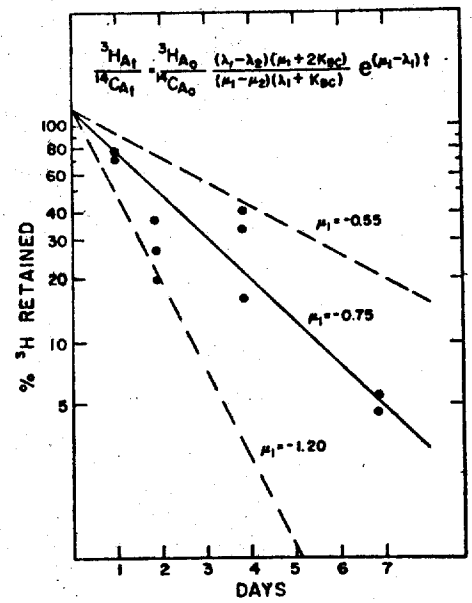


Fig. 1. Semilogarithmic plot of the H^3-retention in cholic acid after different times of enterohepatic circulation of the administered cholic acid-7β-H^3-24-C^{14}.

To solve the kinetic equations one should ideally follow the specific activities of the labeled bile acids in the different pools; this however, does not seem possible for practical reasons. To get an approximation we have therefore assumed that the bile acids are excreted and reabsorbed from the large intestine in proportion to their concentration ($K_{BE} = K_{CE}$; $K_{BA} = K_{CA}$). If a tracer dose of cholic acid 7-β-H^3, 24-C^{14} is introduced into pool A at zero time, the following expression is obtained for the ratio between H^3 and C^{14} of cholic acid in pool A. (The derivation of this equation is shown in the appendix on page 2029.)

$$\frac{H^3_{A_t}}{C^{14}_{A_t}} = \frac{H^3_{A_0}(\lambda_1 - \lambda_2)(\mu_1 + 2K_{BC})}{C^{14}_{A_0}(\mu_1 - \mu_2)(\lambda_1 + K_{BC})} e^{(\mu_1 - \lambda_1)t} \quad (42)$$

The straight lines which represent Equation 42 have been drawn to fit the relative minimal and maximal values for the H^3 retention (Fig. 1) $\mu_1 = -0.55$, $K_{AB} = 0.8$, $K_{BC} = 1.8$, $K_{BA} = 0.9$, $K_{BE} = 0.9$, and $\mu_1 = -1.2$, $K_{AB} = 2.2$, $K_{BC} = 5.2$, $K_{BA} = 4.3$, $K_{BE} = 0.9$, which means that in this series from 32 to 92 per cent of the total amount of cholic acid in the body is converted into deoxycholic acid per day. The parameter for Equation 42, calculated by the method of least squares, gives $\mu_1 = -0.75$, $K_{AB} = 1.24$, $K_{BC} = 2.9$, $K_{BA} = 2.0$, and $K_{BE} = 0.9$; that is, *51 per cent* of the total amount of cholic acid is converted into deoxycholic acid per day.

On the basis of the rate constants obtained in this work (calculated from the line with $\mu_1 = -0.75$, Fig. 1) and the pool sizes, which are known from earlier investigations, some very approximative quantitative information on the enterohepatic circulation of the bile acids in the rat may be obtained. The rate of formation of bile acids in the liver has been found by Bergström and Danielsson (11) to be regulated by the amount of bile acids that reach the liver via the portal blood. A supply of 120 mg. of sodium taurochenodeoxycholate to the liver per day in a 200 gm. bile fistula rat inhibited the cholic acid synthesis

(40 mg. per day) to a level corresponding to that found for the intact animal (about 4 mg. per day). A similar figure for the supply of bile acids to the liver in the rat (144 mg. of cholic acid per day) was obtained by Olivecrona and Sjövall,[3] based on determinations of the bile acid concentration in the portal blood and correlation of these values with the portal blood flow.

If the cholic acid pool in the small intestine, liver, and bile ducts circulates 12 times per day[3]) about *10 per cent* ($K_{AB} \cdot A/12.1) \cdot 100$ of this pool is lost to the large intestine in each circulation. Of the total amount of cholic acid that is transported to the large intestine per day ($K_{AB} \cdot A$) of which *50 per cent* daily is converted into deoxycholic acid ($K_{BC} \cdot B$), *80 per cent* is excreted in the form of various microbial transformation products per day ($K_{BE} \cdot B$) + ($K_{CE} \cdot C$), and *70 per cent* is absorbed from the large intestine and transported to the liver in the form of cholic acid, deoxycholic acid, and their metabolites ($K_{BA} \cdot B$) + ($K_{CA} \cdot C$). If the total cholic acid pool ($A + B$) in a 200-gm. rat amounts to approximately 12 mg.[4] (in all these calculations chenodeoxycholic acid and its metabolites are disregarded since they constitute only a minor component of the bile acids present in the rat (12)) about 120 mg. of cholic acid are absorbed from the small intestine, and 12.2 mg. are transported to the large intestine per day. From this site about 8.4 mg. of bile acids are absorbed and 3.8 mg. are excreted in the feces per day.

SUMMARY

Cholic acid-7β-H^3, 24-C^{14} has been administered to rats and the amount of H^3 and C^{14} determined in the isolated cholic acid after different time intervals. Deoxycholic acid is formed from cholic acid in the large intestine during the enterohepatic circulation by the action of the intestinal microorganisms, but is subsequently 7α-hydroxylated to cholic acid in the liver. With the aid of cholic acid-7β-H^3, 24-C^{14} the reaction mechanism for the deoxycholic acid formation in the intestine has been studied, and the localization of the hydrogen isotope in the isolated deoxycholic acid has been determined through hydroxylation to cholic acid. Taking advantage of the fact that the tritium label in the deoxycholic acid is specifically lost in the hydroxylation reaction, it has been possible to construct a model, by which the amount of deoxycholic acid formed during the enterohepatic circulation may be determined. Furthermore, some approximate quantitative information has been obtained for the enterohepatic circulation of the bile acids. The following results were obtained.

1. The tritium label in cholic acid-7β-H^3, 24-C^{14} was almost completely retained in the molecule during the conversion to deoxycholic acid by the microorganisms in the large intestine.

2. The tritium label in the isolated deoxycholic acid was lost in the 7α-hydroxylation to cholic acid in the liver; this suggests a shift of the H^3-label from the 7β- to the 7α-position.

3. With the proposed model for the metabolism of cholic acid in the rat, it was found that about *50 per cent* of the total cholic acid pool is converted into deoxycholic acid per day during the enterohepatic circulation; the greater part of this acid is subsequently 7α-hydroxylated to cholic acid in the liver.

4. Approximate values for the continuous loss of bile acids from the bile acid pool present in the small intestine, liver, and bile ducts to the pool in the large intestine are given. The rate of absorption of bile acids from the large intestine in the intact rat was found to be much slower than that reported for the absorption process in the small intestine, and figures for the total amount of bile acids that are absorbed from these two sites are given.

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APPENDIX

The following equations may be derived from the model on page 2028.

For tritium:

$$dA/dt = -K_{AB}A + K_{BA}B \tag{1}$$

$$dB/dt = K_{AB}A - (K_{BA} + K_{BC} + K_{BE})B \tag{2}$$

$$dC/dt = K_{BC}B - (K_{CA} + K_{CE})C \tag{3}$$

$$K_{BE} = K_{CE}; \quad K_{BA} = K_{CA}; \quad K_{BC} = K_{CA} + K_{CE}$$

A = 70 per cent, B = 15 per cent and C = 15 per cent (for explanation see the text).

$$dA/dt = -\tfrac{3}{7}K_{BC}A + K_{BA}B \tag{4}$$

$$dB/dt = \tfrac{3}{7}K_{BC}A - 2\,K_{BC}B \tag{5}$$

$$dC/dt = K_{BC}B - K_{BC} \cdot C \tag{6}$$

$A = x$ and $B = y$, $dx/dt = x'$, $dx'/dt = x''$, $dy/dt = y'$, $dy'/dt = y''$.

$$x' = -\tfrac{3}{7}K_{BC}x + K_{BA}y \tag{7}$$

$$y' = \tfrac{3}{7}K_{BC}x - 2\,K_{BC}y \tag{8}$$

$$x' + y' = K_{BA}y - 2\,K_{BC}y \tag{9}$$

$$y'' = \tfrac{3}{7}K_{BC}x' - 2\,K_{BC}y' \tag{10}$$

$$y'' + \tfrac{17}{7}K_{BC}y' - \tfrac{3}{7}K_{BC}(K_{BA} - 2\,K_{BC})y = 0 \tag{11}$$

The roots (μ_1 and μ_2) are:

$$\mu_{1,2} = -\tfrac{17}{14}K_{BC} \pm \tfrac{1}{14}\sqrt{121K_{BC}^2 + 84K_{BA}K_{BC}} \tag{12}$$

and the solution of Equation 11 is:

$$y = L_1e^{\mu_1 t} + L_2e^{\mu_2 t} \tag{13}$$

$$t = 0,\ y = 0 \quad \text{and} \quad L_1 = -L_2 = L$$

$$y = L(e^{\mu_1 t} - e^{\mu_2 t}) \tag{14}$$

$$y' = L(\mu_1e^{\mu_1 t} - \mu_2e^{\mu_2 t}) \tag{15}$$

If the value of y in Equation 14 is substituted for y in Equation 8 then:

$$y' = \tfrac{3}{7}K_{BC}x - 2\,K_{BC}L(e^{\mu_1 t} - e^{\mu_2 t}) \tag{16}$$

$$L(\mu_1e^{\mu_1 t} - \mu_2e^{\mu_2 t}) = \tfrac{3}{7}K_{BC}x - 2\,K_{BC}L(e^{\mu_1 t} - e^{\mu_2 t}) \tag{17}$$

$$\text{at } t = 0;\ L = \tfrac{3}{7}K_{BC}\frac{x_0}{\mu_1 - \mu_2} \tag{18}$$

$$x = \frac{x_0}{\mu_1 - \mu_2}[(\mu_1 + 2\,K_{BC})e^{\mu_1 t} - (\mu_2 + 2\,K_{BC})e^{\mu_2 t}] \tag{19}$$

[3] T. Olivecrona and J. Sjövall, to be published.

[4] S. Eriksson, to be published.

For C^{14}:

$$dA/dt = -K_{AB}A + K_{BA}B + K_{CA}C \quad (20)$$

$$dB/dt = K_{AB}A - (K_{BA} + K_{BC} + K_{BE})B \quad (21)$$

$$dC/dt = K_{BC}B - (K_{CA} + K_{CE})C \quad (22)$$

$$K_{BE} = K_{CE}, K_{BA} = K_{CA}, \text{ and } K_{BC} = K_{CA} + K_{CE}$$

$A = 70$ per cent, $B = 15$ per cent and $C = 15$ per cent (see the text).

$$dA/dt = -\tfrac{3}{7}K_{BC}A + K_{BA}(B + C) \quad (23)$$

$$dB/dt = \tfrac{3}{7}K_{BC}A - 2\,K_{BC}B \quad (24)$$

$$dC/dt = K_{BC}B - K_{BC}C \quad (25)$$

$$d(B + C)/dt = \tfrac{3}{7}K_{BC}A - K_{BC}(B + C) \quad (26)$$

$$A = x, B + C = y, dx/dt = x', dx'/dt = x'', dy/dt = y', dy'/dt = y''.$$

$$x' = -\tfrac{3}{7}K_{BC}x + K_{BA}y \quad (27)$$

$$y' = \tfrac{3}{7}K_{BC}x - K_{BC}y \quad (28)$$

$$x' + y' = (K_{BA} - K_{BC})y \quad (29)$$

$$x' = (K_{BA} - K_{BC})y \quad (30)$$

$$y'' = \tfrac{3}{7}K_{BC}x' - K_{BC}y' \quad (31)$$

$$y'' + \tfrac{10}{7}K_{BC}y' - \tfrac{3}{7}K_{BC}(K_{BA} - K_{BC})y = 0 \quad (32)$$

the roots (λ_1 and λ_2) are:

$$\lambda_{1,2} = -\tfrac{5}{7}K_{BC} \pm \tfrac{1}{7}\sqrt{4\,K_{BC}^2 + 21\,K_{BA}\,K_{BC}} \quad (33)$$

The solution of Equation 32 is:

$$y = L_1e^{\lambda_1 t} + L_2e^{\lambda_2 t} \quad (34)$$

$$t = 0, y = 0 \text{ and } L_1 = -L_2 = L$$

$$y = L(e^{\lambda_1 t} - e^{\lambda_2 t}) \quad (35)$$

$$y' = L(\lambda_1e^{\lambda_1 t} - \lambda_2e^{\lambda_2 t}) \quad (36)$$

If the value of y in Equation 35 is substituted for y in Equation 28 then:

$$y' = \tfrac{3}{7}K_{BC}x - K_{BC}L(e^{\lambda_1 t} - e^{\lambda_2 t}) \quad (37)$$

$$L(\lambda_1e^{\lambda_1 t} - \lambda_2e^{\lambda_2 t}) = \tfrac{3}{7}K_{BC}x - K_{BC}L(e^{\lambda_1 t} - e^{\lambda_2 t}) \quad (38)$$

$$\text{At } t = 0;\ L = \tfrac{3}{7}\cdot K_{BC}/(\lambda_1 - \lambda_2)\cdot x_0 \quad (39)$$

$$x = x_0/(\lambda_1 - \lambda_2)[(\lambda_1 + K_{BC})e^{\lambda_1 t} - (\lambda_2 + K_{BC})e^{\lambda_2 t}] \quad (40)$$

From Equation 19 and Equation 40 the ratio between H^3 and C^{14} of cholic acid in pool A is obtained.

$$\frac{H^3_{At}}{C^{14}_{At}} = \frac{H^3_{A0}\,(\lambda_1 - \lambda_2)\,[(\mu_1 + 2\,K_{BC})e^{\mu_1 t} - (\mu_2 + 2\,K_{BC})e^{\mu_2 t}]}{C^{14}_{A0}\,(\mu_1 - \mu_2)\,[(\lambda_1 + K_{BC})e^{\lambda_1 t} - (\lambda_2 + K_{BC})e^{\lambda_2 t}]} \quad (41)$$

λ_1 is known from earlier investigations to be equal to -0.30 (10). μ_1 is chosen to fit the experimental data. The different K values as well as λ_2 and μ_2 are then obtained from Equations 12 and 33. For $\mu_1 = -0.75$ and $\lambda_1 = -0.30$, μ_2 is -6.25, $\lambda_2 = -3.82$ and $K_{BC} = 2.88$. When these values are used in Equations (19) and (40) it appears that the second term within the brackets may be neglected already after one day. The ratio between H^3 and C^{14} is then:

$$\frac{H^3_{At}}{C^{14}_{At}} = \frac{H^3_{A0}\,(\lambda_1 - \lambda_2)(\mu_1 + 2\,K_{BC})}{C^{14}_{A0}\,(\mu_1 - \mu_2)(\lambda_1 + K_{BC})}\,e^{(\mu_1 - \lambda_1)t} \quad (42)$$

On a semilogarithmic paper, the straight line, which represents this simplified equation, intersects the ordinate at a value of H^3-retention that is higher than 100 per cent.

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THE EFFECT OF NUTRITION ON THE TUMOR RESPONSE IN ROUS CHICKEN SARCOMA

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INTRODUCTION

NUMEROUS observations have confirmed the fact that nutrition may influence the genesis and growth of tumors in rats and mice. The search for requirements in the nutrition of malignant tissue should reveal findings that are valid regardless of the nature of the tumor studied or the kind of animal used, as well as more limited findings. Such information might well include the facts that adequate caloric intake has been shown necessary for tumor growth[1] and that vitamins such as pantothenic acid and riboflavin have been found stimulative,[2] whereas choline may be inhibitory.[3]

EFFECT OF NUTRITION IN ROUS SARCOMA

Little and co-workers[4] showed that folic acid has a marked effect on the tumor response in Rous chicken sarcoma. The effectiveness of folic acid free diets in preventing the tumor response of baby chicks and the rapidity and regularity with which the response occurred in adequately nourished chicks led us to our present study of the effect of each of the different constituents in a synthetic diet for chickens.

The data to be presented confirm observations made on rats and mice to the effect that malignant tissue may utilize vitamins such as pantothenic acid and riboflavin for growth. Our results also indicate that nicotinamide and cholic acid may be stimulative. However, folic acid appears to be the only nutrient required to the extent that tumor response is prevented by its absence.

Method for Demonstrating the Effect of Nutrition.—In our tests, the extent to which each nutrient influenced tumor response was determined by comparing the incidence of tumors in groups of chicks fed the complete diet with the incidence of tumors in groups fed the same diet without the nutrient. By comparing observations made on the eighth, tenth, twelfth, fourteenth, and sixteenth day, the effect of nutrition was evaluated for all stages from the usual time of first appearance of tumors to the time when 90 to 100 per cent of control birds showed tumors. The response with and without nutrient was calculated from the combined results of different tests where more than one test was carried out. The percentage of birds showing tumors when fed diets with the nutrient was divided by the percentage showing tumors when fed the same diet without the nutrient. The nutrients which showed no stimulative effect gave values of approximately 1.0.

Relation of Size of Inoculum to Tumor Response.—An even distribution of tumor responses over the eight days selected for observation of results was readily obtained by controlling the size of the inoculum. As shown in Table I, there is a direct correlation between the size of inoculum and the length of the latent period.

We used homogeneous samples of frozen virus, prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry

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TABLE I. TUMOR RESPONSE OBTAINED WITH HOMOGENEOUS PREPARATION OF INOCULUM KEPT FROZEN FOR VARIOUS PERIODS

AMOUNT OF INOCULUM USED (0.25 ML. DOSE)	INOCULUM KEPT FROZEN (DAYS)	TOTAL NUMBER OF CHICKS USED	NUMBER OF CHICKS THAT GREW TUMORS	PERCENTAGE OF TAKES	AVERAGE LATENT PERIOD (DAYS)
10 mg.	Fresh	21	21	100.0	7.90
	16	16	15	93.7	7.93
1 mg.	Fresh	25	25	100.0	9.56
	16	16	15	93.7	9.66
	24	190	173	90.1	9.53
0.1 mg.	Fresh	25	23	92.0	12.04
	16	17	16	94.1	11.25
	24	190	157	82.6	11.08
0.01 mg.	Fresh	23	13	56.5	12.84
	16	20	8	40.0	14.00

ice chest until used. Preliminary suspensions containing 10 mg. in a 0.25 ml. dose were prepared by adding 50 ml. of 2 per cent peptone solution to 2 Gm. of tumor tissue. A Ten Brock grinder was employed, and further dilutions were made in peptone solution.

In our experiments the desired length of latent period was obtained with 1 mg. of inoculum. The frozen virus was found to be stable for at least a month. It is of interest that Reinhard and co-workers[5] found a similar relationship between the size of inoculum and the length of the latent period for growth of a transplantable mouse adenocarcinoma.

One-day-old New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report[4]) were injected in the right breast with 0.25 ml. of the suspension containing the 1 mg. dose. The feathers were removed from the breast area before inoculation. Groups of ten birds were placed on diets with and without each nutrient tested. Nutrients which were of interest because of possible stimulative effect were retested several times. The chicks were maintained in electric brooders at 90° F.; water and food were supplied ad libitum. Approximately 400 chicks (ten to twenty per group) were used in each experiment.

The basal diet contained 53 per cent Cerelose,* 22 per cent alcohol extracted casein, 4.3 per cent salt mixture, 3 per cent calcium gluconate, 8 per cent gelatin, 4 per cent Ruffex,† 5 per cent soybean oil, 0.25 per cent cholic acid, 0.45 per cent cystine, 200 mg. per cent choline chloride, 3 mg. per cent calcium pantothenate, 3 mg. per cent nicotinamide, 0.5 mg. per cent pyridoxine, 0.3 mg. per cent thiamin chloride, 0.03 mg. per cent biotin, 0.5 mg. per cent riboflavin, 100 mg. per cent inositol, 5 mg. per cent para-aminobenzoic acid, 0.2 mg. per cent folic acid, 5 mg. per cent vitamin E, 0.2 mg. per cent vitamin K, 3,500 units per cent vitamin A, and 200 units per cent vitamin D. The diet with nutrient was usually the complete diet. A commercial chick starter gave the same tumor response as the complete synthetic diet. In studies of substitutes for soybean oil, the diet with nutrient contained the substitute and the basal diet was soybean oil free. In these tests the vitamins A, D, E, and K were added in propylene glycol.

The chicks were observed daily beginning with the eighth day and continuing through the sixteenth day at which time adequately nourished groups showed 90 to 100 per cent tumors. Wing tags applied on the sixth or seventh day were used to identify individual birds. The increasing sizes of tumor were recorded (as previously described) for evidence as to the accuracy of first observations. Tumors recorded as questionable were counted in determining the response whenever this observation was confirmed by subsequent findings.

*Glucose monohydrate. Fisher Scientific Co., Pittsburgh, Pa.

†Purified cellulose containing 70 per cent α cellulose and 30 per cent other celluloses. Fisher Scientific Co., Pittsburgh, Pa.

EXPERIMENTAL RESULTS

Three distinct types of results have been obtained with nutrients which demonstrated a definite stimulative action on tumor growth. While with and without values of approximately 1.0 were obtained in tests on nutrients which did not stimulate tumor growth, (A) constant values of approximately 3.0 were obtained in tests of riboflavin, (B) descending values of 13.0, 6.2, 4.6, 2.6, 2.1 were obtained in tests of nicotinamide, and (C) ascending values of 13.0, 40.0, 63.0, 89.0, 93.0 were obtained in tests of folic acid. While other nutrients gave results similar to those illustrated in A and B, folic acid was the only nutrient giving the result C.

The Effect of Vitamins From Liver.—Table II shows the effect of nine water-soluble vitamins on the tumor response of chicks to Rous sarcoma virus. Folic acid produced the greatest effect and para-aminobenzoic acid and biotin produced the least effect. Nicotinamide and calcium pantothenate influenced the rate of growth more than the final incidence; riboflavin did not change the rate of growth but did influence the incidence at all stages.

TABLE II. EFFECT OF WATER-SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B†)	NUMBER OF TESTS	RESPONSE WITH VITAMIN / RESPONSE WITHOUT VITAMIN				
				8	10	12	14	16
Thiamin*	3 mg./kg.	10/10	1	30.0	1.5	1.3	1.1	1.1
Riboflavin	5 mg./kg.	39/37	4	2.1	3.0	3.1	3.1	3.1
Pyridoxine*	5 mg./kg.	30/30	3	2.0	2.8	1.8	1.4	1.3
Nicotinamide	30 mg./kg.	29/29	3	13.0	6.2	4.6	2.6	2.1
Calcium pantothenate*	30 mg./kg.	29/18	3	24.0	11.0	2.6	2.0	1.8
Inositol	1 Gm./kg.	29/25	3	13.0	1.2	1.1	1.0	1.0
Para-aminobenzoic acid	50 mg./kg.	19/19	2	1.4	1.1	1.0	0.8	0.8
Biotin	0.3 mg./kg.	29/27	3	1.1	1.8	1.2	1.1	1.1
Folic acid	2 mg./kg.	30/32	3	13.0	40.0	63.0	89.0	93.0

*Vitamin deficient chicks were revived with complete diet beginning on the tenth day.

†a/b, Total chicks used to determine effect of diets (a) with and (b) without the vitamin specified.

Since deficiencies of thiamin, pyridoxine, and calcium pantothenate caused serious loss of weight, chicks fed diets without these vitamins were revived with complete diet beginning on the tenth day. In previous work with folic acid it had been shown that the stimulative effect of restoring this vitamin on the tenth day does not become apparent for at least seven days. This practice was adopted in the case of thiamin, pyridoxine, and calcium pantothenate deficiencies to permit survival of the birds for the duration of the test. The responses with and without one of these vitamins for the twelfth, fourteenth, and sixteenth day may or may not be influenced by restoring the vitamin on the tenth day.

Since liver is a rich source of still unidentified vitamins, several fractions of liver were tested in the presence of the nine purified vitamins for possible stimulative effect on the tumor response of chicks to Rous sarcoma virus. None of the liver fractions influenced the rate of growth or incidence of tumor to an extent which would indicate the presence of additional factors for tumor growth.

The Effect of Vitamins A, D, E, and K.—The results of tests of diets with and without vitamins A, D, E, and K are shown in Table III. None of these vitamins stimulated tumor growth when present in the diet. The values 0.8, 0.6, and 0.5 suggest that the oil-soluble vitamins may slightly retard tumor growth. It is of interest that diets with and without the combination of soybean oil and vitamins A, D, E, and K influenced the tumor response to a greater extent than did the diets with and/or without the oil-soluble vitamins alone.

TABLE III. EFFECT OF OIL-SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B*)	NUMBER OF TESTS	RESPONSE WITH VITAMIN / RESPONSE WITHOUT VITAMIN				
				8	10	12	14	16
Combined								
A	35,000 I.U./kg.							
D	2,000 I.U./kg.	19/19	2	0.8	1.0	1.0	1.0	1.0
Combined								
A	35,000 I.U./kg.							
D	2,000 I.U./kg.							
E	50 mg./kg.							
K	2 mg./kg.	10/10	1	5.0	1.3	1.1	1.1	1.1
E	50 mg./kg.	10/ 8	1	0.6	1.2	1.4	1.2	1.2
K	2 mg./kg.	10/10	1	0.5	1.0	1.5	1.1	1.0
Combined								
Soybean oil	5 per cent							
A, D, E, K	As above	20/23	2	10.0	2.6	2.3	2.0	1.6

*A/B, Total chicks used to determine effect of diets (A) with and (B) without the vitamin specified.

The Effect of Various Fats and Oils.—Table IV shows the results of tests in which twelve different substances were tested in a soybean oil free diet. None of these substances stimulated tumor growth. Soybean lecithin, cod-liver oil, and linoleic acid appeared to retard tumor growth when present in the diet. Most of the other substances in the group showed this effect to some degree.

TABLE IV. EFFECT OF FATS AND OILS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT*	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B†)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT / RESPONSE WITHOUT NUTRIENT				
				8	10	12	14	16
Beef liver fat	5	10/10	1	1.0	1.0	0.7	1.0	1.0
Cholesterol	1	54/54	3	1.0	1.2	0.7	0.7	0.8
Coconut oil	5	30/30	3	0.1	1.3	1.1	1.3	1.3
Cod-liver oil	3	10/10	1	1.0	0.7	0.4	0.5	0.6
Corn oil	5	10/10	1	0.5	0.5	0.8	1.0	1.0
Crisco	5	10/10	1	1.0	1.2	0.5	0.8	0.8
Lanolin	3	10/10	1	1.0	0.7	0.5	0.8	0.8
Lard	5	10/10	1	0.0	0.7	0.5	0.6	0.8
Linoleic acid	3	30/30	3	0.0	0.5	0.7	0.8	0.8
Sodium oleate	3	30/30	3	0.1	1.2	1.0	1.2	1.3
Soybean lecithin	1	20/20	2	0.0	0.8	1.3	1.4	1.3
Soybean lecithin	3	10/10	1	0.0	0.5	0.5	0.6	0.6
Soybean oil	5	20/20	2	2.0	1.3	1.0	1.0	1.0

*The basal diet was oil-free; vitamins A, D, E and K were added in propylene glycol.

†a/b, Total chicks used to determine effect of diets (a) with and (b) without the substance specified.

The Effect of Cholic Acid.—Table V shows the results of tests of diets with and without cholic acid, sodium chloride, gelatin, calcium gluconate, and Ruffex. Diets with and without cholic acid influenced tumor growth in much the same way as did diets with and without riboflavin. Three times as many tumors developed when cholic acid was present in the diet.

TABLE V. EFFECT OF CHOLIC ACID IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B*)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT / RESPONSE WITHOUT NUTRIENT				
				8	10	12	14	16
Cholic acid	0.25	20/20	2	30.0	3.2	3.8	3.1	3.1
Sodium chloride	1	15/15	1	0.4	0.6	0.9	0.8	0.9
Gelatin	8	10/10	1	3.0	2.3	1.4	1.2	1.2
Combined								
Gelatin	8							
Calcium gluconate	3	10/10	1	3.0	7.0	1.6	1.6	1.4
Ruffex	4	65/65	4	1.6	1.0	0.9	0.8	0.8

*a/b, Total chicks used to determine effect of diets (a) with an (b) without the substance specified.

SUMMARY

The effect of nutrition on the tumor response in Rous chicken sarcoma was determined by comparing observations of chicks fed synthetic diets with and without each nutrient. Tumor response was stimulated by the presence of folic acid, nicotinamide, calcium pantothenate, riboflavin, and cholic acid in the diet. Folic acid was the only nutrient required to the extent that tumor response was prevented by its absence from the diet.

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Desoxycholic Acid Enhancement of Orally Administered Reserpine

By MARVIN H. MALONE, HOWARD I. HOCHMAN*, and KARL A. NIEFORTH

Quantities of 1:2, 1:4, 1:8, 1:16, and 1:32 molar amounts of reserpine : desoxycholic acid were dissolved in alcohol and/or chloroform, chilled to −5°, and solvents removed under reduced pressure. When administered orally to mice, the presence of desoxycholic acid hastened blepharoptotic activity and increased blepharoptotic potency of reserpine. Maximum enhancement was observed with 1:16 reserpine–desoxycholic acid where 1 mg. of reserpine as the combination was equivalent to 3.8–2.5 mg. of reserpine base using observations 2 and 24 hr. after dosage, respectively. An equivalent physical mixture prepared by trituration was significantly more potent than reserpine base but much less potent than the prepared combination. Desoxycholic acid alone was without blepharoptotic activity.

THE ENHANCED absorption of various medicinal agents upon concomitant administration with desoxycholic acid may be attributed to its ability to reduce interfacial tension and/or to form inclusion or clathrate compounds. Previous work concerning this increased absorption usually has been restricted to the effect of desoxycholic acid on the absorption of the fat-soluble vitamins and closely related analogs where the effect was thought to be due to the surface action of desoxycholic acid. This report is concerned with an increase in the blepharoptotic activity of reserpine when given in intimate combination with desoxycholic acid.

The general role of bile salts in absorption, the ability of desoxycholic acid to form inclusion compounds (choleic acids), and the relative inefficiency of reserpine absorption prompted this study of the effects of combining reserpine and this acid prior to oral administration. Evidence of an interaction between reserpine and desoxycholic acid was reported by Lach and Pauli (1) after the completion of the work reported here. The interaction was demonstrated by an increase in the solubility of reserpine in hydroalcoholic solutions of desoxycholic acid. Simple interfacial effects of desoxycholic acid were not thought to be the sole contributing factor for this increased solubility, and it was suggested that the interaction might result from a combination of micellar solubilization and inclusion formation.

EXPERIMENTAL

Preparation of Reserpine–Desoxycholic Acid Combinations.—The usual method for the formation of desoxycholic acid inclusion compounds (dissolving both desoxycholic acid and the guest component in ethanol and allowing the inclusion compound to crystallize) was not used in this instance due to the poor solubility of reserpine in anhydrous alcohol.

Method A.—The calculated quantity of purified desoxycholic acid (Nutritional Biochemicals Corp., lot 2185) was dissolved in 5 ml. of commercial grade absolute ethanol in a 20-ml. round-bottom flask. The desired amount of reserpine (C grade, Calbiochem, lot 502858) was added and dissolved with slight warming. The flask and its contents were chilled in an ice–salt bath to −5° and the solvent removed under reduced pressure and trapped in a cold finger cooled with a dry ice–acetone bath. The contents of the flask were protected from light as completely as possible. The residues were dried for 12 hr. in a vacuum to remove the last trace of alcohol.

Method B.—This method was essentially the same as above except that 5 ml. of chloroform was used to dissolve the reserpine, and this solution was added to the desoxycholic acid dissolved in 10 ml. of absolute alcohol. The resultant solution was then treated as described under *Method A*.

Method C.—A physical mixture of desoxycholic acid and reserpine was prepared by intimately triturating the two dry powders without previous dissolution in any solvent. Table I summarizes the physical properties of all the test mixtures.

Blepharoptotic Assay.—White mice were obtained from E. G. Steinhilber, Oshkosh, Wis., and maintained in this laboratory on Purina laboratory chow and water *ad libitum* for at least 4 days prior to test. All animals were taken off food 10 hr. prior to dosing and placed back on food after recording the +6 hr. observations. Free access to water was allowed throughout the entire test period. The mice were dosed orally using precision grade syringes and cut-off, blunted, polished 20-gauge hypodermic needles. A constant dosage volume of 30 ml./Kg was maintained for all injections using 0.25% aqueous agar as the dosing vehicle. Test drugs and combinations were suspended by trituration. All test solution suspensions were coded and the animals dosed in a random test pattern using a 3 × 3 assay format with further randomization between the two sexes of mice and the two dosing technicians. Prior to ptotic scoring, each mouse was manually aroused and placed on a screen facing the scorer at the scorer's eye level. Ptosis for each eye was rated following the scale used by Rubin *et al.* (2): 4 = complete, 3 = $^3/_4$, 2 = $^1/_2$, 1 = $^1/_4$ closure of the eyelids. Nonptotic responses were scored as 0. All scores of 4 were checked to insure that the lid was not encrusted shut by eye secretions. The individual metameter was the sum of the ptotic scores for both eyes so that the graded test response could vary from 0–8 per animal.

RESULTS AND DISCUSSION

As shown in Table II, eight balanced log dose response blepharoptotic assays were conducted assaying reserpine (in combination with desoxycholic acid) against reserpine base as a standard. For the assay calculations the reserpine content of

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TABLE I.—PREPARATION OF RESERPINE–DESOXYCHOLIC ACID AND RESERPINE PLUS DESOXYCHOLIC ACID TEST MATERIALS

Identification	Exact molar Ratios	Prepn., Method	Quantities, mg. Reserpine	Quantities, mg. Acid	Melting Range, ° C.
1:2	1:2.06	*B*	59.0	80.0	80–110
1:4	1:4.02	*B*	62.0	161.5	98–125
1:8	1:7.44	*A*	16.2	77.9	114–125
1:16	1:16.31	*A*	29.7	312.6	114–145
1:32	1:31.89	*A*	15.2	311.7	145–148
1 + 16	1 + 17.16	*C*	28.6	316.4	...

TABLE II.—APPARENT ORAL POTENCIES OF RESERPINE IN VARIOUS DESOXYCHOLIC ACID COMBINATIONS AS COMPARED TO RESERPINE BASE ALONE

Identification	hr. After Dosage 2	4	6	10	24
0:0	...	...	...	...	...
1:0	0.781 (0.536–1.138)[a]	1.000 (0.836–1.196)	0.957 (0.793–1.155)	0.928 (0.741–1.162)	1.078 (0.776–1.496)
1:2	2.293 (1.460–3.602)	1.924 (1.482–2.499)	1.658 (1.326–2.073)	1.587 (1.282–1.966)	2.144 (1.672–2.747)
1:4	2.642 (2.182–3.199)	2.542 (1.885–3.428)	2.428 (1.959–3.010)	2.2811 (1.859–2.799)	2.692 (2.135–3.394)
1:8	2.828 (1.858–4.305)	2.349 (1.607–3.433)	2.732 (1.739–4.291)	1.948 (1.393–2.724)	2.056 (1.084–3.901)
1:16	3.779 (2.870–4.974)	3.201 (2.221–4.615)	3.262 (2.645–4.022)	2.594 (2.072–3.247)	2.487 (1.767–3.499)
1:32	3.352 (2.403–4.674)	2.650 (2.075–3.384)	2.471 (1.930–3.165)	2.650 (2.074–3.386)	1.966[b] (1.143–3.382)
1 + 16	1.382 (0.990–1.928)	1.882 (1.393–2.542)	1.876 (1.355–2.596)	1.719 (1.157–2.553)	...[c]

[a] Range of figures within parentheses indicate the calculated 95% confidence limits for the potency. [b] Significant departure from parallelism (P = 0.01–0.05) in 3 × 3 assay; best graphical estimate of potency = 2.49 × reserpine base. [c] Recovery was sufficient so that neither statistical nor graphical estimates of potency could be determined with validity.

the combinations was handled using the "exact" ratios shown in Table I, although the approximate molar ratios are used in this text to identify the various combinations. Doses of 3, 6, and 12 mg./Kg. were used for the reserpine standard (C grade, Calbiochem, lot 502858) and for the reserpine of the 1:2, 1:4, and 1:8 reserpine–desoxycholic acid combinations. Doses of 1.5, 3, and 6 mg./Kg. and 0.75, 1.5, and 3 mg./Kg. were used for the reserpine of the 1:16 and 1:32 combinations, respectively. Where enhancement of the reserpine-like activity did not allow 3 × 3 calculations, a 2 × 2 assay was calculated matching two dosages of the standard with two doses of the combination in the same linear portion of the dose-response curve. Significant departure from parallelism was noted in only one calculation. The statistical treatment for the 3 × 3 (120 mice, 20 animals/dosage level) and 2 × 2 (80 mice) assays involved analysis of variance, factorial analysis, and calculation of potency and its 95% confidence limits using the techniques of Bliss and Calhoun (3). An average λ value (s/b) of 0.27 was obtained for the 32 valid assay calculations reported in Table II, which value agrees well with a λ of 0.29 reported earlier for this assay technique (4) using a different mouse stock, reserpine acetate as the standard, and water as the dosage vehicle.

As shown in Fig. 1, the 1:2 to 1:16 reserpine–desoxycholic acid combinations show a progressive increase in the apparent potency of reserpine with the 1:16 reserpine–desoxycholic acid combination

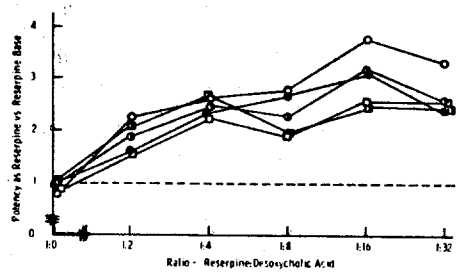

Fig. 1.—Variation of ptotic potency of reserpine when administered orally in the form of various molar ratio coprecipitates with desoxycholic acid. Key: ○, +2 hr.; ◑, +4 hr.; ●, +6 hr.; □, +10 hr.; ◨, +24 hr.

producing the maximum enhancement of activity. The decrease in activity shown by the 1:32 combination would appear to indicate that the maximum attainable activity (1:16) has been physically diluted by excess desoxycholic acid.

As illustrated in Fig. 2, the potency of 1:0 reserpine–desoxycholic acid does not significantly deviate from the theoretical value of 1.0, while maximum distortion of the apparent potency of the 1:2–1:32 combinations was apparent at +2 hr. after oral administration. This appears to indicate that desoxycholic acid increases the speed of absorp-

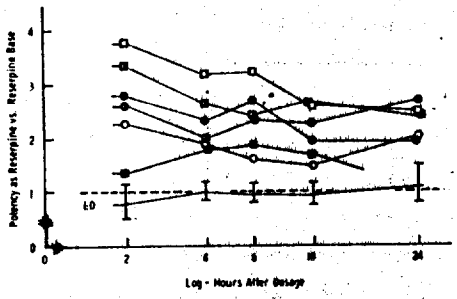

Fig. 2.—Variation with time of the oral potencies of various reserpine–desoxycholic acid molar coprecipitates as compared to reserpine base as a standard. Key: □, 1:16; ◪, 1:32; ●, 1:8; ◑, 1:4; ○, 1:2; ■, 1 + 16; I, 1:0.

tion of reserpine as well as increasing the potency. Considering the 95% confidence limits of potency reported in Table II, reserpine administered as the 1:16 combination behaves equivalent to at least 1.8 mg. of reserpine base (+24 hr.) and possibly equivalent to as much as 5.0 mg. of reserpine (+2 hr.).

A molar equivalent physical mixture of reserpine and desoxycholic acid (1 + 16) produced a slower onset of the period of maximum enhancement (+4–6 hr.), and the potencies were significantly less ($P \leq 0.001$) than those reported for the 1:16 intimate combination. In all cases where calculation was possible, the 1 + 16 mixture was significantly more potent than reserpine base alone (observed P: 0.025–0.05 at +2 hr. and ≤ 0.001 at +4, 6, and 10 hr.).

While there is debate as to whether palpebral ptosis is a peripheral or central manifestation of reserpine-like activity (4–6), this characteristic symptom does indicate absorption of reserpine from the gastrointestinal tract. Coprecipitates of reserpine and desoxycholic acid both increase the potency of reserpine and produce a more rapid onset of reserpine-like activity when administered orally. No attempt has been made here to define the exact physical/chemical nature of the reserpine–desoxycholic acid combination.

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The Incorporation of $S^{35}O_4$ into Bile of Chicks.* (30792)

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The utilization of inorganic sulfate by the chick to synthesize taurine is of interest since it has been previously reported that cysteine-sulfur does not arise from sulfate(1). Further, over one-half of the sulfate-S^{35} administered to the 24-hour embryo was found in the taurine of the day-old chick(2).

The bile acids, cholic and derivatives of it, conjugate with taurine in the chick liver and are passed into the gall bladder. Cholic acid is the limiting factor in formation of taurocholate in dogs(3), and continued feeding appeared to force the synthesis of taurine(4). Feeding cholic acid to young chicks stimulated the incorporation of sulfate-S^{35} into taurocholate(5).

The purpose of this investigation was to ascertain the influence of taurine and the cholic acid derivatives on incorporation of sulfate-S^{35} into the bile of chicks.

Methods. Day-old chicks were fed *ad libitum* a simplified basal ration of cerelose (glucose-hydrate) and isolated soybean protein with supplements of vitamins, minerals, and corn oil. These birds were maintained on this diet with the taurine and bile acid supplements indicated in the respective Tables for 14 days or as long as indicated. Following the feeding period the isotope was administered and the chicks sacrificed as described in the Tables.

Sulfate-S^{35} was administered orally by means of a 1 ml pipette or injected subcutaneously. Each dose contained 20 μc of carrier free $H_2S^{35}O_4$ in dilute HCl solution. Bile fluid was removed from the gall bladder or the chicks and pooled for each treatment.

* This manuscript is published with permission of the Director of West Virginia University, Agri. Exp. Station, Morgantown, as Scientific Paper No. 696. Radioactive materials were obtained from Oak Ridge National Laboratory on allocation from U. S. Atomic Energy Commission. This work was completed under contract No. AT-(30-1)-2766 between West Virginia Univ. College of Agri. and Atomic Energy Commission.

TABLE I. Influence of Taurine on Incorporation of S^{35} from Injection Sulfate-S^{35} into Bile Fluid of New Hampshire Chicks. The isotope was injected subcutaneously at 1 day of age. Each value is pooled average of 5 chicks.

Supplemental taurine to diet	Days after subcutaneous injection of sulfate-S^{35}								
	1	2	5	7	9	12	14	16	19
	% S^{35} dose/ml bile fluid								
—	1.92	2.16	2.01	1.33	.94	.62	.31	.24	.18
.1%	1.31	1.15	1.19	1.02	.60	.44	.28	.20	.17

TABLE II. Effect of Dietary Taurine on Weight, Feed Efficiency, Protein Efficiency Ratio (PER), Quantity of Bile, and Concentration of S^{35} in Bile of 14-Day-Old New Hampshire Chicks. These chicks were sacrificed 24 hours after oral dose of $S^{35}O_4$.

Dietary taurine (%)	14-day wt (g)	Feed efficiency, gain/feed	PER	Avg bile vol (ml)	% orally dosed S^{35}/ml bile
—	121.6	.66	3.29	.06	1.02
.1	142.2	.78	3.92	.15	.44

From these pooled samples, an aliquot was diluted with distilled water, 0.1 ml of fluid was dried on stainless steel planchets and counted under gas flow geiger tube. Dosing standards were counted and all S^{35} activity reported as per cent of the S^{35} dose.

Whole and hydrolized bile samples were chromatographed to ascertain the presence of taurine-S^{35}. Activity was observed at the R_f of commercial taurine in three different solvent systems (80% aqueous phenol; butanol-acetic acid-water, 50:12:50 v/v; and collidine-lutidine:water, 1:1:1 v/v). When whole bile and n-butanol extracted bile was chromatographed, activity was observed at the R_f of commercial taurocholate (butanol-3% acetic acid, 1:1; Whatman No. 4 paper pretreated in 70% acetic acid).

Results and discussion. The supplementation of taurine to the diet diminished the amount of S^{35} present in chick bile fluid at periods during the first 14 days after injection (Table I). The S^{35} activity was about one-half that of the chicks not receiving dietary taurine during the first 5 days, suggesting that taurine synthesis from sulfate occurs even in the presence of exogenous taurine. When sulfate-S^{35} was given orally to the chicks which had been fed the basal ± taurine for 14 days (Table II), the concentration of bile-S^{35} was again decreased by dietary taurine. The bladder volume of bile was increased by dietary taurine as was the body weight, feed efficiency and protein efficiency ratio (PER). This is probably due, in part, to the lower level of dietary methionine from the soybean protein, since taurine can be formed from methionine.

Interestingly, when chicks were fed a casein-purified diet with optimal supplementations, little growth response to taurine or cholic acid was observed. When the soybean protein purified diet was fortified with methionine, less growth promotion attributed to taurine or cholic acid supplementation was observed. However, the presence of methionine supplied as the free amino acid or the feeding of casein as the only nitrogen source did not prevent or retard the use of sulfate to synthesize taurine in the chick liver.

Feeding cholic acid alone (Table III) further enhanced the bile-S^{35} concentration while cholic with taurine equaled that of the control chicks. The marked difference in bile volume, a 2-fold increase with taurine or taurine plus cholic, and a 3-fold increase with cholic, reflects a highly significant increase in the bile-S^{35} per chick receiving cholic acid. Those fed cholic with taurine had a 2-fold increase over the control of taurine supplemented chicks. This trial shows that cholic acid, and to a lesser extent taurine, stimulate the synthesis of the sulfur-containing bile-fluid components.

Realizing that the bile volume could fluctuate due to the individual metabolic pattern, a trial was made to ascertain bladder volume at hourly intervals after oral adminis-

tration of sulfate-S^{35} to the chick. The amount of sulfate-S^{35} and of taurocholate-S^{35} was measured in these bile samples (Table IV). The volume of bile with time appears relatively constant. Since consumption of food or the placement of substance in the digestive tract often effects the release of the bile fluid into the intestine, the chicks were usually deprived of food a few hours before dosing and sacrifice.

Even with the continual efflux of bile fluid, the amount of S^{35} in the bile increases through 6 hours after dosing. After dosing, an increasing quantity of the total S^{35} is incorporated in organic molecules as evidenced by the increased amount of taurocholate-S^{35}.

With the chick, addition of cholic, dehydro-, and deoxycholic acids was associated

TABLE III. Effect of Dietary Taurine, Cholic Acid or Both on Incorporation of S^{35} from Oral Doses of Sulfate-S^{35} into Bile of Chicks. Subsequent to sacrifice, 24 hours after dosing, the bile from each chick per diet group was pooled. Each dietary group contained 25 chicks.

Supplement to basal	14-day body wt (g)	Avg vol bile fluid/chick (ml)	% S^{35} dose/ml bile fluid	% S^{35} dose in bile/chick
—	118	.07	2.12	.15
Taurine (.1%)	126	.15	1.07	.16
Cholic acid (.34%)	124	.22	4.98	1.10
Taurine (.1%) + cholic acid (.34%)	125	.17	1.99	.34

TABLE IV. Incorporation of Orally Administered Sulfate-S^{35} into n-Butanol Solution-S^{35} of Bile Fluid from 14-Day-Old Chicks at Short Intervals After S^{35}-Dosing. Chicks were dosed at 8 A.M. and deprived of food between dosing and time of sacrifice.

	Hours after dosing					
	1	2	3	4	5	6
Avg vol of bladder-bile (ml)	.07	.10	.08	.08	.09	.11
n-butanol soluble-S^{35} (% of total bile-S^{35})*	8.74	9.52	10.38	11.67	11.93	13.49

* Taurine and conjugated taurine are separated by n-butanol extraction as taurocholate is selectively absorbed by the alcohol(6).

TABLE V. Influence of Bile Acid and Taurine Supplementation on Distribution of Sulfate-S^{35} in Liver and Bile of Chicks 6 Hours After Oral Administration of the Isotope.

Supplement to basal*	Avg 14-day wt	Liver wt (g/100 g body wt)	% S^{35} dose in 1 g liver	% S^{35} activity in liver soluble in n-butanol†	Avg vol bladder bile/chick (ml)	% S^{35} dose in 1 ml bile	% S^{35} activity in bile soluble in n-butanol†
—	92.1	4.51	.12	1.92	.07	.87	13
Cholic	99.5	4.47	.06	10.34	.20	1.89	32
Lithocholic	72.8	11.28	.23	.94	.02	.25	17
Dehydrocholic	99.2	4.54	.19	3.09	.15	2.62	29
Deoxycholic	114.2	4.32	.08	6.25	.18	1.43	25
Taurine	96.2	5.03	.06	3.57	.13	.49	14
Cholic and taurine	121.6	4.26	.02	7.69	.21	.43	23
Lithocholic and taurine	77.8	12.29	.12	8.93	.03	.17	14
Dehydrocholic and taurine	91.2	4.57	.04	11.76	.17	.46	26
Deoxycholic and taurine	126.2	4.45	.05	6.25	.26	.39	23

* Taurine supplemented at 0.1% of diet; bile acids were supplemented equimolar to taurine
† Taurocholate is selectively absorbed by n-butanol(6).

with a slight growth response and enhanced bile-S^{35} concentration over the control while lithocholic acid was growth depressing and toxic at the same dietary level (Table V). The livers of the birds fed lithocholic acid were abnormally large and contained more S^{35} per gram than did the livers of the other dietary groups of chicks. The liver taurocholate-S^{35} 6 hours after dosing was highest when cholic and deoxycholic were fed alone and when dehydrocholic was supplemented with taurine.

Studies are in progress concerning the chick liver reactions whereby sulfate-sulfur is converted to taurine-sulfur without passing through cysteine, which is a normal reaction pathway of mammals.

Summary. Chicks utilize sulfate-S^{35} to synthesize taurine. Dietary cholic acid, taurine or both are associated with increased volumes of bile fluid. Cholic acid feeding enhances sulfate-S^{35} incorporation into the bile while taurine decreases such reactions.

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Thin-Layer Chromatography of Steroid Ketones and Their Derivatives

IV. TLC of Bile Acids, Keto-bile Acids and Their Derivatives

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The polarity of the bile acids are similar to that of the corticoids. Concerning the thin-layer chromatographic separation of the bile acids and their peptides, only few detailed data are found in the literature (*1,3,5*). The most efficient solvents used in the separation are strong hydrophylic ones such as alcohols and water.

The paper chromatographic separation and quantitative determination of the bile acids has been initially investigated by Sjövall (*4*).

In order to separate the ketoxime isomers of the bile acids from the starting material, keto-bile acids and their hydrolysis products, experiments have been carried out with solvents which have been previously used for the separation of these compounds.

MATERIALS AND METHODS

Compounds. 3α-, 6α-dihydroxy-cholanic acid, mp 197°C (I); 3α-, 6α-dihydroxy-methyl-cholanate, mp 71–72°C (II); 3α-hydroxy-6-keto-methyl-cholanate, mp 130–31°C (III); 3α-acetoxy-6-keto-methyl-cholanate, mp 99–100°C (IV); 3α-acetoxy-6-one-oxime-methyl-cholanate, mp 160°C (V); 3α-acetoxy-cholanic-acid-6-one-oxime, mp 110–112°C (VI); 3α-acetoxy-6-one-oxime-methyl-cholanate, mp 166–167°C (VII); 3α-, 7α-, 12α-trihydroxy-cholanic acid, mp 198°C (VIII); 3α-, 7α-, 12α-trihydroxy-methyl-cholanate, mp 151–152°C (IX); 3α-, 12α-, dihydroxy-7-keto-methyl-cholanate, mp 144–145°C (X); 3α-, 12α-diacetoxy-7-keto-methyl-cholanate, mp 115°C (XI); 3α-, 12α-dibenzoyloxy-7-keto-methyl-cholanate, mp 91°C (XII); 3α-, 12α-diacetoxy-methyl-cholanate-7-one-oxime, mp 99–100°C (XIII); 3α-, 12α-dibenzoyloxy-methyl-cholanate-7-one-oxime, mp 97°C (XIV); 3α-, 7α-dihydroxy-12-keto-cholanic acid, mp 179–187°C (XV); 3α-, 7α-dihydroxy-12-keto-methyl-cholanate, mp 154–155°C (XVI); 3α-, 7α-

diacetoxy-12-keto-methyl-cholanate, mp 174–175°C (XVII); 3α-, 7α-diacetoxy-methyl-cholanate-12-one-oxime, mp 98–100°C (XVIII); 3α-, 12α-dihydroxy-cholanic acid, mp 136–137°C (XIX); 3α-, 12α-dihidroxy-methyl-cholanate, mp 80–81°C (XX); 12α-hydroxy-3-keto-cholanic acid, mp 100–110°C (XXI); 12α-hydroxy-3-keto-methyl-cholanate, mp 144–145°C (XXII); 12α-acetoxy-3-keto-methyl-cholanate, mp 118–119°C (XXIII); 12α-acetoxy-methyl-cholanate-3-one-oxime, mp 74–75°C (XXIV).

Solvent systems. (1) Cyclohexane–chloroform–acetic acid (70:20:10; v/v); (2) benzene–methanol (95:5; v/v); (3) benzene–methanol (90:10; v/v); (4) toluene–acetic acid–water (50:5; v/v); and (5) toluene acetic–acid–water (5:1:1; v/v).

Spraying reagents. (A) Orthophosphoric acid (sp gr 1.70)–water (1:1; v/v); (B) J_2-vapor; and (C) Phosphotungstic acid reagent (fresh solution of 1.5 g phosphotungstic acid in 100 ml ethanol); drying: 5 minutes at 120°C.

Method. The same technique was used as detailed earlier (2).

DISCUSSION

The results are shown in Table 1. The thin-layer chromatography (TLC) data concerning the derivatives of different bile acids are separated into groups. The series of the TLC data I–VII related to 3α-, 6α-dihydroxy-cholanic acid (hyodeoxycholic acid) and its derivatives; VIII–XVIII refer to 3α-, 7α-, 12α-trihydroxy cholanic acid (cholic acid) and its derivatives; and XIX–XXIV are connected with 3α-, 12α-dihydroxycholanic acid (deoxycholic acid) and its derivatives.

As shown in Table 1, solvent system 3 is most suitable for the separation of hyodeoxycholic acid and its derivatives. Where no figure is given, the R_f value was zero or there was no movement of the compound on the thin-layer.

The data presented in Table 1 shows the effectiveness of the separations achieved with different solvent systems. For example, compounds XIII and XVIII are not separated in systems 2 and 4, but are separated in systems 1 and 5. For the separation of deoxycholic acid and its derivatives, system 3 was the best suited.

The simplest and best system for these separations consists of a mixture of a nonpolar and a polar solvent, e.g., benzene:methanol,

TABLE 1

SEPARATION OF BILE ACIDS, KETO-BILE ACIDS, AND THEIR DERIVATIVES

Compounds	R_f values. Solvent systems 1	2	3	4	5
I	0.05	—	0.07	0.62	—
II	0.08	0.07	0.26	0.53	—
III	0.13	0.27	0.40	0.55	0.09
IV	0.46	0.96	0.95	0.92	0.32
V	0.29	0.88	0.92	0.72	0.29
VI	0.10	—	0.08	0.60	0.07
VII	0.32	0.46	0.62	0.77	0.27
VIII	—	—	—	0.46	—
IX	—	0.07	0.21	0.52	—
X	—	0.13	0.27	0.58	—
XI	0.24	0.81	0.87	0.70	0.27
XII	0.23	0.78	0.87	0.72	0.28
XIII	0.26	0.50	0.62	0.70	0.22
XIV	0.27	0.51	0.74	0.71	0.17
XV	—	—	—	0.47	—
XVI	0.07	0.12	0.25	0.54	—
XVII	0.36	0.62	0.89	0.68	0.28
XVIII	0.38	0.50	0.75	0.65	0.27
XIX	0.08	—	—	0.59	—
XX	0.23	0.20	0.38	0.61	—
XXI	0.13	—	0.05	0.60	—
XXII	0.21	0.15	0.34	0.65	—
XXIII	0.41	0.77	0.90	0.67	0.26
XXIV	0.42	0.44	0.63	0.69	0.22

benzene:acetone, etc., where the non-polar percentage is much higher (90–95%) than that of the polar. These systems were found to be satisfactory for the separation of homo-aza-bile acid lactams.

In summary, from our results it has been possible to compile a comprehensive table of thin-layer separations of the bile acids and their derivatives.

Solvent system 4 was found to be the most suitable for the TLC separation of most of the derivatives. At any rate, each of the enumerated systems, 1–5 may be used to separate the different groups of bile acids and their derivatives.

SUMMARY

Solvent systems are described for the thin-layer separation of bile acids and their derivatives. Mixtures of nonpolar and polar solvents, containing a high percentage of nonpolar solvent, are most suitable.

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EXPERIMENTAL ATHEROSCLEROSIS

VI. EFFECTS OF VARIOUS BILE ACIDS ON CHOLESTEROL LEVELS

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The manner in which fats are absorbed from the intestinal tract has long been a controversial subject. Although it is agreed that neutral fat is hydrolyzed by lipase prior to absorption,[1] the nature of the processes by which fatty acids, insoluble in water, are brought into solution and transported across the intestinal wall is not clearly understood. At first it was assumed that the insoluble fatty acids combined with the alkaline pancreatic juice to form soaps and as such diffused into the intestinal epithelium. It has recently been shown, however, that the intestinal contents are slightly acid in reaction; the role of the bile salts and bile acids in the absorption of fat has therefore attracted renewed attention.

According to Verzár and von Kuthy,[2] during the digestion of fat the bile salts and acids become adsorbed to the intestinal epithelium and by virtue of their hydrotropic action are able to attract and pass the fatty acids into the epithelial cells. These workers found bile salts to be good solvents for oleic, palmitic and stearic acids. Fürth and Scholl[3] reported that both saponified and nonsaponified fats require bile for their absorption from the intestines. Riegel and his co-workers[4] studied the absorption of oleic acid in intestinal loops of dogs and showed that the absorption of this fatty acid was greatly increased in the presence of sodium taurocholate in the loop and somewhat less increased in the presence of hepatic or gallbladder bile. They ascribed the action of taurocholate to surface phenomena since minute concentrations of the bile salt facilitated the absorption of relatively large amounts of fatty acid.

Another hypothesis which deserves mention is the so-called "choleic acid principle" proposed by Wieland and Sorge.[5] They found that desoxycholic acid formed stable water-soluble molecular compounds with higher fatty acids and cholesterol and ascribed an important function to this phenomenon in the transporting of insoluble substances through the intestinal wall. Bashour and Bauman[6] studied the solubility of cholesterol in various solutions of bile salts, cholesterol was found to be most soluble in solutions of desoxycholate and least soluble in those of taurocholate. Spanner and Bauman[7] had shown previously that sodium desoxycholate was the most effective of the bile salts studied in keeping cholesterol in solution. The addition of sodium oleate to desoxycholate almost doubled its solvent properties for cholesterol. Likewise, Schönheimer[8] found that desoxycholic acid hastens the absorption of cholesterol from the intestinal tract of mice and rabbits.

Nedswedski[9] found that in the presence of bile salts, cholesterol may be esterified with palmitic, stearic or oleic acids by the action of lipase. Frölicher and Süllmann[10] noted that in rabbits fed cholesterol, free and combined cholesterol in the intestinal lymph rose to about the same extent, indicating that partial esterification of free cholesterol takes place in the intestinal wall. Hummel[11] reported that while bile acids fed to mice caused a moderate increase in the cholesterol content of the liver, the simultaneous feeding of bile salts and cholesterol resulted in considerable deposition of this ster-

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10. Frölicher, E., and Süllmann, H.: Biochem. Ztschr. **274**:21, 1934.

11. Hummel, R.: Ztschr. f. physiol. Chem. **185**:1[illegible] 1929.

in the liver. Loeffler[12] described somewhat similar results in mice and also concluded that greater storage of lipid occurs in the liver when bile acids are fed along with cholesterol.

The primary purpose of the present investigation was to determine the effect of a selected bile acid when fed with cholesterol on the level of the blood cholesterol and on the amount of this sterol deposited in the aorta. Additional data on the cholesterol content of the liver were also obtained in two experiments.

MATERIAL AND METHODS

In all, 75 female rabbits, 3 to 5 months old, were used in this study. Thirty-nine served as controls. Each animal was fed a basic ration of Ralston Purina rabbit chow[13] daily and 1 Gm. of cholesterol was mixed with the food three times weekly for a period varying from twelve to fourteen weeks. Thirty-six rabbits received in addition to the chow and cholesterol 0.5 Gm. of dehydrocholic acid, cholic acid, hyodesoxycholic acid (whole hog bile), desoxycholic acid or glycocholic acid[14] three times weekly. The bile acid was also mixed intimately with the chow, especial care being exercised that the rabbits consumed their ration of cholesterol and bile acid completely.

The bile acids were investigated consecutively rather than concurrently because of lack of animal space. For each group of experimental animals studied, a control series was examined simultaneously; the total duration of the experiment was therefore sixty-six weeks.

The total and the ester cholesterol in the whole blood were determined at regular intervals by a modified Bloor method.[15] At the end of the experimental period (twelve to fourteen weeks), the rabbits were killed. The procedure used for killing the animal, removing the aorta and determining its cholesterol content has been described elsewhere.[16] In two experiments the cholesterol content of the liver was also estimated.

RESULTS

Dehydrocholic Acid.—Table 1 shows the results obtained with this bile acid. For each two week period, the total cholesterol in the whole blood was moderately higher in the experimental group (average 675 mg. per hundred cubic centimeters at the fourteenth week) as compared with the controls (average 529 mg. per hundred cubic centimeters at the fourteenth week). The rate of increase of ester cholesterol in the whole blood was similar in both groups. Little or no difference was observed in the amount of cholesterol deposited in the aorta in control and in experimental animals.

Cholic Acid.—The results obtained with this bile acid are given in table 2. In rabbits receiving cholic acid and cholesterol, not only was the concentration of total cholesterol in the whole blood significantly higher (average 649 mg. per hundred cubic centimeters, compared with 401 mg. per hundred cubic centimeters in the controls, at the fourteenth week), but the per cent of ester cholesterol in the blood was greater than in rabbits fed cholesterol alone (43 per cent in the experimental group compared with 30 per cent in the controls at the eighth week). Moreover, considerably more cholesterol was deposited in the aorta in rabbits

TABLE 1.—*Effect of Feeding Cholesterol With and Without Simultaneous Feeding of Dehydrocholic Acid on the Total and the Ester Cholesterol Content of the Whole Blood and on the Cholesterol Content of the Aorta*

	Cholesterol in Whole Blood												Cholesterol in Aorta, Mg. per 100 Gm. Dry Weight
	Total, Mg. per 100 Cc.								Esters, % of Total				
Rabbit No.	0 Wk.	2 Wk.	4 Wk.	6 Wk.	8 Wk.	10 Wk.	12 Wk.	14 Wk.	0 Wk.	4 Wk.	8 Wk.	14 Wk.	
Control Group—1 Gm. of Cholesterol Three Times Weekly for Fourteen Weeks													
12	96	114	121	191	371	625	564	636	10	19	51	59	614
13	99	148	130	188	336	472	441	586	15	32	47	48	856
14	72	107	177	280	387	625	507	493	15	25	49	63	1,751
15	96	101	105	235	441	521	472	625	12	13	54	52	563
17	136	163	199	487	559	595	455	412	21	..	51	67	1,374
21	88	123	214	521	487	417	452	528	13	38	50	71	1,511
23	98	...	295	457	559	494	306	298	14	48	51	54	1,648
26	96	...	452	493	487	893	815	658	18	50	45	73	4,589
Average	98	126	212	345	479	581	512	529	15	32	49	61	1,588
Experimental Group—1 Gm. of Cholesterol and 0.5 Gm. of Dehydrocholic Acid Three Times Weekly for Fourteen Weeks													
1	164	155	200	164	457	595	484	507	31	20	51	58	793
2	84	341	318	670	516	707	605	700	15	32	66	43	2,391
3	90	103	89	190	126	395	452	514	12	23	31	65	811
4	107	139	193	252	156	291	344	500	19	..	39	53	946
7	128	131	202	483	383	494	401	431	20	31	48	61	310
9	121	481	551	735	1,042	1,271	1,316	1,200	19	51	60	52	1,809
10	89	191	383	670	884	997	1,042	875	14	31	54	71	3,354
Average	112	220	277	449	509	677	664	675	19	31	50	58	1,488

12. Loeffler, K.: Ztschr. f. physiol. Chem. **178**:186, 1928.

13. This chow contains a mixture of grains and alfalfa hay supplemented by vitamins and minerals.

14. The dehydrocholic acid was obtained from E. R. Squibb & Sons, the cholic acid from Sandoz Chemical Works, Inc., the hyodesoxycholic acid (whole hog bile) from Parke, Davis & Company and the desoxycholic acid and glycocholic acid from Sandoz Chemical Works, Inc. and from the private stocks of Dr. Carl H. Greene. The glycocholic acid fed was a mixture of equal parts of the free acid and sodium glycocholate. The material supplied by Dr. Greene was made by C. H. Boehringer Sohn, Germany.

15. Bloor, W. R., and Knudson, A.: J. Biol. Chem. **27**:107, 1916.

16. Bruger, M., and Fitz, F.: Arch. Path. **25**:637, 1938.

fed cholic acid (average 3,389 mg. per hundred grams of dry aorta in the experimental group compared with 1,398 mg. per hundred grams of dry aorta in the controls).

It may be of interest to mention that at necropsy the gallbladders of the rabbits fed cholic acid and cholesterol were considerably enlarged. The control animals showed no such change. Histologic studies of these enlarged gallbladders made by Dr. Henry Spitz, of the Department of Pathology of the New York Post-Graduate Medical School and Hospital, showed pronounced thickening of the wall.

Hyodesoxycholic Acid (Whole Hog Bile).—Table 3 details the results obtained with hyodesoxycholic acid. The feeding of this bile acid and cholesterol was accompanied by no greater concentration of total or of ester cholesterol in the whole blood and by no greater deposit of this sterol in the aorta than was the feeding of cholesterol alone.

Desoxycholic Acid.—The results obtained with this bile acid are given in table 4. Like hyodesoxycholic acid, desoxycholic acid was without influence on the cholesterol content of the whole blood or on that of the aorta. This bile acid did not increase the cholesterol content of the liver.

Glycocholic Acid.—Table 5 details the findings with glycocholic acid. Like cholic acid, glycocholic acid when fed simultaneously with cholesterol augmented markedly the total cholesterol content of the whole blood (average 1,054 mg. per hundred cubic centimeters in the experimental group, compared with 576 mg. per hundred cubic centimeters in the controls at the eighth week) and of the aorta (average 4,799 mg.

TABLE 2.—*Effect of Feeding Cholesterol With and Without Simultaneous Feeding of Cholic Acid on the Total and the Ester Cholesterol Content of the Whole Blood and on the Cholesterol Content of the Aorta*

Rabbit No.	Cholesterol in Whole Blood: Total, Mg. per 100 Cc.						Cholesterol in Whole Blood: Esters, % of Total				Cholesterol in Aorta, Mg. per 100 Gm. Dry Weight
	0 Wk.	2 Wk.	4 Wk.	6 Wk.	8 Wk.	14 Wk.	0 Wk.	4 Wk.	8 Wk.	14 Wk.	
Control Group—1 Gm. of Cholesterol Three Times Weekly for Fourteen Weeks											
111	89	96	276	262	150	605	8	34	17	53	2,303
112	111	313	408	903	500	682	9	45	31	44	721
113	120	375	347	384	347	664	10	38	41	34	1,061
114	134	188	148	423	230	145	8	21	28	31	1,061
115	98	375	323	750	544	620	9	33	42	53	4,855
116	95	195	357	245	250	364	7	42	23	51	500
117	115	375	278	250	255	185	8	35	29	35	981
118	94	202	150	129	168	395	8	34	27	52	884
119	102	208	213	388	107	107	9	38	31	24	1,082
120	85	112	217	352	239	240	7	29	29	47	531
Average	104	244	272	409	279	401	8	35	30	42	1,398
Experimental Group—1 Gm. of Cholesterol and 0.5 Gm. of Cholic Acid Three Times Weekly for Fourteen Weeks											
102	99	695	1,103	1,136	987	750	10	56	43	48	4,700
103	79	276	507	750	815	806	8	42	50	47	2,731
104	121	625	815	688	815	652	7	55	51	43	4,437
105	82	363	341	707	586	528	6	42	43	55	1,662
106	117	625	875	1,102	873	615	13	47	42	47	5,682
107	106	329	473	595	247	324	8	45	29	51	1,440
109	107	625	650	1,000	962	806	11	44	53	49	5,621
110	104	507	213	433	391	707	8	25	33	52	837
Average	102	506	622	801	710	649	9	45	43	49	3,389

TABLE 3.—*Effect of Feeding Cholesterol With and Without Simultaneous Feeding of Hyodesoxycholic Acid (Whole Hog Bile) on the Total and the Ester Cholesterol Content of the Whole Blood and on the Cholesterol Content of the Aorta*

Rabbit No.	Cholesterol in Whole Blood: Total, Mg. per 100 Cc.					Cholesterol in Whole Blood: Esters, % of Total					Cholesterol in Aorta, Mg. per 100 Gm. Dry Weight
	0 Wk.	3 Wk.	6 Wk.	9 Wk.	12 Wk.	0 Wk.	3 Wk.	6 Wk.	9 Wk.	12 Wk.	
Control Group—1 Gm. of Cholesterol Three Times Weekly for Twelve Weeks											
211	98	240	173	670	781	16	34	29	42	47	951
212	94	368	551	987	833	16	38	44	41	51	2,229
214	71	114	...	750	708	18	25	..	38	41	1,843
216	114	148	245	750	915	18	31	31	50	54	1,376
218	89	144	193	694	750	14	26	31	54	45	814
220	100	350	371	551	708	15	32	35	41	43	790
Average	94	227	307	734	753	16	31	34	44	47	1,334
Experimental Group—1 Gm. of Cholesterol and 0.5 Gm. of Hyodesoxycholic Acid Three Times Weekly for Twelve Weeks											
201	87	184	551	1,042	893	18	28	40	50	53	1,292
202	98	226	315	658	781	16	26	34	50	56	1,261
203	108	235	514	605	...	18	37	39	34	..	1,560*
204	93	313	475	507	647	23	28	42	41	44	1,142
206	89	798	387	568	750	22	36	38	40	46	1,505
207	107	175	208	...	...	21	28	35	..	..	566†
Average	97	322	408	676	768	20	31	38	43	50	1,221

* The rabbit died at the eleventh week.

† The rabbit died at the tenth week.

per hundred grams of dry aorta in the experimental group, compared with 2,932 mg. per hundred grams of dry aorta in the controls). The percentage of ester cholesterol in the whole blood appeared to be influenced little by this bile acid. The ingestion of glycocholic acid and cholesterol resulted in a pronounced deposition of cholesterol in the liver in experimental rabbits (average 1,375 mg. per hundred grams of wet liver) compared with animals fed cholesterol alone (average 726 mg. per hundred grams of wet liver).

TABLE 4.—*Effect of Feeding Cholesterol With and Without Simultaneous Feeding of Desoxycholic Acid on the Total and the Ester Cholesterol Content of the Whole Blood and on the Cholesterol Content of the Aorta and of the Liver*

Rabbit No.	Cholesterol in Whole Blood: Total, Mg. per 100 Cc. 0 Wk.	3 Wk.	7 Wk.	11 Wk.	13 Wk.	Cholesterol in Whole Blood: Esters, % of Total 0 Wk.	3 Wk.	7 Wk.	11 Wk.	13 Wk.	Cholesterol in Aorta, Mg. per 100 Gm. Dry Weight	Cholesterol in Liver, Mg. per 100 Gm. Wet Weight
Control Group—1 Gm. of Cholesterol Three Times Weekly for Thirteen Weeks												
312	81	147	781	917	686	21	48	43	47	43	745	631
316	96	225	559	418	481	19	50	31	30	38	1,207	858
317	76	139	347	221	568	16	49	38	51	44	648	558
318	96	431	813	1,136	988	21	55	56	48	53	1,923	782
319	121	596	833	682	852	17	61	42	48	36	1,196	815
320	99	431	750	1,250	1,250	21	47	44	43	39	647	928
Average	95	328	681	770	779	19	51	42	45	42	1,061	762
Experimental Group—1 Gm. of Cholesterol and 0.5 Gm. of Desoxycholic Acid Three Times Weekly for Thirteen Weeks												
301	142	332	765	873	1,072	31	54	46	45	..	853	876
302	100	469	625	...	682	27	42	35	..	47	957	406
303	117	403	568	833	721	25	44	45	51	39	600	1,209
305	119	260	765	682	938	24	48	47	42	32	724	1,077
307	125	408	625	708	577	30	51	39	37	46	965	938
308	107	399	938	815	682	26	54	43	49	41	3,125	750
309	100	815	586	457	...	25	51	53	45	..	631	695
311	120	255	682	...	694	26	40	38	..	42	931	462
322	118	149	332	...	190	25	36	52	..	42	633	392
Average	116	387	654	728	695	26	47	44	45	41	1,047	756

TABLE 5.—*Effect of Feeding Cholesterol With and Without Simultaneous Feeding of Glycocholic Acid on the Total and the Ester Cholesterol Content of the Whole Blood and on the Cholesterol Content of the Aorta and of the Liver*

Rabbit No.	Cholesterol in Whole Blood: Total, Mg. per 100 Cc. 0 Wk.	2 Wk.	4 Wk.	6 Wk.	8 Wk.	10 Wk.	13 Wk.	Cholesterol in Whole Blood: Esters, % of Total 0 Wk.	2 Wk.	4 Wk.	6 Wk.	8 Wk.	10 Wk.	13 Wk.	Cholesterol in Aorta, Mg. per 100 Gm. Dry Weight	Cholesterol in Liver, Mg. per 100 Gm. Wet Weight
Control Group—1 Gm. of Cholesterol Three Times Weekly for Thirteen Weeks																
511	100	175	344	361	658	962	872	..	..	..	..	..	..	..	2,641	699
520	130	303	250	217	253	242	596	..	..	..	..	..	..	..	645	452
532	110	383	550	938	815	893	625	21	54	46	51	50	48	40	9,615	943
533	105	222	391	326	417	658	658	17	39	47	23	42	50	30	1,645	1,009
534	137	289	500	596	682	765	833	25	37	44	40	41	42	41	2,840	642
535	99	205	347	417	750	1,103	1,103	34	54	44	47	41	41	51	3,375	509
536	127	341	178	154	278	260	266	22	56	53	47	50	36	36	934	442
539	99	371	625	670	625	987	1,136	25	41	62	38	50	47	36	3,538	723
540	85	257	266	421	708	531	...	22	49	66	47	51	29	..	1,156	1,116
Average	110	283	384	455	576	714	761	24	47	52	42	46	42	39	2,932	726
Experimental Group—1 Gm. of Cholesterol and 0.5 Gm. of Glycocholic Acid Three Times Weekly for Thirteen Weeks																
522	98	216	436	682	852	735	735	26	54	54	51	44	42	49	5,953	1,219
523	123	183	115	765	1,339	962	987	29	53	50	44	49	39	34	2,568	1,275
524	199	431	417	721	852	1,100	...	43	44	46	44	36	..	..	8,000	1,893
530	211	605	938	1,014	1,500	1,014	815	38	56	45	48	43	31	35	3,720	1,674
541	117	276	264	323	1,072	...	...	19	48	32	39	43	..	..	757	1,639
543	131	132	452	383	708	852	893	29	38	48	46	43	34	49	7,796	580
Average	136	346	397	648	1,054	933	856	31	49	46	45	43	37	42	4,799	1,375

COMMENT

The foregoing experiments on rabbits indicate that feeding of cholic acid or glycocholic acid and cholesterol increases markedly the cholesterol content of the whole blood and of the aorta (feeding of cholesterol without bile acid was the control). Dehydrocholic acid, hyodesoxycholic acid and desoxycholic acid do not possess this property. Moreover, in a limited study it was shown that the ingestion of glycocholic acid and cholesterol is followed by deposition of large amounts of cholesterol in the liver whereas desoxycholic acid produces no such result. Again, feeding of cholic acid, unlike that of the other bile acids tested, is accompanied by increased concentration of combined (ester) cholesterol in the whole blood; it is conceivable that cholic acid may stimulate esterification of cholesterol in the wall of the gut.

Cholic acid and glycocholic acid probably increase the absorption of cholesterol from the intestinal tract, thus producing high concentrations of this sterol in the blood. The deposition of cholesterol in the aorta and (as shown in the experiment with glycocholic acid) in the liver is thereby enhanced. It is interesting to note that the other bile acids tested produce no such effects.

This selective action on cholesterol metabolism of some of the bile acids may be gleaned from the in vitro experiments of Bauman and his co-workers [17]; these investigators noted that the solubility of cholesterol was different in various solutions of bile salts. Loeffler [13] found in mice that the liver contained greater concentrations of cholesterol when cholic acid was fed as compared with the levels obtained after the feeding of desoxycholic acid. Hummel [11] subsequently confirmed these observations, using cholic acid; dehydrocholic acid was shown to have no such effect.

17. Bashour and Bauman.[6] Spanner and Bauman.[7]

SUMMARY

Cholic or glycocholic acid fed with cholesterol to the rabbit increases markedly the cholesterol contents of the whole blood and the aorta as compared with the levels obtained following the ingestion of cholesterol alone.

Dehydrocholic acid, hyodesoxycholic acid and desoxycholic acid do not possess this property.

In two series of experiments in which desoxycholic acid and glycocholic acid were used respectively, the latter was shown to augment the cholesterol content of the liver; the former was without effect in this regard.

The feeding of cholic acid, unlike that of any other bile acid tested, is accompanied by increased concentration of combined (ester) cholesterol in the whole blood.

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Biological Studies of Dihydrocholesterol

IV. Effect of Bile Acids and Other Choleretic Agents on Dihydrocholesterol-Induced Cholelithiasis in the Rabbit

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Introduction

Previous studies have shown that the feeding of dihydrocholesterol to rabbits consistently leads to inflammation of the biliary tract and deposition of concrements in the bile ducts and gallbladder.[1] It was further demonstrated that the formation of these concrements, which consist largely of bile salts, could be prevented entirely or in part by the simultaneous administration of dehydrocholic acid.[2] These results suggested that the experimentally induced cholelithiasis could be affected by changes in either the pH of the bile or the rate of bile flow. Since the administration of dehydrocholic acid produced no significant changes in the pH of the bile,[12] it was pertinent to study the effect of naturally occurring bile acids (cholic acid, deoxycholic acid, hyodeoxycholic acid) and other choleretic agents (dehydrocholic acid, florentyrone [Zanchol], Gallogen, Dreole and MA-C2 *) upon the development of the dihydrocholesterol-induced cholecystitis and cholelithiasis and upon the tissue concentrations of cholesterol and dihydrocholesterol.

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* The chemical formulas and the manufacturers of these compounds are listed in Table 1.

Methods

Eighty-nine male chinchilla type or albino rabbits were maintained for four weeks on the different dietary regimens indicated in Table 1. The animals were kept in individual cages and had access to food at all times. The basic diet was Purina Rabbit Chow Pellets to which olive oil, U. S. P., had been added to obtain a final concentration of 12% olive oil. When required, the food was treated with dihydrocholesterol, bile acids, or both, as described previously.[1,2] The choleretic drugs were thoroughly mixed with the food, together with the olive oil, by use of a mechanical mixer.

At the end of four weeks the animals were killed by intravenous injection of pentobarbital. During this time the animals had either maintained their weight or gained weight. There were no obvious toxic effects attributable to any of the choleretic drugs tested, although the dosages used were as high as or higher than those known to produce adequate choleresis in acute animal experiments[4,6] † or in clinical trials.[7]

The severity of the cholelithiasis was estimated by measurement of the dry weight of the concrements present in the gallbladder and by histologic examination. The concentrations of cholesterol and dihydrocholesterol in liver, muscle, and serum were determined at the end of the experimental period. The histologic and biochemical methods[1,2] and the procedure for estimating the pathologic involvement of the biliary tract[8] have been described previously.

Results

Table 1 summarizes the average weight of the rabbits at the start of the experiment, the average dry weight of the gallstones, and the average pathologic involvement of the 16 groups of animals used in this study.

† None of the drugs used in this study had been tested in rabbits. Charlier and Vandersmissen[6] administered single doses of Dreole amounting to 300 mg. per kilogram to guinea pigs, without observing harmful effects.

TABLE 1.—*Degree of Cholelithiasis and Pathologic Involvement of Rabbits*

Group	Dietary Additions: Dihydrocholesterol, 0.5%	Dietary Additions: Choleretic Agent, 0.25%	Animals, No.	Weight of Animals, Kg.	Dry Weight of Gallstones: Average, Mg.	Dry Weight of Gallstones: Range, Mg.	Pathologic Involvement (Arbitrary Units)*
A	+	None	18	2.7	180	0-360 †	10.4
B	—	None	7	2.3	0	—	0.9 ‡
C	—	Deoxycholic acid	4	2.9	0	—	2.5
D	+	Deoxycholic acid	6	2.5	0	—	3.3
E	—	Cholic acid	3	2.1	0	—	1.7
F	+	Cholic acid	4	2.1	0	—	2.8
G	—	Dehydrocholic acid	5	2.1	0	—	1.0
H	+	Dehydrocholic acid	10	2.6	10	0-90	5.1
I	—	Hyodeoxycholic acid	5	3.4	0	—	4.0
J	+	Hyodeoxycholic acid	6	3.2	270	0-460 §	7.3
K	—	Gallogen ‖	3	2.3	0	—	0.7
L	+	Gallogen ‖	4	2.7	150	20-290	6.7
M	+	Zanchol ¶	4	2.8	220	30-580	9.3
N	+	MA-C2 ∮	4	2.8	220	0-330 §	9.0
O	+	Dreole **	6	2.1	100	0-270 ††	4.5

* For method of estimation see Reference 3.

† One animal in this group had no gallstones.

‡ Three rabbits in this group showed an increase in periportal cellular infiltration. The gallbladders and extrahepatic bile ducts were normal.

§ One animal in this group had a rudimentary gallbladder containing no concrements.

‖ Gallogen (Massengill) is the diethanolamine salt of mono-(+)-camphoric acid ester of 2, 4-dimethylbenzyl alcohol.

¶ Zanchol (Searle) is γ-oxo- γ-fluoranthoylbutyric acid.

∮ MA-C2 (Ames) is β-[4(1,2,3,10b-tetrahydrofluoranthoyl)]-propionic acid.

** Dreole (Lilly) is *p*-hydroxyphenylsalicylamide.

†† Two animals in this group had no gallstones.

All but 1 of the 18 animals fed 0.5% dihydrocholesterol for four weeks (Group A) had gallstones. On the basis of previous studies, a 100% incidence of cholelithiasis was expected in this group. It is not known why one of the rabbits proved resistant.

The gross appearance of the biliary tract of animals fed 0.5% dihydrocholesterol for three weeks has been described in an earlier paper.[2] The present control group (Group A) had received 0.5% dihydrocholesterol for four weeks and had lesions of similar

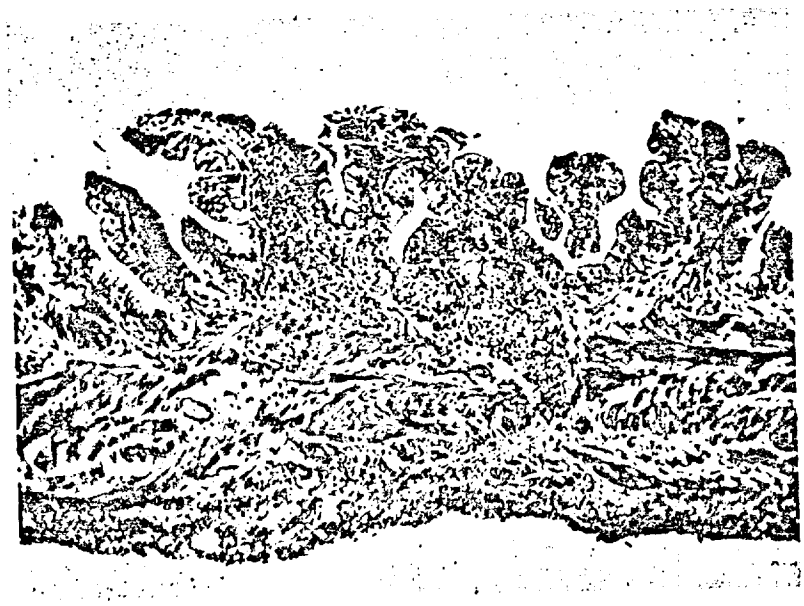

Fig. 1.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol for four weeks (Group A). Stones weighing 203 mg. (dry weight) were present in the fundus. The mucosal folds are thick and adherent. Cellular infiltration of lymphocytes and histiocytes extends throughout the wall. Both the lamina propria and serosa are thickened by fibrous tissue. Hematoxylin and eosin; reduced 10% from mag. × 100.

severity. The 17 animals of this group which developed gallstones (average dry weight 180 mg.) showed the expected congestion and thickening of the walls of the gallbladder. In about one-half of the animals the intra- and extrahepatic ducts contained concrements.

The histologic changes in this group were similar to those described in animals receiving 0.25%-1% dihydrocholesterol for three to six weeks [1-3] and consisted briefly of edema, fibrosis, and cellular infiltration of the biliary tract (Fig. 1).

On the average, control Group A had the greatest pathologic involvement, 10.4+. This value is very close to that of 11.5+ obtained when 1.0% dihydrocholesterol was fed for four weeks.[3]

The results listed for Group H (0.5% dihydrocholesterol plus 0.25% dehydrocholic acid), in which 8 of 10 animals were found to be free of gallstones, are likewise in accord with an earlier observation [2] that dehydrocholic acid inhibits or prevents the formation of gallstones. However, in the present experiment the inflammatory reaction was severer than that reported in the previous study. This is probably due to the fact that in the present experiment the animals received dihydrocholesterol plus dehydrocholic acid for a period of four weeks, i. e., for one week longer than in the earlier study.

The data of Table 1 further show that Zanchol, MA-C2, Gallogen, and hyodeoxycholic acid were ineffective in suppressing the inflammatory manifestations and gallstone formation. The gallbladders of the animals receiving dihydrocholesterol plus 0.25% of these substances (Groups J, L, M, and N) could not be distinguished from those of Group A. The lowered pathologic involvement observed in Groups J and L was due to the absence of calculi from the intra- and extrahepatic bile ducts, suggesting that these agents did exert some choleretic effect.

In contrast, deoxycholic acid and cholic acid suppressed the development of the inflammatory lesions and completely prevented the formation of concrements (Groups D and F) (Fig. 2). These two naturally occurring bile acids proved to be even more effective than dehydrocholic acid in preventing the dihydrocholesterol-induced irritation of the biliary tract.

The six animals receiving Dreole plus dihydrocholesterol (Group O) had an average pathologic involvement of 4.5+; only four of these animals had gallstones (average dry weight 100 mg.). Both the weight of the gallstones and the over-all pathologic involvement were distinctly diminished in comparison with the control group receiving dihydrocholesterol alone. The lowered pathologic involvement resulted largely from suppression of the inflammatory reaction of the biliary tract in animals receiving dihydrocholesterol plus Dreole (Fig. 4).

Hyodeoxycholic acid fed without dihydrocholesterol was found to be an irritant of

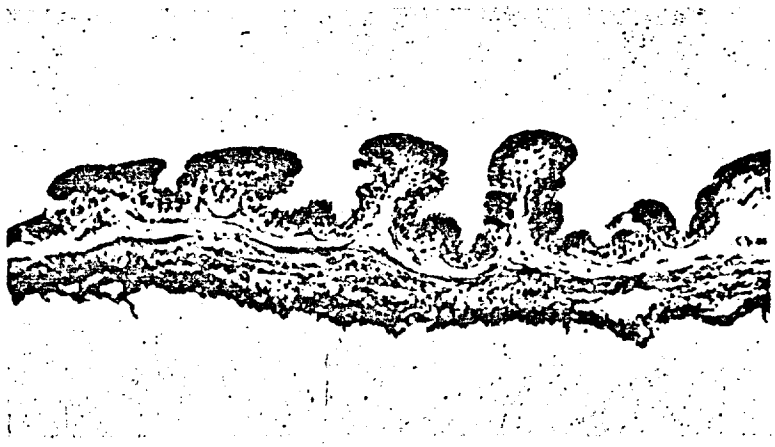

Fig. 2.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol and 0.25% cholic acid for four weeks (Group F). No calculi were found in the biliary tract. The mucosal folds are thin. The lamina propria is delicate. Only an occasional lymphocyte is present. The histology is that of a normal gallbladder. Hematoxylin and eosin; reduced 10% from mag. × 100.

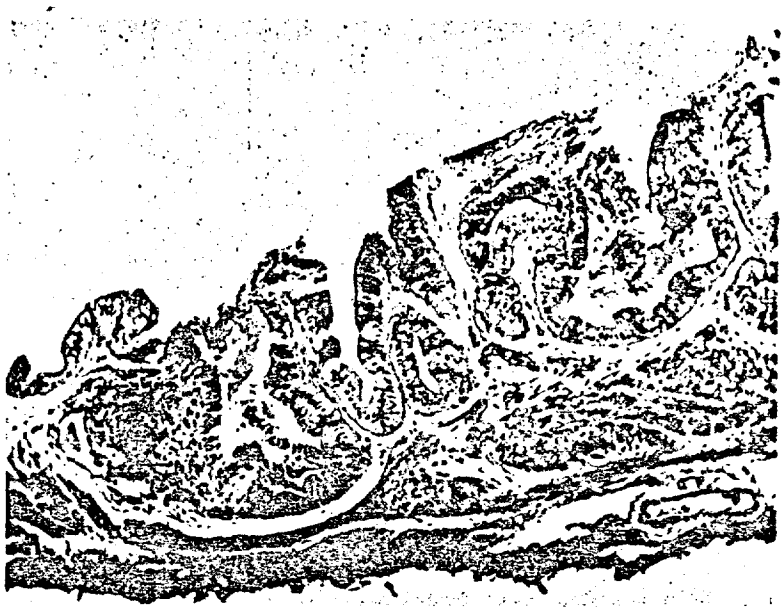

Fig. 3.—Gallbladder of a rabbit fed 0.25% hyodeoxycholic acid without dihydrocholesterol for four weeks (Group I). The mucosal folds, the laminal propria, and the muscularis are edematous. Scattered lymphocytes and histiocytes are present throughout the wall, suggesting that this cholanic acid exerted an irritating effect. Hematoxylin and eosin; reduced 10% from mag. × 100.

the biliary tract in that all five animals of Group I (0.25% hyodeoxycholic acid) showed a slight inflammation without calculus formation (Fig. 3).

Table 2 lists the initial serum cholesterol concentrations and the average serum, liver, and muscle sterol concentrations of the 16 groups of animals at the end of the four-week feeding period. In the present study some increases in serum cholesterol levels were observed in several groups of experimental animals. These increases were most pronounced in the rabbits receiving deoxycholic acid with or without dihydrocholesterol but were also observed in the rabbits fed dihydrocholesterol plus cholic acid, dehydrocholic acid, Zanchol, or Gallogen. There have been reports that cholic acid, but not deoxycholic acid, can produce an increase of serum cholesterol concentrations in cholesterol-fed rabbits.[11] The present study indicates that deoxycholic acid is considerably more effective in producing elevated serum sterol levels than cholic acid, even when the animals were maintained on dihydrocholesterol-free diets (Group C). In addition, deoxycholic acid feeding produced increases of liver sterol concentrations both with and without the addition of dihydrocholesterol to the diet (Groups C and D).

As observed previously,[2,3] the administration of dihydrocholesterol led to the replacement of about one-quarter to one-third of

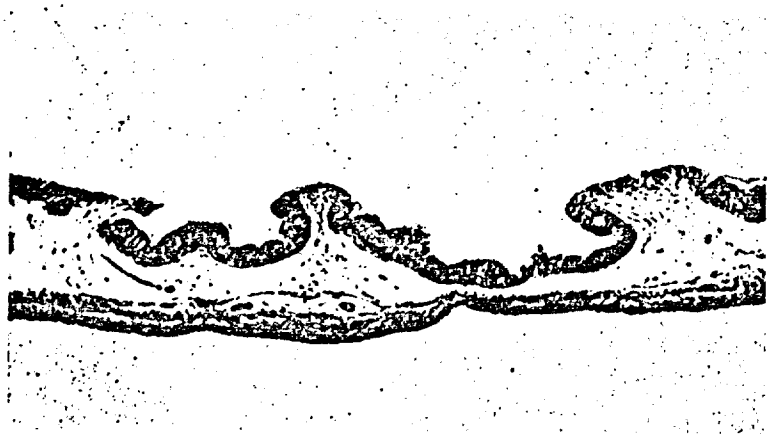

Fig. 4.—Gallbladder of a rabbit fed 0.5% dihydrocholesterol and 0.25% Dreole for four weeks (Group O). The fundus contained concrements weighing 214 mg. (dry weight). The mucosal folds are somewhat flattened due to distention of the gallbladder by bile and concrements. There is edema of the lamina propria but little cellular infiltration. Hematoxylin and eosin; reduced 10% from mag. × 100.

TABLE 2.—*Tissue Sterol Concentrations of Rabbits When Killed*

Group	Dietary Additions: DHC,* 0.5%	Dietary Additions: Choleretic Agent, 0.25%	Initial Serum Cholesterol, Mg. %	Final Serum Sterol: Total Sterol, Mg.%	Final Serum Sterol: Cholesterol, Mg.%	Final Serum Sterol: DHC in Total Sterol, %	Final Liver Sterol: Total Sterol, Mg/Gm.	Final Liver Sterol: Cholesterol, Mg/Gm.	Final Liver Sterol: DHC in Total Sterol, %	Final Muscle Sterol: Total Sterol, Mg/Gm.	Final Muscle Sterol: DHC in Total Sterol, %
A	+	None	46	184	90	51	6.88	3.48	49	0.63	32
B	—	None	42	51	48	6	3.31	2.81	15	0.62	19
C	—	Deoxycholic acid	62	164	145	12	4.82	3.94	18	0.58	3
D	+	Deoxycholic acid	48	291	170	42	18.2	7.72	58	0.66	33
E	—	Cholic acid	40	85	81	5	3.44	2.87	17	0.65	14
F	+	Cholic acid	42	196	101	48	7.87	3.93	50	0.72	42
G	—	Dehydrocholic acid	74	97	97	0	3.11	2.83	9	0.68	19
H	+	Dehydrocholic acid	39	206	94	54	5.81	2.72	53	0.62	31
I	—	Hyodeoxycholic acid	35	47	40	15	2.99	2.72	9	0.62	20
J	+	Hyodeoxycholic acid	28	92	37	60	7.63	3.38	56	0.66	41
K	—	Gallogen †	56	48	50	0	3.84	3.21	16	0.62	23
L	+	Gallogen †	32	149	77	48	5.82	2.86	51	0.55	31
M	+	Zanchol ‡	24	186	92	51	5.16	2.87	44	0.63	33
N	+	MA-C2 §	34	128	66	48	5.31	2.79	48	0.67	39
O	+	Dreole ‖	46	125	73	42	4.14	2.29	45	0.73	26

* DHC indicates dihydrocholesterol.
† Diethanolamine salt of mono-(†)-camphone acid ester of 2,4-dimethylbenzyl alcohol.
‡ γ-oxo- γ-fluoranthoylbutyric acid.
§ β-[4(1,2,3,10b-tetrahydrofluoranthoyl)]-propionic acid.
‖ *p*-hydroxyphenylsalicylamide.

muscle sterol by dihydrocholesterol. None of the choleretic agents studied produced additional increases in either the concentration of dihydrocholesterol or the total sterol in muscle tissue.

Comment

The major cholanic acid constituents of rabbit bile are deoxycholic and cholic acid, with the former predominating. These two bile acids were effective in preventing dihydrocholesterol-induced cholecystitis and cholelithiasis. Dehydrocholic acid, the triketo derivative of cholic acid, inhibited gallstone formation fairly well (8 of 10 animals had no cholelithiasis) but did not protect the biliary tract against the irritating effect of dietary dihydrocholesterol. The other cholanic acid tested was hyodeoxycholic acid (3α,6α-dihydroxycholanic acid), the major bile acid of hog bile. This bile acid produced an irritation of the biliary tract of the rabbit, even when no dihydrocholesterol was administered (pathologic involvement 4.0+), and had little or no protective effect when fed together with this sterol. The failure of hyodeoxycholic acid to inhibit the formation of biliary calculi can be ascribed to the fact that this bile acid is relatively ineffective in promoting the flow of bile.[8,9] Three of the four non-bile acid choleretics tested did not inhibit the experimentally induced cholelithiasis, although the dosages used were generally as high as or higher than those recommended in the literature.[4-7] The failure of Zanchol and MA-C2 to exert significant protective effects was disappointing, because these substances are fluoranthenes, the structure of which bears a superficial similarity to that of the phenanthrene nucleus of the steroids. The results obtained with rabbits fed Dreole (*p*-hydroxyphenylsalicylamide) plus dihydrocholesterol suggest that this compound provided some protection against the irritating effect of dihydrocholesterol. Since Dreole is a salicylate, it may well possess anti-inflammatory properties, as well as choleretic activity.

The present investigation confirms the results of earlier studies indicating that the concentration of dihydrocholesterol in the tissues bears no simple relationship to the intensity of the lesions produced in the biliary tract. In fact, while the animals receiving deoxycholic acid plus dihydro-

cholesterol (Group D) had serum and liver sterol concentrations which were much higher than those found in any other group, these animals had no biliary calculi and exhibited minimal inflammation of the biliary tract. This finding suggests that there are two possible explanations for the absence of biliary calculi in rabbits receiving dihydrocholesterol plus cholic, deoxycholic, or dehydrocholic acids in their diet.

1. The administration of bile acids inhibits the conversion of sterols to cholanic acids by the liver.[10] This would explain the accumulation of sterols in the livers of rabbits fed dihydrocholesterol plus bile acids (particularly deoxycholic acid) and would also account for the fact that the conversion of dihydrocholesterol to bile salt-like substances is inhibited. This hypothesis would further serve to explain why the species-specific cholanic acids proved to be the most effective in preventing the formation of biliary calculi.

2. Deoxycholic, cholic, and dehydrocholic acids are more effective choleretic agents than the other substances studied.

If the second explanation is found to be valid, dihydrocholesterol-fed rabbits would prove useful for the evaluation of choleretic drugs. Presumably, a choleretic drug of the same order of activity as deoxycholic acid or cholic acid should inhibit the formation of biliary calculi and the development of the inflammatory reaction. It is important to point out that in such comparative studies the dosages used would require detailed investigation.

Summary

The effect of cholanic acids and other choleretics upon the biliary tract of dihydrocholesterol-fed rabbits was investigated. It was shown that deoxycholic acid and cholic acid completely prevented the dihydrocholesterol-induced cholelithiasis, while dehydrocholic acid was slightly less effective. Hyodeoxycholic acid and three of the four non-bile acid choleretics tested did not suppress the formation of biliary calculi or the development of the inflammatory reaction of the biliary tract; however, Dreole (p-hydroxyphenylsalicylamide) partially protected the biliary tract of the rabbits against the irritating effect of dihydrocholesterol.

The dihydrocholesterol was supplied by the Schering Corporation. Deoxycholic, cholic, and dehydrocholic acids were supplied by the Ames Company, Inc., and hyodeoxycholic acid by Canada Packers, Ltd., and the Wilson Laboratories. Eli Lilly & Company, G. D. Searle & Co., the S. E. Massengill Company, and the Ames Company, Inc., supplied the choleretic drugs used in this study.

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Faecal excretion products of cholic acid in man*†

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The two primary bile acids formed from cholesterol in man are cholic and chenodeoxycholic acids. These bile acids are attacked by microbial enzymes in the intestine and excreted in the faeces as completely transformed metabolites. Hydrolysis of conjugates, oxidation of hydroxyl groups to ketones, removal of the C_7-hydroxyl group and inversion of the 3α-hydroxyl group to the 3β position are the main reactions performed by micro-organisms. The subject has been reviewed by Bergström, Danielsson & Samuelsson (1960). The main faecal bile acids in man are 3,12-dihydroxycholanic, 3-hydroxy-12-ketocholanic and 3-hydroxycholanic acids, appearing as a mixture of the 3α- and the 3β-hydroxy isomers (Fischer, 1911; Carey & Watson, 1955; Heftmann, Weiss, Miller & Mosettig, 1959; Danielsson, Eneroth, Hellström, Lindstedt & Sjövall, 1963).

As faecal bile acid excretion is one of the measures of cholesterol degradation, interest in the factors controlling this excretion is increasing. However, the literature contains several contradictory reports on the bile acid excretion in man. Many of these discrepancies are probably due to the fact that certain methods used for measuring faecal bile acids are not completely satisfactory. As the investigation described here does not concern itself with the quantitative aspects of bile acid excretion, a discussion of those problems is omitted. A recent review is that of Danielsson (1964).

The turnover of cholic acid can be calculated by determining the rate of faecal isotope excretion after administration of ^{14}C-labelled cholic acid. Such determinations in the rat have demonstrated that several factors influence the elimination of cholic acid. The slowest faecal excretion rate of labelled cholic acid has been observed in germ-free rats and in rats treated with phthalylsulphathiazole and oxytetracycline (Gustafsson, Bergström, Lindstedt & Norman, 1957; Lindstedt & Norman, 1956), the estimated biological half-life of cholic acid being at least three times that in conventional rats. Portman & Murphy (1958) have demonstrated that the type of diet has a pronounced influence on the turnover of cholic acid: with a commercial rat diet 'largely based on corn germ' (Portman, 1962), the half-life of cholic acid was 2·0 days, whereas with a standard low-residue, semi-purified diet with sucrose as the source of carbohydrate and with the same diet supplemented with 20% of cellulose the half-lives were 4·2 and 1·4 days, respectively.

* This paper is no. 137 of a series of papers on *Bile acids and steroids* published by a group led by Professor Sune Bergström, Stockholm. Paper no. 136: *J. biol. Chem.* (1963), 238, 3846.

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Intestinal bacteria and dietary factors play important parts in the elimination of bile acids by influencing their reabsorption in the enterohepatic circulatory system. A common mechanism of action cannot be excluded, however, as changes in the composition of the diet induce changes in the intestinal flora. Decreased reabsorption of bile acids might be caused by changes in the transit time of the intestinal contents, formation of less absorbable derivatives or binding of bile acids to micro-organisms or indigestible food residues.

The physical state of the bile acids varies in different parts of the intestine (Gustafsson & Norman, 1962). After administration of [24-^{14}C]cholic acid to rats it was shown that the labelled bile acids in the small intestine were present in the supernatant fluid after centrifugation of the small intestinal contents at 25000 *g*. In contrast, half of the labelled bile acids in the caecum were not readily soluble in water and could only be recovered by acetone extraction of the sediment. This difference has been ascribed to the action of intestinal micro-organisms, since the caecal bile acids of germ-free rats were found to occur mainly in a water-soluble form.

The experiments to be described form part of investigations on the influence of the intestinal flora on the metabolism and turnover of bile acids in man. The extractability and nature of the excretory products of [24-^{14}C]cholic acid are reported.

EXPERIMENTAL AND RESULTS

General

The efficiency of various extraction and dialysation procedures was tested by applying them to faecal homogenates to which bile acids had been added. These procedures were then applied to the faeces of patients to whom labelled cholic acid had been given. Bile acid metabolites in faeces were separated by diethyl ether extraction and chromatography. Finally, the chemical nature of metabolites separated by extraction procedure and by dialysis was determined.

[24-^{14}C]cholic acid (15 μc) was given as the sodium salt dissolved in water to three patients, who were in hospital during the experiment and received the ordinary hospital diet. The patients were suffering from neurological diseases and had no clinical or laboratory signs of disease of the liver, gall-bladder or gastro-intestinal tract.

Fractionation of crude faeces extracts

Approximately 20 g faeces were homogenized in 40 ml saline with a Virtis Homogenizer (The Virtis Company Inc., Gardiner, New York) with propeller, for 1 min at 5000 rev/min and fractionated according to the scheme shown in Fig. 1. The faecal homogenate could be separated by centrifugation at 25000 *g* for 1 h into a top layer, a clear subnatant liquid and a sediment. The first two were combined in Extract A and the sediment was treated as shown in Fig. 1. All operations were done at 0–5°.

Dialysis of faecal homogenate

Dialysis was performed in regenerated cellulose tubing (Visking Company). The faecal homogenate was dialysed with five volumes of 1/15 M-phosphate buffer, pH 7·4, which was renewed every 6 h.

Separation of bile acids

Extracts A, B, C and the residue after evaporation of Extracts D and E were acidified to pH 1 with hydrochloric acid and extracted first with diethyl ether and then with butanol.

The labelled bile acids, which were almost entirely recovered in the ether extract, were separated by reversed-phase partition chromatography. The following phase systems were used:

Phase system	Moving phase	ml	Stationary phase	ml
C 1 (Norman, 1953)	Methanol:water	150:150	Chloroform:isooctanol	15:15
F 1 (Sjövall, 1953)	Methanol:water	165:135	Chloroform:heptane	45:5
F 2 (Sjövall, 1953)	Methanol:water	180:220	Chloroform:heptane	45:5
G (Norman, 1953)	Methanol:water	255:45	Heptane	50
HD (Danielsson, 1958)	Isopropanol:water	165:135	Chloroform:heptane	10:40

Columns for phase systems C 1, F 1, F 2 and G consisted of 4 ml stationary phase supported on 4·5 g hydrophobic Hyflo Super-Cel (Johns-Manville, New York).

Columns for phase system HD consisted of 3 ml stationary phase on 4·5 Hostalene (polyethylene powder; Farbwerke Hoechst, G.m.b.H., German Federal Republic).

A 4·5 g column was used when the amount of material to be analysed was less than 50 mg. For larger amounts, the columns were correspondingly increased. To facilitate comparison, the effluent volumes shown in the figures have been corrected to correspond to a 4·5 g column. The fractions collected from the columns were titrated with methanolic sodium hydroxide, and the isotope was determined in portions after plating in a Frieseke–Hoepfner methane gas-flow counter.

3α,12α-dihydroxycholanic and 3β,12α-dihydroxycholanic acids were separated on columns of Woelm aluminium oxide, grade V, as described by Danielsson *et al.* (1963). Thin-layer chromatography was performed as described by Eneroth (1963).

Extraction of bile acids from faeces

[24-^{14}C]*cholic and deoxycholic acids added to faecal homogenate.* A mixture of ^{14}C-labelled cholate and deoxycholate was added to portions of a faecal saline homogenate and, after further homogenization, extracted according to the scheme shown in Fig. 1. The amounts of labelled cholic and deoxycholic acids in the different extracts were determined after chromatographic separation. The distribution of isotope in the different extracts, shown in Table 1, indicates that both bile acids were adsorbed to the faecal residue. However, adsorption occurred to a much greater extent with deoxycholate than with cholate. For complete recovery of isotope, extraction with organic solvents was necessary. The acetone extracts contained the main part of labelled bile acids remaining after the phosphate buffer extraction, and the last small

quantity of isotope was recovered by extraction for 48 h with chloroform/methanol 1:1. The latter extraction method has been demonstrated to give quantitative recovery of bile acids from faeces (P. Eneroth, K. Hellström & J. Sjövall, 1964, to be published).

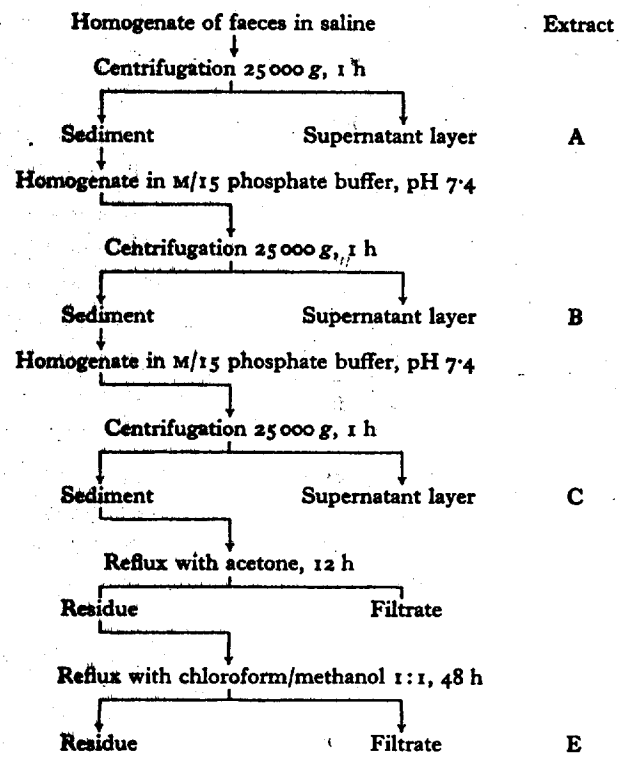

Fig. 1. Procedure for fractionation of faeces.

Table 1. *Extraction according to scheme shown in Fig. 1 of labelled cholate and deoxycholate added to a homogenate of human faeces*

	0·05 mg cholate, 0·05 mg deoxycholate, 10 g faeces homogenate			5 mg cholate, 5 mg deoxycholate, 10 g faeces homogenate	
		Percentage distribution of recovered ^{14}C-labelled:		Percentage distribution of recovered ^{14}C-labelled:	
Extract	Lipids soluble in ether (mg)	Cholate	Deoxycholate	Cholate	Deoxycholate
A	7·2	62·8	14·9	58·9	21·1
B	3·5	18·8	12·7	23·6	21·8
C	3·1	8·4	19·1	11·2	14·6
D	50·7	8·4	52·5	6·1	41·0
E	13·5	1·6	0·8	0·2	1·5

Labelled metabolites of orally administered [24-^{14}C]*cholic acid.* Three patients were given [24-^{14}C]cholic acid, and faeces were collected daily for 1 week. After homogenization in saline, portions of the homogenate were extracted according to the scheme shown in Fig. 1. Results of the differential extraction of labelled metabolites

from the faeces on the 5th day after the administration of [24-^{14}C]cholic acid are shown in Fig. 2. Of the metabolites of cholic acid, 10–23% were extractable with saline (Extracts A). In addition, 10–22% of the metabolites were loosely bound to the faecal residue and could be extracted with phosphate buffer (Extracts B + C). Most of the isotope was found in the acetone extracts (D), but even with this method there was incomplete recovery, as 5–15% of the labelled compounds were obtained later in the chloroform/methanol Extracts (E).

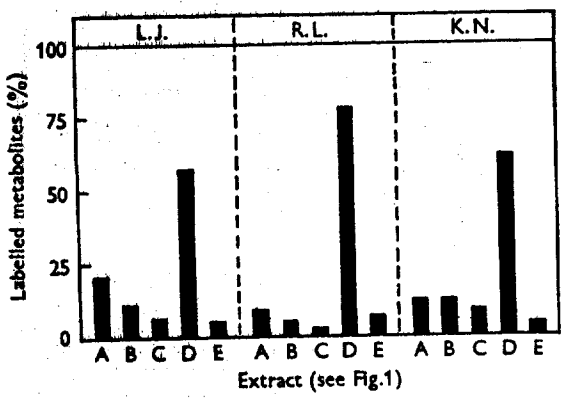

Fig. 2. Percentage distribution of recovered isotope after extraction according to scheme in Fig. 1 of samples of faeces 5 days after administration of [24-^{14}C]cholic acid to patients L.S., R.L. and K.N.

Table 2. *Percentage distribution of the dialysable and non-dialysable labelled compounds recovered by dialysis of faecal homogenates against* 1/15 M*-phosphate buffer, pH* 7·4. *After dialysis, the contents of the dialysis sack were centrifuged for* 60 *min at* 25000 g

	Dialysable compounds		Non-dialysable compounds			
			Supernatant layer		Sediment	
Material	Lipids soluble in ether (mg)	Labelled compounds (%)	Lipids soluble in ether (mg)	Labelled compounds (%)	Lipids soluble in ether (mg)	Labelled compounds (%)
10 g faeces homogenate and 0·05 mg [24-^{14}C]cholate and 0·05 mg [24-^{14}C]deoxycholate	16	97	2	2	81	1
10 g faeces homogenate and 5·0 mg [24-^{14}C]cholate and 5·0 mg [24-^{14}C]deoxycholate	—	96	—	2	—	2
10 g faeces homogenate from patient L.J. 3 days after administration of [24-^{14}C]-cholate	12	50	8	4	172	44
10 g faeces homogenate from patient K.N. 3 days after administration of [24-^{14}C]-cholate	12	61	2	2	107	37

Dialysis of bile acids in faecal homogenates

[24-^{14}C]*cholic and deoxycholic acids added to a faecal homogenate.* ^{14}C-labelled cholate and deoxycholate were added to faecal homogenates, which, after further homogenization, were transferred to dialysis bags. After dialysis, the contents were centrifuged for 1 h at 25000 *g* and the supernatant layer was separated from the sediment. The isotope was extracted from the dialysate and the supernatant layer by ether extraction after acidification, and from the sediment by chloroform/methanol extraction. Recoveries of added isotope in five similar experiments were between 94 and 98%. The upper half of Table 2 gives the percentage distribution of recovered labelled compounds, showing that practically all of the added labelled cholate and deoxycholate was separated from the faecal residue by dialysis.

Table 3. *Combined Extracts A, B, C, D, and E from homogenates of faeces of patients 5 days after administration of* [24-^{14}C]*cholic acid, extracted first with ether and then with butanol*

	Ether extract		Butanol extract	
Patient	mg/g faeces	% of recovered isotope	mg/g faeces	% of recovered isotope
L.J.	48	91	14	9
R.L.	53	98	12	2
K.N.	36	95	23	5

Labelled metabolites of [24-^{14}C]*cholic acid.* The results of the dialysis of faecal homogenates from patients K.N. and L.J. 3 days after administration of [24-^{14}C]cholic acid are shown in the lower half of Table 2. They indicate that at least a third of the excreted faecal metabolites of cholic acid are non-dialysable and are present in the sediment after centrifugation.

Dialysis of Extracts A, B and C of faeces from these patients showed that all the labelled metabolites in those extracts are dialysable. The residue after phosphate buffer extraction, however, also contained a large amount of labelled metabolites that could be separated from the residue by dialysis.

Separation of metabolites of [24-^{14}C]*cholic acid according to extractability with ether*

Unconjugated bile acids and glycine conjugates of mono- and di-hydroxycholanic acids can be extracted with ether from an acidified aqueous solution, but glycine conjugates of trihydroxycholanic acids and all taurine conjugates must be extracted with butanol. Extracts A, B and C and the residue, after evaporation, of Extracts D and E were combined, acidified, and extracted first with ether and then with butanol. As shown in Table 3, most of the labelled material was recovered in the ether extracts, and only 2–9% of the isotope was present in the subsequent butanol extracts. Thus a maximum of 2–9% of cholic acid was excreted in the faeces as either taurine conjugates or glycocholic acid.

As the ether extracts contained almost all of the labelled metabolites, and less

impurities than the butanol extracts, they were used for the subsequent column chromatographic separations of labelled metabolites. No analysis of the labelled material in the butanol extracts was done.

Column chromatographic separations of labelled metabolites of [24-^{14}C]cholic acid

Partition column chromatography was used to obtain group separations of [24-^{14}C]-cholic acid metabolites. In chromatography with phase system F 1, 3,7,12-trihydroxycholanic and 3,12-dihydroxy-7-ketocholanic acids appear with or just after the front, and separated from 3,12-dihydroxycholanic and 3-hydroxy-12-ketocholanic acids which appear in effluent fractions 40–60 ml and 80–110 ml respectively. 3,12-diketocholanic acid is retained in the stationary phase. However, no separation of the 3α- and 3β-hydroxy isomers is obtained.

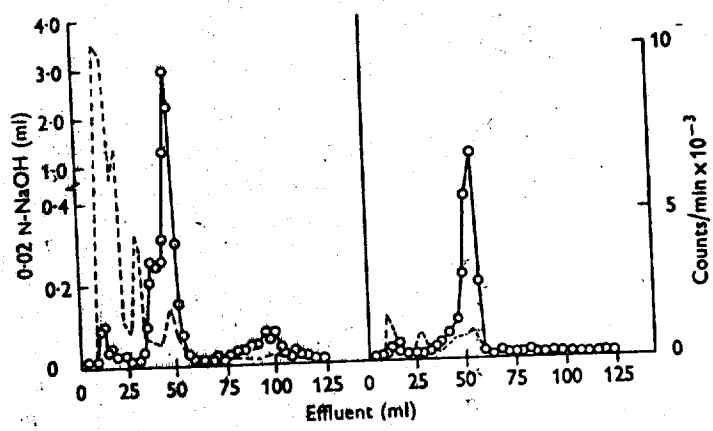

Fig. 3. Separation of labelled material from faeces 3 days after administration of [24-^{14}C]cholic acid to patient K.N. Phase system F 1 (see p. 175).

Left, combined ether extracts (A–E) (see Fig. 1); right, rechromatographed after saponification of labelled products retained in the column after the chromatograph shown in the left part; - - -, defined by left-hand legend to ordinate; o—o, defined by right-hand legend to ordinate.

The combined ether extracts of A, B, C, D and E were chromatographed with phase system F 1 and a representative chromatogram is shown on the left in Fig. 3. Labelled material representing four bile acid regions was obtained.

Fraction (I). Minor amounts of labelled compounds appeared with or just after the front. Rechromatography of Fraction (I) with phase system C showed that it contained only small amounts of unchanged cholic acid, and instead several more-polar and less-polar metabolites of cholic acid were separated.

Fraction (II). This fraction, which represents the deoxycholic acid band, contained most of the labelled products. It was methylated and further fractionated on a column of aluminium oxide, Woelm, grade V with increasing concentrations of ethyl acetate in benzene. The chromatographic fractions were analysed by subjecting small portions to thin-layer chromatography and by determination of isotope content (Fig. 4). Two

labelled compounds were isolated; the major one consisted of methyl 3α,12α-dihydroxycholanate and the minor one of methyl 3β,12α-dihydroxycholanate.

Fraction (III). After the deoxycholic acid band minor radioactive bands appeared, the quantitatively most important one corresponding to 3-hydroxy-12-ketocholanic acid (80–110 ml).

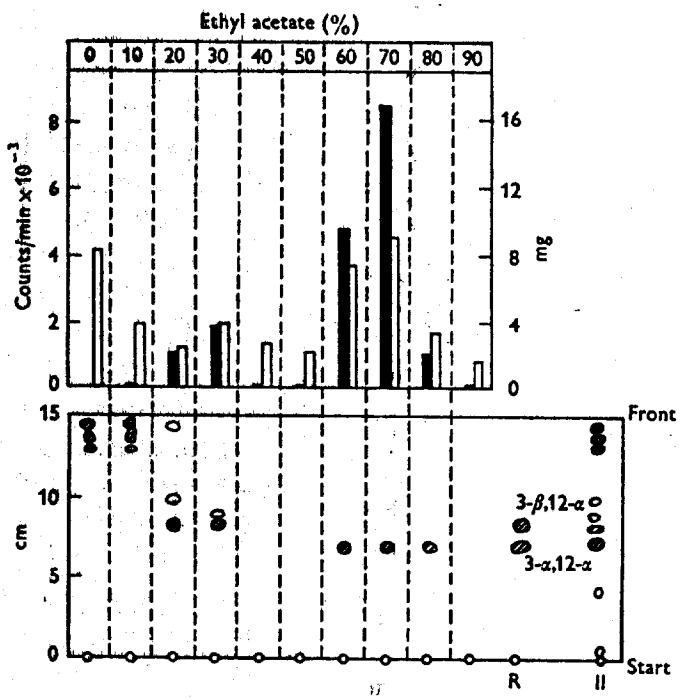

Fig. 4. Alumina fractionation of the 'deoxycholic acid band' from the chromatograph shown in Fig. 3, left. Column, 10 g aluminium oxide; fractions, 100 ml. The composition of the eluate is given as the percentage of ethyl acetate in benzene. □, amount of eluted solids (mg); ■, amount of isotope (counts/min × 10^{-3}).

The lower part of the figure shows the thin-layer silica gel chromatograph of the different fractions. R, reference substances: methyl 3α,12α-dihydroxycholanate and 3β,12α-dihydroxycholanate; II, portion of Fraction II before chromatography. Moving phase: trimethylpentane/ethyl acetate/acetic acid 10:20:0·2. Spots revealed by spraying with sulphuric acid and heating. Intensity of spots shown by differences in cross-hatching.

Fraction (IV). This fraction represents the labelled material retained in the stationary phase after chromatography with phase system F 1. It contained 25–50% of the labelled compounds and more than 90% of the lipids in the extracts used for chromatography. Rechromatography with phase system F 2 showed that only trace amounts of isotope were eluted at the place of 3,12-diketocholanic acid and almost all of the isotope remained in the stationary phase. By means of the phase system G, with which cholanic acid is eluted at 60–100 ml (Fig. 5*A*), part of the labelled compounds was eluted in the front fractions and part as a peak at 100–130 ml (Fig. 5*C*). As shown by the titration curve, the main part of the acidic products, consisting of fatty acids, was eluted with this phase system. The stationary phase, however, still contained the majority of the labelled products. For their separation, phase system HD, described

by Danielsson (1958) and used for the separation of cholesterol and related neutral steroids, was used. With phase system HD, as shown in Fig. 5*D*, the remaining labelled products were eluted in effluent fractions 70–150 ml just after cholesterol (Fig. 5*B*). No radioactive material was retained in the stationary phase after elution of the column with 150 ml of moving phase.

Thus Fraction (IV) consisted of at least three different labelled compounds. After heating in M-methanolic sodium hydroxide at 37° for 2 h, and chromatography with phase system F 1, all the isotope in this fraction could be eluted as a single peak at the place of 3,12-dihydroxycholanic acid as illustrated on the right in Fig. 3. Chromatography of the deoxycholic acid band on a column of aluminium oxide showed that the main labelled compound consisted of 3β,12α-dihydroxycholanic acid. Hence, Fraction (IV) consisted of saponifiable derivatives of mainly 3β,12α-dihydroxycholanic acid.

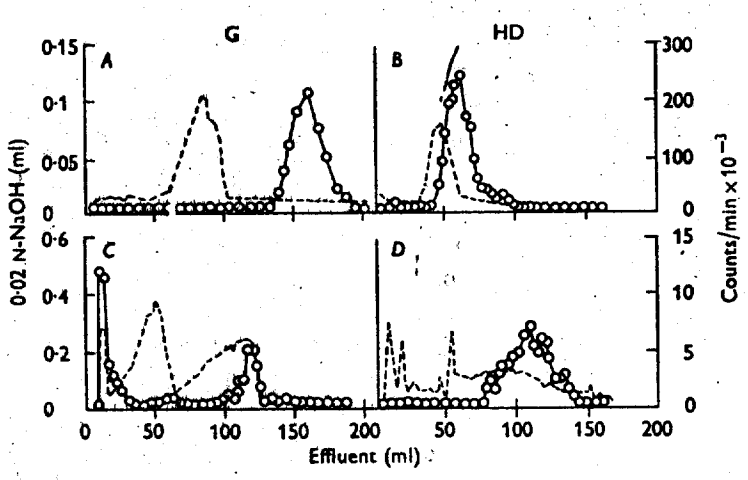

Fig. 5. *A* and *B*: separation of 4 mg cholanic acid and a tracer dose of [4-^{14}C]cholesterol with phase system G and HD (see p. 175). *C* and *D*: subsequent separation of labelled material retained in the column after the chromatograph shown in Fig. 3, left. - - -, defined by left-hand legend to ordinate; o—o, defined by right-hand legend to ordinate.

Correlation between chemical nature of metabolites of [24-^{14}C]cholic acid and their extractability

The ether extracts of (A), (B+C) and (D+E) from faeces 2, 3 and 4 days after administration of [24-^{14}C]cholic acid, were subjected to chromatography with phase system F 1 and HD. Representative chromatograms of labelled metabolites of Extracts (A) and (D+E) are shown in Figs. 6 and 7. The percentage distribution of isotope in each of Fractions (I), (II), (III) and (IV) for the three patients was determined. The results, summarized in Fig. 8, showed that the composition of the labelled bile acids in the saline (A) and phosphate buffer (B+C) extracts was quite different from that in the acetone and chloroform–methanol extracts (D+E). The extractability of Fractions I–IV was as follows:

Fraction (I) and (III) were mainly present in Extracts (A) and (B+C).

Fraction (IV) was not extracted by saline or phosphate buffer.

Fraction (II) was mainly recovered in Extracts (D + E) but was also present in the aqueous Extracts (A + (B + C)). The labelled 3α,12α-dihydroxycholanic and 3β-, 12α-dihydroxycholanic acids in Fraction (II) from samples 4 days after administration of [24-^{14}C]cholic acid were separated on columns of aluminium oxide. As shown in

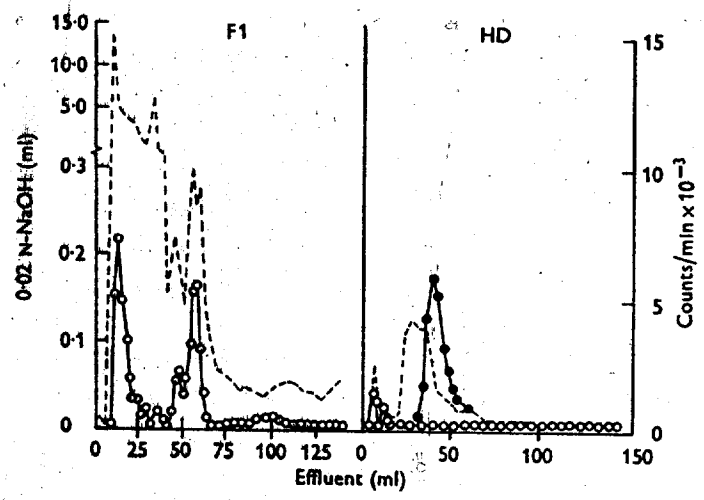

Fig. 6. Separation of unhydrolysed labelled material in Extract A (see Fig. 1) from faeces 3 days after administration of [24-^{14}C]cholic acid to patient E.L. Extract A was first chromatographed with phase system F 1 (see p. 175). The material remaining in the stationary phase was rechromatographed with phase system HD. - - -, titration values (defined by left-hand legend to ordinate); o—o radioactivity (defined by right-hand legend to ordinate); •—•, extinction values in the Tschugaeff colour reaction. Carrier: 2 mg cholesterol.

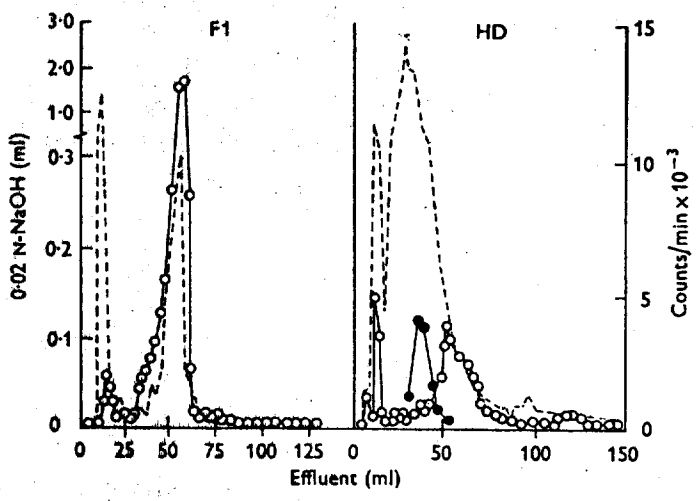

Fig. 7. Separation of unhydrolysed labelled material in Extract (D + E) (see Fig. 1) from faeces 3 days after administration of [24-^{14}C]cholic acid to patient E.L. For explanation see legend to Fig. 6.

Fig. 9, the percentage of labelled 3β isomer is about the same in Fractions (II) of Extracts (A), (B + C) and (D + E), but is less than half of that in the deoxycholic acid band isolated after hydrolysis of the saponifiable labelled products (Fraction (IV)).

As also is seen in Fig. 8, the distribution of the isotope between the different extracts showed that the percentage of isotope in Extracts (D+E) increases from the 2nd to the 4th day after the administration of [24-^{14}C]cholic acid. Parallel with these changes, the percentage of labelled bile acids in Fraction (I) decreased and the percentage of labelled saponifiable derivatives of 3,12-dihydroxycholanic acid increased.

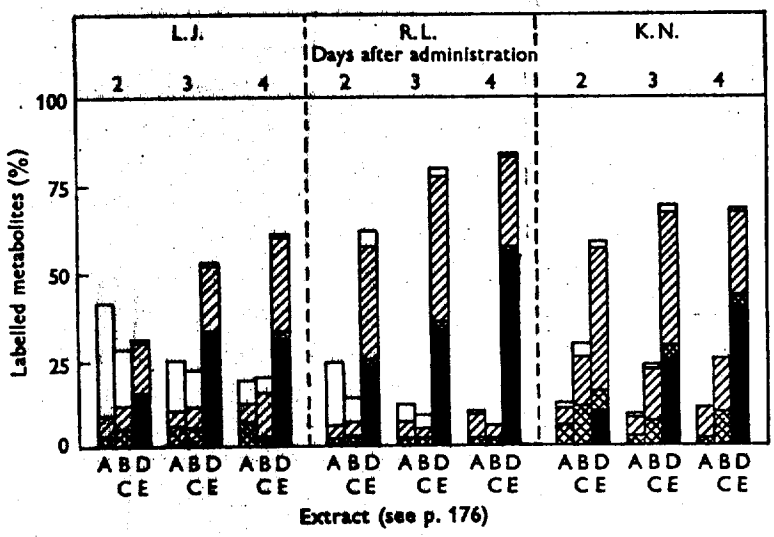

Fig. 8. Distribution, according to extractability and chromatographic behaviour, of labelled metabolites in Fractions I, II, III and IV (see p. 179) of extracts of faeces of patients L.J., R.L. and K.N. after administration of [24-^{14}C]cholic acid. □, Fraction I; ▧, Fraction II; ▩, Fraction III; ■, Fraction IV.

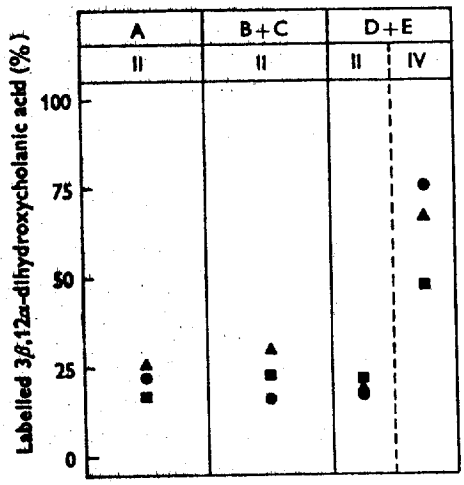

Fig. 9. Percentage of 3β,12α-dihydroxycholanic acid in Fractions II and IV (see p. 179) after chromatographic separation of Extracts A, (B+C) and (D+E) (see Fig. 1) of faeces of patients L.J. (▲), R.L. (●) and K.N. (■) 4 days after administration of [24-^{14}C]cholic acid.

Composition of dialysable and non-dialysable metabolites of [24-^{14}C]cholic acid

The dialysable and non-dialysable labelled products from the experiment illustrated in Table 2 were each chromatographed with phase system F 1. The results showed that all the labelled products in Fractions (I) and (III) and more than 90% of the labelled 3,12-dihydroxycholanic acid in Fraction (II) could be separated from the faecal residue by dialysis. The non-dialysable bile acids consisted of the saponifiable derivates of 3,12-dihydroxycholanic acid and small amounts of 3,12-dihydroxycholanic acid.

DISCUSSION

Extraction methods

Various extraction procedures for faecal bile acids have been described: 80% aqueous ethanol, ethanol, chloroform, methylal/methanol 4:1 and chloroform/methanol 1:1 have been proposed as extraction solvents. The subject has been reviewed by Danielsson (1964). P. Eneroth, K. Hellström & J. Sjövall (1964, to be published) have shown that chloroform/methanol 1:1, but not ethanol, gave complete recovery of isotope from human faeces after administration of labelled bile acids. The main metabolites of cholic acid after hydrolysis of faecal extracts, deoxycholic and 3-hydroxy-12-ketocholanic acids, are as sodium salts very soluble in water. It therefore seemed peculiar that those metabolites had to be extracted with chloroform–methanol.

The investigation now described showed that only 20–30% of the labelled metabolites of cholic acid were extractable with saline and phosphate buffer. Most of the remaining isotope was recovered by acetone extraction, but extraction with chloroform–methanol 1:1 was found necessary for quantitative recovery. One explanation of why faecal bile acids have to be extracted by organic solvents is that 3,12-dihydroxycholanic acid is adsorbed to the residue in a saline homogenate of faeces. Another explanation was found when the different unhydrolysed extracts were analysed by chromatography. The main parts of the labelled products left after the extraction with saline and phosphate buffer were saponifiable derivatives of 3,12-dihydroxycholanic acid. These compounds were found to be less soluble in water.

An intracellular localization of bile acids also could explain the need to extract bile acids with organic solvents. However, deoxycholic acid formed in vitro in subcultures of anaerobic intestinal micro-organisms is present extracellularly (Norman & Shorb, 1962). In addition, 3,12-dihydroxycholanic acid in human faeces is mainly present in a dialysable form, a fact that makes an intracellular localization less likely.

Metabolites of cholic acid

Owing to the limited number of subjects it is impossible to draw conclusions about variations from one individual to another. In all patients studied, deoxycholic acid is the main microbial metabolite of cholic acid. Small amounts of other metabolites were also isolated. These results are in accordance with the findings of other investigators who have studied the nature of the metabolites of cholic acid after hydrolysis of the

primary extracts. There are, however, no reports on the presence of saponifiable derivates of 3,12-dihydroxycholanic acid in human faeces. These compounds are probably formed by the action of microbial enzymes. Labelled products with the same chromatographic behaviour are formed in cultures of human faecal micro-organisms from [24-^{14}C]deoxycholic acid (Norman, 1964, to be published). Investigations on the chemical structure of these compounds are in progress.

General

Human bile contains the conjugates of cholic, deoxycholic and chenodeoxycholic acids. Deoxycholic acid is formed in the intestine by microbial enzymes in the process of enterohepatic circulation. However, other metabolites of cholic and chenodeoxycholic acids, i.e. different 3β-hydroxy isomers and lithocholic acid, do not appear in bile in appreciable amounts. These findings could be explained by differences in absorbability of different bile acid metabolites themselves or by their formation at more distal levels in the intestine where the intestinal capacity to absorb them is reduced.

Borgström (1960) has separated human intestinal contents from the proximal part of the small intestine by centrifugation at high speed (above 45000 *g*) into an oily top layer, a clear subnatant layer and a sediment. The clear subnatant layer contained many different lipids together with the bile acids, which were present as conjugates. In more distal parts of the small intestine and in the colon the microbial transformations of the bile acids change their physical state by the formation of bile acid derivatives which are either poorly soluble in water or easily adsorbed to the residue. Assuming that the bile acids are absorbed only in a water-soluble form, such alterations can influence the absorption of the bile acids. Thus, for an elucidation of factors influencing the removal of bile acids from the enterohepatic circulation, the chemical and physical state of the bile acids in the intestinal contents as well as the ability of the intestinal wall to absorb these compounds should be investigated at various levels in the intestine.

SUMMARY

1. The faecal excretory products of cholic acid have been studied in man by analysing the labelled products after oral administration of [24-^{14}C]cholic acid.

2. Sequential extractions of faeces with saline, phosphate buffer (pH 7·4), acetone and chloroform/methanol 1:1 were performed. Of the labelled metabolites, 20–45% appeared in the aqueous extracts. Most of the remaining isotope was recovered in the acetone extracts, but the last 5–15% required extraction with chloroform/methanol.

3. More than half of the labelled compounds in faecal homogenates were dialysable.

4. Column chromatographic separations of the labelled unhydrolysed metabolites of cholic acid showed that only small amounts of conjugated bile acids and unchanged cholic acid were left. The main metabolites were 3,12-dihydroxycholanic acid and 3-hydroxy-12-ketocholanic acid. At least 25% of the labelled metabolites were present as saponifiable derivatives of 3,12-dihydroxycholanic acid, mainly the 3β isomer.

5. The bile acid composition of the different extracts varied. Labelled compounds more polar than 3,12-dihydroxycholanic acid and 3-hydroxy-12-ketocholanic acids were recovered in the aqueous extracts. 3,12-dihydroxycholanic acid was about equally distributed between the different extracts. The saponifiable derivatives of 3,12-dihydroxycholanic acid were present only in the organic solvent extracts.

6. The non-dialysable bile acids mainly consisted of saponifiable derivatives of 3,12-dihydroxycholanic acid. More than 90% of the 3,12-dihydroxycholanic acid was dialysable.

7. The results show that chemical transformations of cholic acid by intestinal micro-organisms also change the physical state of the bile acids in the intestinal contents by forming derivatives which are either poorly soluble or easily adsorbed to the residue.

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Metabolism of Glycocholic Acid in Man

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Norman, A. 1970: Metabolism of Glycocholic Acid in Man. *Scand. J. Gastroenterol.* 5, 231-236.

Bile from patients given an oral dose of [24-^{13}C] cholyl [1-^{13}C] glycine was extracted and the labelled conjugates chromatographically separated. The results indicate that 43-75% of the remaining [24-^{13}C] labelled bile acids was present as double-labelled glycocholic acid 25-26 hours after administration of the isotope. During the same period, 3-6% of the remaining labelled bile acid had been 7α-dehydroxylated. The glycodeoxycholic acid isolated only contained small amounts of isotope in the glycine compared with that in the steroid nucleus, indicating that unconjugated cholic acid rather than conjugated cholic acid is the main substrate for 7α-dehydroxylation in the intestinal tract.

Key-words: Bile acids; deconjugation

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In man, cholic and chenodeoxycholic acids are the bile acids that are formed primarily from cholesterol, and they are excreted in bile conjugated with taurine or glycine (1). In the duodenum and upper part of the jejunum, the bile acids are present in a conjugated form (4). Since the faecal bile acids are mainly unconjugated (12), an extensive splitting by microorganisms must occur during passage through the intestinal tract. Part of the unconjugated bile acids are probably absorbed, but as only conjugates are seen in bile from normal humans (15), the unconjugated bile acids are conjugated prior to excretion into bile. In addition to splitting of conjugates, intestinal microorganisms are able to carry out various transformations of the steroid nucleus, resulting in formation of secondary bile acids, which take part in the enterohepatic circulation. One of the most important of these transformations in man is 7α-dehydroxylation of cholic acid to form deoxycholic acid (7).

The steatorrhoea in the blind loop syndrome has been ascribed to the influence on fat absorption of an excessive splitting of conjugated bile acids in the duodenum or upper part of the jejunum (6). A varying amount of unconjugated bile acids has been detected in intestinal contents aspirated from the upper jejunum of patients with the stagnant blind loop syndrome (13, 16).

The aim of the present investigation was to investigate the extent of deconjugation and 7α-dehydroxylation of glycocholic acid in humans with an intact enterohepatic circulation. This was achieved by analysing the labelled products in bile after administration of glycocholic acid labelled with ^{13}C in both the bile acid and the amino acid part. The clinical applications of the method have been studied in gastrectomized patients, and are described in a subsequent paper (9).

MATERIAL AND METHODS

Patients. The study includes eight women 45-60 years old, who were admitted to the hospital for cholecystectomy. Cholecystography, 1-2 months previous to admission, had shown that all had a single stone, which filled the gall

Table I. Abbreviations, trivial names, and systematic names of bile acids described

Abbreviation		Trivial name	Systematic name
G	C	[24-^{14}C] cholyl [1-^{14}C] glycine	[24-^{14}C] 3α, 7α, 12α-trihydroxy-5β-cholanoyl [1-^{14}C] glycine
G	C	[24-^{14}C] cholyl glycine	[24-^{14}C] 3α, 7α, 12α-trihydroxy-5β-cholanoyl glycine
G	D	[24-^{14}C] deoxycholyl [1-^{14}C] glycine	[24-^{14}C] 3α, 12α-dihydroxy-5β-cholanoyl [1-^{14}C] glycine
G	D	[24-^{14}C] deoxycholyl glycine	[24-^{14}C] 3α, 12α-dihydroxy-5β-cholanoyl glycine
T	C	[24-^{14}C] cholyl taurine	[24-^{14}C] 3α, 7α, 12α-trihydroxy-5β-cholanoyl taurine
T	D	[24-^{14}C] deoxycholyl taurine	[24-^{14}C] 3α, 12α-dihydroxy-5β-cholanoyl taurine
	C	[24-^{14}C] cholic acid	[24-^{14}C] 3α, 7α, 12α-trihydroxy-5β-cholanoic acid
	D	[24-^{14}C] deoxycholic acid	[24-^{14}C] 3α, 12α-dihydroxy-5β-cholanoic acid
	G	[1-^{14}C] glycine	
	T	taurine	

bladder to only a small extent, and the gallbladder bile showed a normal concentration of x-ray contrast. The patients had no clinical signs of cholecystitis or icterus. They had no abdominal pain during the month before admission.

Labelled bile acids. [24-^{14}C] cholyl [1-^{14}C] glycine and cholyl [1-^{14}C] glycine were synthesized as described earlier (11) from [24-^{14}C] cholic acid or [1-^{14}C] glycine (New England Nuclear Corp., Boston Mass). The specific activity of each of these compounds was 250 μC/mM.

Administration of labelled bile acids. These were given at 8 a. m. to the fasting patients. Six patients received 6 μC of [24-^{14}C] cholyl [1-^{14}C] glycine, while 2 patients received 5 μC cholyl [1-^{14}C] glycine, all administered as the sodium salts dissolved in water. Afterwards, the patients ate the regular hospital food. Gallbladder bile was obtained 25-26 or 73-74 hours after administration of the labelled bile acids, during the operation for cholecystectomy.

Fractionation of labelled compounds in bile. The steps used for the extractions and chromatographic separations of these compounds are outlined in Fig. 1.

Hydrolysis of conjugated bile acids. The material to be hydrolysed was transferred into long test tubes. After evaporation, the residue was dissolved in 1 ml of 20 per cent KOH in ethylene glycol, and the tubes were then inserted into a sandbath for 20 minutes at 220°C (5).

Chromatographic technique. Columns for reversed phase partition chromatography consisted of 4 ml stationary phase supported on 4.5 g of hydrophobic Hyflo Super-Cel, as described earlier (12). The phase systems shown in Table II were used.

The fractions collected from the columns were titrated with 0.02 N NaOH in methanol.

Thin-layer chromatography (TLC) of unconjugated bile acids was performed using the technique and phase systems described by Eneroth (4).

Isotope determination. Aliquots of the extracts were subjected to oxygen-flask combus-

Table II. Phase systems used

	Phase system Cl (ml)	Phase system Fl (ml)
Moving phase	methanol: water 150:150	methanol: water 165:135
Stationary phase	chloroform: isooctanol 15:15	chloroform: heptane 45:45

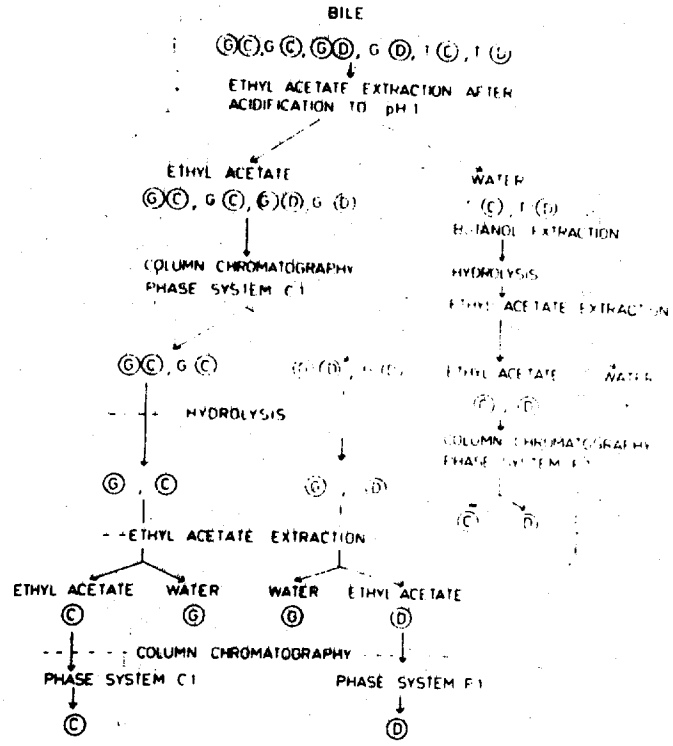

Fig. [illegible] tion and chromatographic separation of labelled bile acids in rats. The abbreviations used are listed in Table I.

tion, and the ^{14}C in the resultant carbon dioxide was determined by liquid scintillation (2). Aliquots of the chromatographic fractions were counted in a Packard Tri-Carb liquid scintillation spectrometer. After TLC, the radioactive spots were located by autoradiography.

Determination of double-labelled glycocholic and glycodeoxycholic acid. Column chromatography with phase system C1 separates glycocholic acid from glycodeoxycholic and glycochenodeoxycholic acids, the latter being eluted in the same fractions. The μmoles of bile acids in the two bands were determined by titration of the chromatographic fractions to enable the c.p.m. per μmoles of conjugated bile acids to be calculated. After hydrolysis the resulting unconjugated bile acids were chromatographically separated, as outlined in Fig. 1, and the c.p.m. per μmole of unconjugated bile acids was then calculated. The ratio of [24-^{14}C] cholyl [1-^{14}C] glycine/[24-^{14}C] cholyl glycine and the ratio [24-^{14}C] deoxycholyl [1-^{14}C] glycine/[24-^{14}C] deoxycholyl glycine could then be calculated.

RESULTS

[24-^{14}C] cholyl [1-^{14}C] glycine. The labelled compounds in bile were extracted and separated as shown in Fig. 1. All isotope in bile was recovered in the ethyl acetate extracts and the butanol extracts. The water phase after the butanol extraction was found to contain no isotope. The ethyl acetate extracts were chromatographically separated, and a representative chromatogram is shown in Fig. 2. Isotope was demonstrated in the glycocholic acid band (25-45 ml effluent), and in the combined glycodeoxycholic and glycochenodeoxycholic acid band (130-200 ml effluent). Less than 0.5 per cent of isotope was eluted in the front band at the places where taurine conjugates should appear. The isolated glycocholic acid always contained isotope both in the steroid and in the glycine moiety, whereas [illegible] isotope could

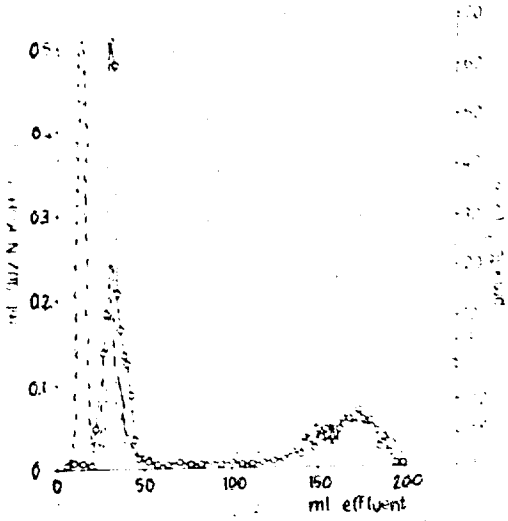

Fig. 2. Column chromatography using phase system C1 of labelled bile acids extracted with ethyl acetate from bile 25 hours after administration of [24-^{14}C] cholyl [1-^{14}C] glycine to patient 1.

phase system C1. After hydrolysis, the unconjugated bile acids can be chromatographically separated using the same phase system. In patients 1-4, less than 0.5 per cent, and in patients 5-6 less than 1.5 per cent of the labelled unconjugated bile acids were recovered at the places of these two metabolites. TLC of aliquots of the fractions showed that the isotope was eluted at places where the known reference compounds of these metabolites were found.

Cholyl [1-^{14}C] glycine. In the two patients given cholyl [1-^{14}C] glycine, the bile obtained at 24 and 26 hours after administration of the isotope was fractionated according to Fig. 1. Labelled compounds were only recovered in the ethyl acetate extracts, and chromatographic separation of these extracts showed that 0.3 and 0.5 per cent of the isotope respectively were eluted at the place of glycodeoxycholic acid, whereas the remaining isotope appeared at the place of glycocholic acid.

be demonstrated in the glycine of glycodeoxycholic acid. The results of analysis of the labelled compounds are summarized in Table III. They indicate that 43-75 per cent of the isotope in the bile acids was present as double-labelled glycocholic acid after 25-26 hours of enterohepatic circulation. After 73-74 hours circulation, only 5-6 per cent of the bile acid conjugates were present as double-labelled glycocholic acid.

The glycine conjugates of 3α, 12α-dihydroxy-7-keto-5β-cholanoic and 3α, 7β, 12α-trihydroxy-5β-cholanoic acid are eluted together with glycocholic acid after chromatography with

DISCUSSION

The main transformations of glycocholic acid shown to occur during the enterohepatic circulation in man are summarized in Fig. 3. The present investigation using double-labelled [24-^{14}C] cholyl [1-^{14}C] glycine showed that an extensive deconjugation occurs normally, during the passage of bile acids through the intestinal tract. The results also indicate that unconjugated cholic acid is the major substrate for 7α-dehydroxylation, since only trace amounts of isotope were recovered in glycine in the glycodeoxycholic acid isolated

Table III. Labelled conjugates in bile after oral administration of [24-^{14}C] cholyl [1-^{14}C] glycine

Patient	Hours after administration	% of [24-^{14}C] isotope isolated from					
		T C*	T D	G C	G C	G D	G D
1	25	16	2	18	60	4	0
2	26	21	2	30	43	4	0
3	26	10	1	18	69	2	0
4	25	8	1	14	75	2	0
5	73	8	14	31	6	41	0
6	74	9	30	14	5	42	0

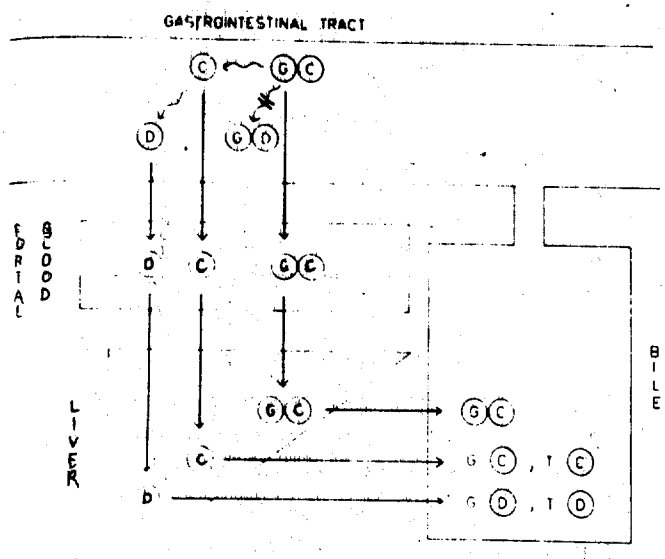

Fig. 3 Reactions shown in the conversion of [24-14C] cholyl [1-14C] glycine during [illegible] hepatic circulation in man. For abbreviations see Table I. reaction catalyzed by microbial enzymes.

The trace amount of isotope in the deoxycholyl [1-14C] glycine that was obtained after administration of cholyl [1-14C] glycine may be explained by the fact that labelled glycine, formed by splitting of glycine conjugates in the intestine, is absorbed and used in de novo conjugation of bile acids with glycine in the liver. A minor transformation of cholic acid into 3α, 12α-dihydroxy-7-keto-5β-cholanoic acid, and 3α, 7β, 12α-trihydroxy-5β-cholanoic acid occurs, but this was quantitatively unimportant when compared with the main reactions, i.e. deconjugation and 7α-dehydroxylation.

In vitro splitting of conjugates is a very common property of both aerobic and strictly anaerobic microorganisms (3, 8). Human jejunal contents normally contain microorganisms capable of splitting conjugates in vitro. The gastric acidity, emptying of the gallbladder, diet and other conditions that may change the transit time of intestinal contents, can influence the gastrointestinal microflora, and thus exert a secondary influence on the deconjugation of conjugated bile acids in the gastrointestinal tract. It is therefore reasonable to assume that the extent to which glycine conjugates are split can vary greatly during enterohepatic circulation under normal conditions. The extent to which taurocholic acid is split need not be the same as that observed for glycocholic acid. It has been shown that intestinal microorganisms vary in their capacity to split turine and glycine conjugates in vitro (8). Furthermore, the taurine conjugates seem to be more extensively split than glycine conjugates, in the intestinal contents of patients with the stagnant-loop syndrome (16).

ACKNOWLEDGEMENTS

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Thin-layer Chromatography of Bile Acids on Lipophilic Sephadex

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In a previous paper the preparation of a methylated Sephadex derivative was described.[1] As thin-layer gel filtration is now an established technique,[2,3] the possibility of using methylated Sephadex in thin-layer chromatography (TLC) of lipids has been briefly investigated in this laboratory. Because of the volatility of most organic solvents, a closed developing chamber had to be used, in which the thin-layer plate also was kept during the sample application.

Experimental. Thin-layer plates are prepared on glass-plates, 5 × 20 cm, in the following way: 5 g of Sephadex G-25 superfine (Pharmacia, Uppsala, Sweden), methylated as described previously,[1] is mixed with 25 ml of chloroform or dioxane. The slurry is poured on the glass plate and spread into a thin layer with a glass rod. A proper thickness of the layer is obtained by having two stainless steel wires, 0.5 mm diameter, placed across the plate, parallel to and near the two long sides. The glass rod is pressed against the wires while the slurry is spread with a single movement. The plate is then allowed to dry for a few seconds until the gel surface seems dry but still swollen. The stainless steel wires are removed and the plate

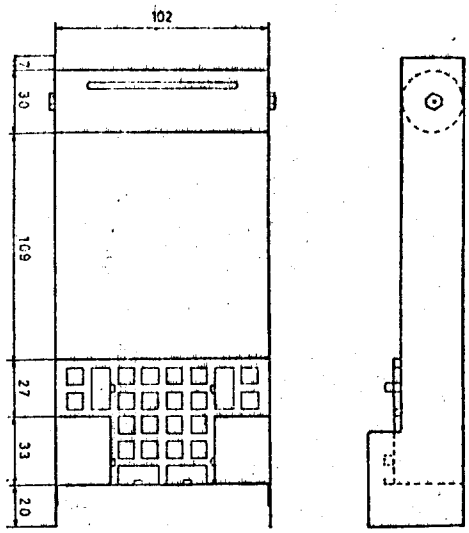

Fig. 1. Stainless steel frame, see text.

placed into the stainless steel frame shown in Fig. 1. At the top of the frame is a cylindrical solvent reservoir filled with the solvent to be used in the chromatography. The reservoir has a narrow slit opening, through which a filter paper strip, serving as a solvent bridge, is inserted. The other end of the strip is folded around the upper part of the thin-layer plate and kept in place with a short piece of teflon tubing, which has been slit open lengthwise. The frame is fitted into a glass jar, 275 × 120 × 55 mm. The front of the jar is covered with a glass plate, held in place with stainless steel clamps. The thin-layer plate is equilibrated for 2 h with descending solvent. The jar is then tilted so that the plate lies in a horizontal position, and the samples are injected with a Hamilton syringe (in 1 – 5 μl) onto the starting line through three silicone rubber stoppers fitted into three 6 mm holes in the covering glass plate. The jar is tilted to the appropriate angle (30° – 80° depending on the solvent used) and the plate is developed by a descending technique. Immediately after the development the damp plate is covered with a filter paper just soaked in chloroform. This paper is pressed against the moist gel layer by means of another glass plate. The paper with the transferred lipids is then sprayed with an appropriate reagent, 5 % phosphomolybdic acid in ethanol being suitable for the detection of the bile acids investigated in this experiment.

Results and discussion. Because of the volatility of the solvents used, exact information on the procedure of preparing the plates and transferring the lipids can only be gained by experience. It might be of advantage to check the transfer onto the filter paper by using a coloured substance. As the dry gel surface has a cracked appearance and is extremely fragile, the plate cannot be sprayed directly. By using the transfer technique for revealing the spots, the methylated Sephadex might also be collected and used in another experiment.

Some of the results are given in Table 1 and Fig. 2. As the molecular weights of the bile acids are relatively similar, no effort has been made to demonstrate the presence of a gel filtration effect. Rather, the solvent systems were designed to give a pronounced liquid-gel partition or adsorption effect. Thus, methylated Sephadex is known to swell strongly in solvents of medium polarity, such as dioxane and chloroform, whereas aliphatic hydrocarbons are not incorporated in the gel. A mixture of chloroform and heptane will give a straight phase liquid-gel partition system. Further-

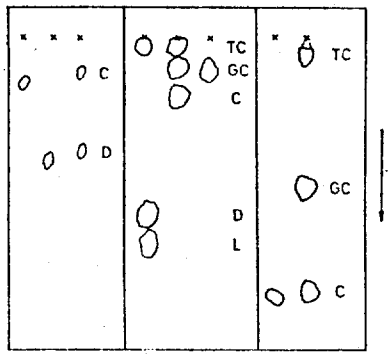

Fig. 2. Descending TLC of bile acids on methylated Sephadex G-25, superfine. *Left chromatogram.* Solvent system: chloroform 32, heptane 16, acetic acid 1 (v/v/v). Developed 2.5 h, angle 80°. *Middle chromatogram.* Solvent system: chloroform 66, 2,2,4-trimethylpentane 33, acetic acid 1. Developed 0.75 h, angle 80°. *Right chromatogram.* Solvent system: dioxane 24, 2,2,4-trimethylpentane 24, acetic acid 1. Developed 3.5 h, angle 30°. The samples, cholic (C), deoxycholic (D), lithocholic (L), glycocholic (GC) and taurocholic (TC) acids were applied in 4 μl of solvent. 4 μg of each substance were used.

Table 1. TLC mobilities of bile acids on methylated Sephadex G-25 superfine in different solvent systems.

Solvent	(v/v)	Angle[a]	Time h.	TC[b]	Distance in mm from origin GC	C	D	L
Chloroform Heptane	2 1	80°	10.5	—	—	17	67	—
Chloroform Heptane Acetic acid	33 16 1	80°	2.5	—	—	16	50	—
Chloroform Heptane Acetic acid	39 10 1	80°	0.9	—	12	23	43	58
Chloroform TMP[c] Acetic acid	66 33 1	80°	1.0	4	13	25	76	89
Chloroform TMP Acetic acid	66 33 1	80°	4.0	13	45	89	—	—
Dioxane TMP Acetic aci	25 25 1	30°	3.5	16	65	109	—	—

[a] Angle between the thin-layer plate and the horizontal plane.
[b] TC = taurocholic acid; GC = glycocholic acid; C = cholic acid; D = deoxycholic acid; L = lithocholic acid.
[c] TMP = 2,2,4-trimethylpentane.

more, the presence of adsorption to the gel network is indicated by the greatly increased mobility of polar compounds when small amounts of acetic acid are added to neutral solvent systems.

Solvent systems giving efficient separations of bile acids and bile acid conjugates in TLC on silica gel have been given by Enerothi[4] and Hofman.[5] TLC on lipophilic Sephadex does not seem to offer any advantages over silica gel TLC as far as separation of bile acid isomers is concerned. Nevertheless, TLC on lipophilic Sephadex might be a useful complementary technique. Thus, free bile acids can be chromatographed in neutral solvent systems without tailing. The inert nature of the methylated Sephadex makes it possible to separate labile compounds. Column experiments indicate that recovery of compounds from the thin-layers should be complete. Finally it is suggested that TLC on lipophilic Sephadex could be used as a method for rapid screening of suitable solvent systems to be used in column chromatography.

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Enterohepatic Circulation of Cholic Acid and Cholesterol Metabolism in the Rat[1]

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ABSTRACT

A role for cholic acid in the regulation of body cholesterol levels is emphasized by the following experiment and findings. Fifteen rats with biliary and duodenal fistulae were divided into three groups. 4-^{14}C-cholesterol was admistered intravenously to all animals on day 1. Two groups received a duodenal infusion of either sodium taurocholate or of cholesterol at the same time and thereafter daily. Bile samples were collected daily for 15 consecutive days. Cholic, chenodeoxycholic acid and cholesterol were the major labeled compounds in the bile. Labeled cholic acid disappeared from the bile of control animals after day 8 while it persisted in the group receiving unlabeled cholic acid up to day 15. The decrease of specific radioactivity of labeled biliary cholic acid in the rats receiving unlabeled cholesterol corresponded to that of control animals. A significant increase in the concentration of cholesterol was found in the plasma of animals receiving cholic acid and in the liver of those receiving unlabeled cholesterol.

INTRODUCTION

The processes leading to the formation of cholesterol are well explored (1) but less is known about the mechanisms regulating the rate of its synthesis and degradation. Working with an in vitro system, Siperstein and Fagan (2) demonstrated inhibition of β-hydroxy-β-methylglutaryl reductase in the liver of rats kept for a short time on a diet supplemented with small amounts of cholesterol, thus confirming the earlier observations of Gould and Popjak (3), Bucher et al. (4) and Siperstein and Guest (5). Identical mechanisms have been observed in the liver of other mammals including man (6), but an inhibition of the regularly occurring extrahepatic mevalonate synthesis (7) by dietary cholesterol has not been demonstrated in any species. It has been proposed that cholesterol can inhibit the reduction of β-hydroxy-β-methylglutarate in the liver only after cholesterol passes through the intestinal wall and forms a specific cholesterol lipo-protein complex since this inhibition cannot be shown after parenteral administration of cholesterol or if body cholesterol is elevated following increased endogenous production of the sterol (6).

Due to the aforementioned limitations of the control of cholesterol biosynthesis at the step of the conversion of β-hydroxy-β-methylglutarate to mevalonate, it does not seem likely that this control is the sole mechanism regulating concentration and distribution of cholesterol in plasma and tissues. Cholesterol content of plasma and tissues appears to depend upon the balance between the intake (exogenous) and biosynthesis on one side, and degradation and excretion on the other side. Only a small portion of cholesterol is excreted unchanged and a negligible amount is transformed into steroid hormones. Bile acids are the principal end products of cholesterol metabolism (8). The loss of these acids via the intestinal tract will increase their formation from cholesterol and as a consequence plasma and tissue cholesterol will decrease (9). A relation between the rate of formation of bile acids and cholesterol levels in plasma and tissues exists in several mammalian species (10) and studies in vitro indicate a selective effect of different bile acids on cholesterol metabolism (11). Experimental data reported by Dietschy (12) suggest that bile salts affect cholesterogenesis in the small bowel by, presumably, inhibiting the formation of mevalonic acid. It has been found that following intravenous injection of 4-^{14}C-cholesterol to guinea pigs with biliary fistulae, labeled cholic (3α,7α,12α-trihydroxycholanic) acid appears slowly in the bile and undergoes a prolonged enterohepatic circulation compared with the relatively rapid excretion of labeled chenodeoxycholic (3α,7α-dihydroxycholanic) and 7-ketolithocholic (3α-hydroxy,7-ketocholanic) acids observed in such animals (13). The half-life of labeled cholic acid is prolonged and its formation from cholesterol reduced in patients with hypercholesterolemia (14). No elevation of plasma cholic acid can, however, be measured in this patient category (15).

The present study concerns the relation be-

[1]A preliminary report was presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1965.

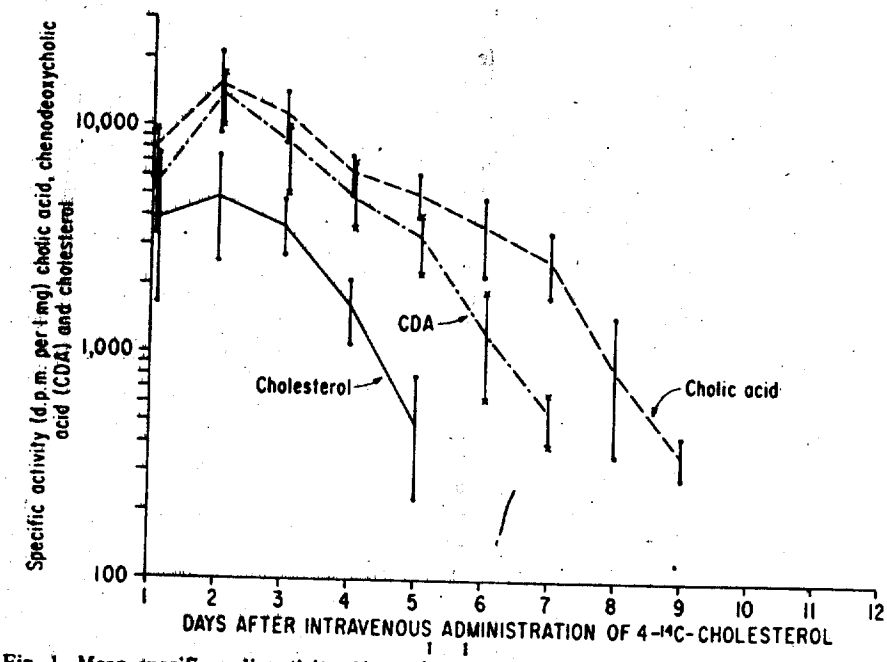

Fig. 1. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in control animals given 4-^{14}C-cholesterol intravenously on day 1. ● ●, ○ ○, x x, range of values.

tween concentration of cholic acid in enterohepatic circulation and the rate of conversion of cholesterol into bile acids in the rat.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats weighing 300-400 g were used. Animals were fed Purina rat chow and water ad lib. Biliary fistulae were performed in a manner similar to that previously described in guinea pigs (13), but the surgical procedure was modified because of the smaller caliber of the bile duct and the absence of the gallbladder in the rat. One polyethylene catheter (PE-10) was inserted into the proximal and another into the distal portion of the common bile duct which was then ligated and cut between the two sites of the insertion of the catheters. The free ends of the catheters were passed under the skin to the back of the animal, pulled through a cutaneous opening and connected to allow enterohepatic circulation. Animals showing loss of weight, bacterial growth in cultures of bile, obstruction of catheters by biliary precipitates or any gross or microscopic evidence of disease at the post mortem examinations were excluded from this study.

Fifteen healthy animals were divided into three equal groups. Each of the 15 animals received 0.1 μc (6.6 μg) 4-^{14}C-cholesterol, dissolved in 0.2 ml 40% ethanolic saline per 100 g body weight into the tail vein 48 hr after the completion of the surgical procedure. The animals of two groups received at the same time and thereafter daily a duodenal infusion of 2 mg sodium taurocholate or 2 mg cholesterol respectively, dissolved or suspended in 2 ml 12.5% ethanolic saline per 100 g body weight. Suspensions of cholesterol were very fine and remained stable for several hours. The five control rats received only 12.5% ethanolic saline by duodenal infusion. All infusions were performed with a manually operated syringe over a 10 min period. Continuous enterohepatic circulation was maintained through the experiment except for short daily interruptions between 8 and 9 a.m. During this time bile samples of about 0.5 ml each were collected. Thus the loss of bile throughout the experiment was minimal. The animals were killed with a neck clamp 15 days following the injection of labeled cholesterol. Blood was withdrawn by cardiac puncture, and liver, kidneys and aorta were collected. Bile, plasma and tissues were frozen immediately and kept frozen until analyzed.

Bile acids were determined in the bile essentially as previously described (13,16). All samples were diluted to 50 ml with 1N sodium hydroxide, boiled 5 hr, acidified to pH 1 with conc. hydrochloric acid and extracted 3 times with 5 volumes ethyl ether each time. The pooled extracts were washed with water until

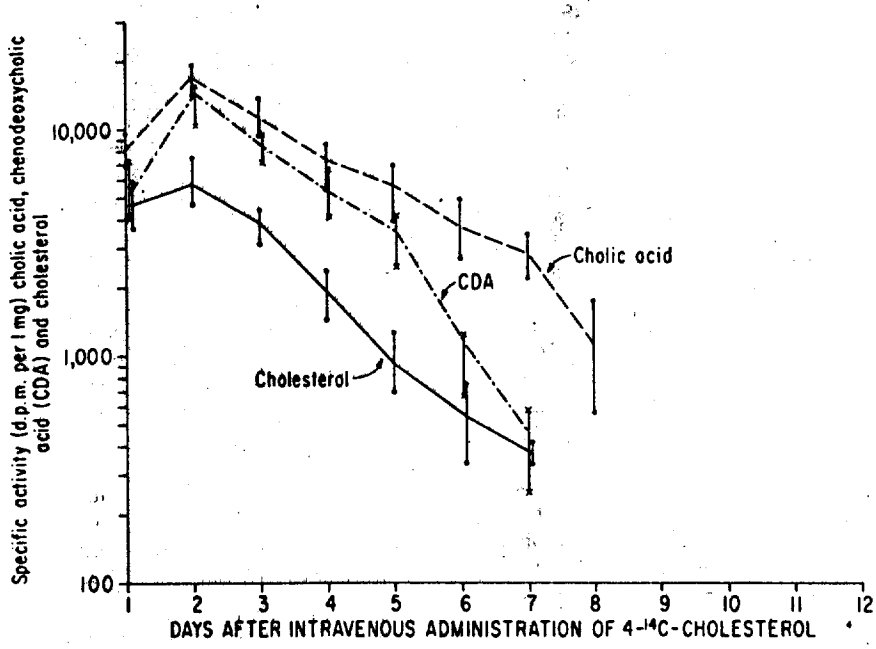

Fig. 2. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in animals receiving unlabeled cholesterol by duodenal infusion. The symbols are the same as in Figure 1.

free of hydrochloric acid and the ethyl ether evaporated to dryness. The content of free bile acids in these samples was evaluated by paper chromatography (17). Chromatographic mobility of individual bile acids in the extract was compared to that of standard compounds, chromatographed on duplicate strips and sprayed with 10% phosphomolybdic acid in 70% ethanol following chromatography. The paper strips used for biological samples were scanned for radioactivity. The radioactive zones of these strips corresponding to phosphomolybdic acid positive spots on the standard strips were eluted with chloroform-methanol (1:1) and the eluate evaporated to dryness. The residue was treated with 65% sulfuric acid and the resulting chromogen measured by spectrophotometry (18). The cholesterol content of bile, plasma and homogenized tissues was determined spectrophotometrically following previously described extraction and purification procedures (19).

In order to determine purity of the radioactive products, the isolated bile acids and cholesterol were mixed with authentic compounds and crystallized several times: cholic acid from aqueous methanol, chenodeoxycholic acid from mixture of ethyl acetate and petroleum ether, cholesterol from acetone and from aqueous methanol. Specific activity (d.p.m./mg) remained constant through repeated crystallizations. Radioactivity of the samples was measured in a scintillation counter.

RESULTS

Isolated ^{14}C-cholic and ^{14}C-chenodeoxycholic acid contained 80-95% of the total radioactivity present in the bile while 10-20% of the radioactivity in the biliary samples chromatographed like cholesterol. No other labeled material was present in sufficient amount to be identified. Biliary excretion of all labeled compounds determined (cholic, chenodeoxycholic acid, cholesterol) was most abundant in all animal groups on the second day after the intravenous admistration of 4-^{14}C-cholesterol. The data are presented in Figures 1-3 as specific activity (d.p.m./mg) of cholic, chenodeoxycholic acid and cholesterol isolated from bile.

Specific radioactivity of cholic and chenodeoxycholic acid in control animals (Fig. 1) decreased rapidly following the admistration of 4-^{14}C-cholesterol; labeled cholic acid disappeared from the bile after the ninth day and labeled chenodeoxycholic acid after the seventh day. Specific radioactivity of biliary cholesterol in control animals was considerably lower than that of the two bile acids. The last samples containing labeled cholesterol were collected on the fifth day after the injection of 4-^{14}C-cholesterol.

Specific radioactivity of cholic acid, chenodeoxycholic acid and cholesterol in the group receiving duodenal infusion of unlabeled cholesterol (Fig. 2) did not differ significantly from

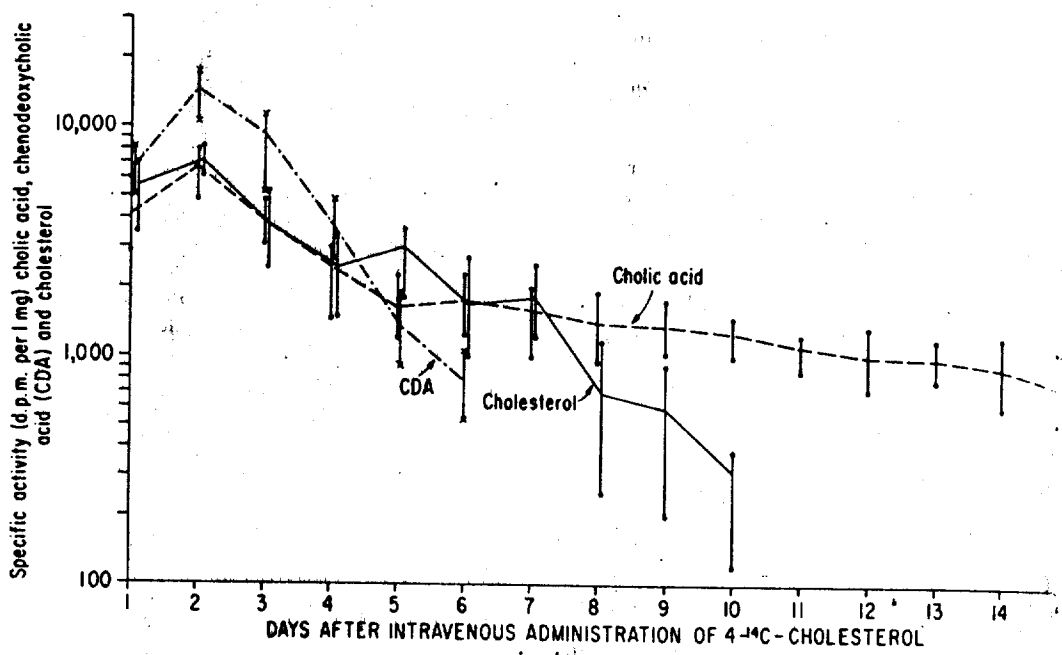

Fig. 3. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in animals receiving unlabeled cholic acid by duodenal infusion. The symbols are the same as in Figure 1.

that observed in the control group.

In the animals receiving non-labeled cholic acid by duodenal infusion (Fig. 3) the daily excretion of labeled cholic acid was never as high as in the control group. Its specific radioactivity decreased rather rapidly until the fifth day of the experiment, thereafter the decrease per day was very moderate. Labeled cholic acid persisted in the bile of this group until the 15th day following the administration of labeled cholesterol. This was the only group where labeled cholic acid could be measured nine days after the administration of 4-^{14}C-cholesterol. The decrease of specific radioactivity of chenodeoxycholic acid in this group was practically identical to that in the control group. The specific radioactivity of cholesterol in the bile of animals with supplement of cholic acid was initially higher than in the other two groups but samples obtained after the 10th day contained only small and quite variable amounts of labeled cholesterol.

Cholesterol concentrations in plasma, liver, kidneys and aorta are listed in Table I. A significant increase was noted in the plasma of the group receiving cholic acid and in the liver of the group receiving unlabeled cholesterol.

DISCUSSION

The rate of formation of bile acids from cholesterol is inversely proportion to their concentration in the enterohepatic system (20). The present study demonstrates a delay of conversion of exogenous cholesterol to cholic acid, a prolonged biliary excretion of the formed cholic acid and elevated plasma levels of cholesterol when the amount of cholic acid in enterohepatic circulation is increased. No changes in the extent of conversion of labeled cholesterol to cholic acid or in plasma concentrations of cholesterol were seen in the animals receiving daily supplements of unlabeled cholesterol. Increase of hepatic cholesterol in this group may be due to an accumulation of dietary cholesterol which could not be compensated by suppression of hepatic biosynthesis demonstrated by Siperstein and Fagan (2). This control of cholesterol biosynthesis operates by inhibiting the reduction of β-hydroxy-β-methylglutarate to mevalonate in the liver and it is triggered by high ingestion of cholesterol. It is also known that this mechanism functions only when cholesterol is absorbed via the intestinal tract (6).

A role of cholic acid concerning physiologic or pathologic regulation of body cholesterol levels or both has been shown in mice (10) and is also suggested by the prolonged enterohepatic cycling of this compound in the guinea pig (13). Dietschy's (12) and present data indicate that this is also the case in the rat. The changes observed in animals with increased amount of cholic acid in the enterohepatic cir-

TABLE I

Plasma and Tissue Concentration of Cholesterol in Control Animals and After 15 Days of Daily Duodenal Administration of Cholesterol or Cholic Acid

Experimental group	Plasma mg/100 ml	Liver g/100 g	Kidneys g/100 g	Aorta g/100 g
Control	73.1 ± 8.6[a]	0.38 ± 0.03	0.41 ± 0.04	0.23 ± 0.01
Cholesterol	71.1 ± 10.2	0.69 ± 0.04	0.44 ± 0.07	0.29 ± 0.02
Cholic Acid	103.0 ± 11.2	0.36 ± 0.06	0.44 ± 0.09	0.25 ± 0.06

[a]Mean value ± 1 SD.

culation (Fig. 3) strongly suggest reduction of metabolic transformation of cholesterol to cholic acid and could serve as experimental models for diseases associated with hypercholesterolemia. Similar conditions exist in rats treated with intestinal antibacterial chemotherapeutics (21) and such animals show prolonged excretion of labeled cholic acid in feces (22).

The cholic acid molecule is normally dehydroxylated at the 7 position in the sterol nucleus by the action of bacteria in the intestinal tract of the rat. It is thus transformed into deoxycholic (3α,12α-dihydroxycholanic) acid. The compound is reabsorbed in this latter form and rehydroxylated in the liver before it is excreted (8). When animals are treated with antibacterial chemotherapeutics, intestinal bacterial growth is retarded, bacterial action is less effective or even abolished and dehydroxylation may not occur. In such case cholic acid is reabsorbed unchanged and possibly in larger quantity than its dihydroxy derivative because it is more polar. This sequence of events may explain the prolonged enterohepatic circulation of cholic acid associated with a possible alteration of the intestinal bacterial flora. Prolonged enterohepatic circulation of cholic acid is also observed in rats receiving cholic acid supplements (this study) and in patients with hypercholesterolemia (14). An excessive production of cholic acid by the liver may be responsible for the genesis of non-dietary hypercholesterolemia in these patients (14). Also, changes in the genetic control of cholesterol biosynthesis may be a contributory factor (23). High cholesterol levels in tissues and plasma may be followed by enhanced formation of bile acids. Dihydroxy acids may be rapidly excreted via the intestinal tract while cholic acid may be reabsorbed in large quantities and inhibit its own synthesis from cholesterol. Such inhibition may moreover lead to abnormal accumulation of cholesterol because its main path of excretion is blocked.

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ACTION OF BILE SALTS ON THE METABOLISM AND TRANSPORT OF WATER-SOLUBLE NUTRIENTS BY PERFUSED RAT JEJUNUM *IN VITRO**

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SUMMARY

The highly purified, crystalline conjugated bile salts glycocholate and taurocholate at 8 mM did not affect: (1) the transport of glucose, sorbose, urea, lysine, leucine, acetate and sodium across perfused segments of rat jejunum; (2) the incorporation of leucine and lysine into protein, or of acetate into lipid by perfused jejunal segments; or (3) the uptake of oxygen and the conversion of glucose into triglyceride by jejunal rings. However, they did inhibit adenosine triphosphatase activity of jejunal mucosal homogenates and brush border preparations, and at 4 mM significantly decreased the P:O ratios of respiring liver mitochondria. Deoxycholate, a common contaminant of conjugated bile salt preparations, at 0.4 mM inhibited the transport of all substances examined, except sorbose, and depressed the other metabolic functions studied. Cholate was a weaker inhibitor than deoxycholate. Long-chain fatty acids, another common contaminant of bile salt preparations, caused an increased incorporation of glucose into triglyceride.

Thus previous reports that conjugated bile salts stimulate or inhibit the absorption or intracellular metabolism of water-soluble nutrients by the gut may well have resulted from contamination of the bile salt preparations with deoxycholate or fatty acids.

INTRODUCTION

The important role of bile in the intestinal absorption of lipids has generally been attributed to the action of the constituent bile salts in stimulating the hydrolysis of triglycerides and in solubilizing their breakdown products[1,2]. In addition, bile salts enhance the uptake and cleavage of β-carotene by intestinal slices[3], the uptake and esterification of palmitate[4] and oleate[5], the absorption of cholesterol[6,7] and the uptake of the fat-soluble vitamins D (ref. 8) and K (ref. 9). Some lipid-cleaving enzymes are also affected by bile salts. The activities of pancreatic lipase[10], cholesterol esterase[11]

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and pancreatic vitamin A esterase[12] are increased by bile salts, and the former two enzymes are stabilized by bile salts as well[11,13,14].

In recent years conjugated bile acids have also been reported to influence the intestinal transport and intracellular metabolism of water-soluble metabolites. Conjugated bile salts (usually glycocholate or taurocholate) have been reported to inhibit the absorption and transport of glucose by jejunal segments under some conditions[16–18], to enhance it under others[17] or to be without effect[4]. In addition conjugated bile salts have been reported to inhibit the transport of acetate, leucine, sodium, sorbose and urea by the jejunum[16,19]. Although intestinal ATPase activity, which is presumably linked to the active transport of some of these substances[20], is inhibited in homogenate systems by taurocholate and glycocholate[19], the concentration of ATP in intestinal cells is reduced rather than enhanced by taurocholate and glycocholate[18,21].

Conjugated bile salts have also been reported to inhibit the oxygen uptake of jejunal rings[19], to inhibit the incorporation of leucine into protein and of acetate into lipid (non-saponifiable fraction) by perfused jejunal segments[16] and to increase the incorporation of glucose into triglyceride glycerol by jejunal rings[4,22]. Also glycocholate and taurocholate are reported to decrease the P:O ratios of respiring liver mitochondria[23] and of intestinal homogenates[24].

In view of these many unrelated and often disparate reports, we hoped to develop a unifying hypothesis which might rationalize the various effects of conjugated bile acids on the uptake and intracellular metabolism of water-soluble metabolites by the intestinal mucosa. In using extremely pure taurocholate and glycocholate, it soon became apparent that many of the reported effects of conjugated bile acid on water-soluble nutrients could not be duplicated. In the present paper we have compared the effects on intestinal metabolism of commercial bile salt preparations and of pure crystalline products, and have concluded that the majority of these effects are not caused by the conjugated bile salts themselves, but rather by small amounts of deoxycholate or fatty acids present in the preparations of conjugated bile salts.

MATERIALS AND METHODS

Radioactive substrates

D-[$^{14}C_6$]glucose (Calbiochem), D,L-[2-^{14}C]leucine, D,L-[2-^{14}C]lysine and ^{22}NaCl (Volk Radiochemical Co.) and sodium [1-^{14}C]acetate (New England Nuclear Corp.) were used without further purification.

Reagents and solvents

Reagents and solvents other than those involved in the preparation and purification of bile salts were obtained as reagent quality materials when possible and were used without further purification. In all experiments glucose was added to the incubation or perfusion media as a solid (Dextrose anhydrous, Baker Analyzed Reagent) less than 1 h before incubations were started.

Bile acids

Cholic and deoxycholic acids (Nutritional Biochemicals Corp.) were purified by HOFMANN's procedure[25], which was modified to include a 2-min period of refluxing the

bile acid with 5 g of activated charcoal (Norit A) in 100 ml of ethanol. Cholic acid was recrystallized twice and deoxycholic acid once. The glycine and taurine derivatives of cholic acid were synthesized as the sodium salts by the method of NORMAN[26]. Sodium glycocholate was recrystallized once and sodium taurocholate at least six times. Cholic and deoxycholic acids were converted to the sodium salts by dissolving in an equivalent amount of NaOH solution. Also commercial preparations of sodium glycocholate (Pfanstiehl, Pure) and sodium taurocholate (Sigma, Grade II) were used in many experiments without further purification. Cholate and deoxycholate were usually added to the incubation or perfusion media as 0.1 M stock solutions. Glycocholate and taurocholate were added as the solids less than 1 h before the incubation was started.

The melting point ranges of recrystallized cholic and deoxycholic acids and the synthesized glycocholate and taurocholate agreed with those in the literature[26,27]. The constituent bile acids present in several bile salt preparations were determined by thin-layer chromatography on silicic acid[28]. Cholic and deoxycholic acids contained only faint traces of contaminating bile acids. No impurity was detected in the synthetic taurocholate and only faint traces of cholate in the synthetic glycocholate. On the other hand, each commercial preparation of glycocholate or taurocholate showed at least six component bile acids by thin-layer chromatography. Both contained taurocholate, glycocholate and taurodeoxycholate as major components, and cholate and deoxycholate as minor components. Other commercial preparations which were tested were similarly heterogeneous*.

The commercial preparations of taurocholate and glycocholate were analyzed for contaminating lipids by the methods given in the next section. Both contained faint traces of triglycerides, cholesterol and esterified cholesterol. Commercial taurocholate also contained 1.4% (by wt.) of fatty acids, of which 34% was palmitic, 12% stearic, 20% oleic and 25% linoleic acids. The commercial glycocholate contained 4.5% (by wt.) of fatty acids, of which 50% was palmitic, 19% stearic and 23% oleic acids.

Assay of lipid contaminants in commercial bile salt preparations

The lipid contaminants were extracted with hexane from an acidified ethanol–water solution of the bile salt. An aliquot of the water-washed hexane extract was separated by thin-layer chromatography according to the procedure of BROWN AND JOHNSTON[29] in which *n*-hexane–ether–acetic acid (90:10:1, v/v/v) was used as the developing solvent. The lipids present in the fatty acid spot and in the remainder of the developed area were each eluted with acidified ethanol–hexane and the hexane solutions were washed with water.

Aliquots of the washed hexane solutions of the fatty acids and of the total lipids, together with appropriate blanks, were titrated with aqueous 0.100 M NaOH (thymol blue end-point) in the presence of sufficient isopropanol to give a single phase during the titration. Other aliquots of the washed hexane solutions of the fatty acids and of the total lipids were oxidized with the permanganate–periodate oxidant of LEMIEUX AND RUDLOFF[30].

Suitable aliquots of the extracted fatty acids and total lipids, both before and

* T. M. PARKINSON, unpublished observations.

after oxidation with permanganate, were methylated by treatment with diazomethane in ether–methanol (90:10, v/v)[31].

The methyl esters of the fatty acids were separated on a 4-ft column of 3% SE-30 methyl silicone rubber gum on 100 to 120 mesh Gas-Chrom P (Applied Science Laboratories, Inc.). The instrument used was a Model 600 Research Specialties Co. gas chromatograph.

Liquid-scintillation spectrometry

Aqueous samples (1 ml) were added directly to 15 ml of toluene–ethanol scintillation fluid, which consisted of 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxyazolyl)-benzene and 4 g of 2,5-diphenyloxazole dissolved in a solution of 573 ml of ethanol and 426 ml of toluene. Well-mixed samples were counted in a Tri-Carb liquid-scintillation spectrometer (Packard Instrument Co.). Lipid samples were evaporated to dryness in a counting vial, and the residue was dissolved in 15 ml of toluene–ethanol scintillation fluid. BRAY's scintillation fluid[32] was used for counting lipids which had been separated by thin-layer chromatography. Protein specimens were suspended in a 3% suspension of Cab-O-Sil (Packard Instrument Co.) in toluene–ethanol scintillation fluid by means of a Ten Broeck tissue grinder, and the suspension was counted. Counts were corrected for background and for quenching when necessary.

Colorimetric analyses

Glucose analysis was carried out by a slight modification of the method of SEIFTER *et al.*[33]. The resorcinol method of ROE[34], originally developed for the analysis of fructose, was used for the determination of sorbose. Urea was determined by the procedure of RICHTER AND LAPOINTE[35]. Inorganic phosphate was determined by the GOMORI modification[36] of the Fiske and SubbaRow method. The biuret method of GORNALL, BARDAWILL AND DAVID[37] was used for protein determination.

Histological technique

Selected intestinal segments were flushed out and then fixed for at least 18 h in a 10% formalin–1% $CaCl_2$ solution[38]. Small pieces (2 to 3 mm long) of fixed tissue were dehydrated at 56° for 4 h in a 1:1 mixture of Carbowax 4000 and Carbowax 1590 (Union Carbide Chemicals Co.), were embedded in Carbowax and were then cooled in a refrigerator for 30 min. The hardened tissue block was wrapped in Saran wrap and stored for future use, or immediately trimmed and sectioned on a Spencer "820" microtome. The sections were floated on an aqueous solution containing 0.02% potassium dichromate and 0.02% gelatin, picked up on glass microscope slides, dried at room temperature and stained with Harris' hematoxylin and aqueous eosin Y.

Experimental animals; securing of tissues

Male albino rats (Rolfsmeyer Farm, Madison, Wisc.), weighing 150–300 g, were maintained on commercial rat diets and were fasted 16–24 h prior to use.

The animals were anesthetized with ether and about 15 cm of the upper jejunum adjacent to the duodenum were removed through a ventral midline incision. The excised tissue was rinsed, and the intestinal contents were flushed out with 0.9% NaCl. The washed jejunal segments were used as a source of mucosa for ATPase experiments, as a source of everted jejunal rings for the measurement of oxygen uptake and of the

incorporation of glucose into lipids, and as a source of the 6-cm segments used with the perfusion apparatus. Liver mitochondria were prepared by the technique of LEE AND WHITEHOUSE[23], except that nuclei and cell debris were removed by centrifugation for 10 min at 1000 × g.

Perfusion and transport studies

The intestinal perfusion apparatus of SMYTH AND WHALER[39], modified by the addition of a side arm to the serosal fluid reservoir to facilitate sampling, was used. A 6-cm segment of the upper jejunum immediately adjacent to the duodenum was quickly attached to the apparatus and immersed in 35 ml of serosal fluid. Within 3 min after removal of the segment from the animal, the perfusion fluid (25 ml) was added and flow was started. Krebs–Ringer bicarbonate (pH 7.4) equilibrated with 95 % O_2–5 % CO_2 (see ref. 15) was used for both serosal and perfusion fluids, and substrates, inhibitors and bile salts were added to the perfusion fluid only. When substrates other than glucose were used, however, 250 mg % glucose was present in both perfusion and serosal fluids. Unless otherwise stated, perfusions were carried out at 37° for a period of 1 h. Aliquots of the serosal fluid, and occasionally of the perfusion fluid, were removed at various time intervals after the start of the perfusion. Increase in the concentration of a substance in the serosal fluid, usually during the 30 to 60-min period of perfusion, was taken as a measure of its transport across the gut section.

Isotopic tracers were used for measuring the transport of L-lysine, L-leucine, acetate and sodium by incorporating 10^6 disintegrations/min of D,L-[2-^{14}C]lysine, D,L-[2-^{14}C]leucine, sodium [1-^{14}C]acetate or ^{22}NaCl into the perfusion fluid.

Measurement of the incorporation of lysine and leucine into protein

After perfusion for 60 min at 37° with Krebs–Ringer bicarbonate (pH 7.4) equilibrated with 95 % O_2–5 % CO_2 (see ref. 15) containing 2.5 mg of glucose per ml, 2 mM L-lysine or L-leucine, and 10^6 disintegrations/min of D,L-[2-^{14}C]lysine or D,L-[2-^{14}C]leucine, the intestinal segment was rinsed with 0.9 % NaCl and homogenized in 8 ml of water in a Ten Broeck tissue grinder. The protein was precipitated by the addition of 2 ml of 50 % trichloroacetic acid and the precipitated protein was washed once with 10 ml of water. The washed protein was suspended and counted as described above.

Measurement of the incorporation of acetate into tissue lipid

After perfusion for 60 min at 37° with Krebs–Ringer bicarbonate (pH 7.4) containing 2.5 mg of glucose per ml, 1 mM sodium acetate and 10^6 disintegrations/min of sodium [1-^{14}C]acetate, the intestinal segment was removed from the apparatus, rinsed with 0.9 % NaCl and heated under reflux with 9.4 ml of ethanol and 0.6 ml of 33 % KOH for 30 min. Ten ml of water were added to the digest. The cooled, diluted digest was extracted by shaking with 20 ml of hexane. The hexane extract (non-saponifiable fraction) was washed once by shaking with 20 ml of water, transferred to a scintillation vial and evaporated to dryness. The aqueous alcoholic residue remaining after hexane extraction was acidified with 5 M H_2SO_4 and extracted by shaking with 20 ml of hexane. The hexane extract (fatty acid fraction) was washed once by shaking with 20 ml of water, transferred to a scintillation vial and evaporated to dryness. The material in the scintillation vial was then counted as described above.

ATPase assay

Washed jejunal segments were slit longitudinally, spread on waxed paper and the mucosa was scraped off with a razor blade. The mucosa was weighed and homogenized at 0° to 2° in a Ten Broeck tissue grinder in 100 volumes of 30 mM histidine buffer (pH 7.3). This buffer, which contained 1 mM ethylenediaminetetraacetonitrile, was used in preparing all solutions used in this assay.

Mucosal homogenates were incubated in the presence and absence of bile salts for 15 min at 37° in 2 ml of a medium containing 3 mM ATP, 4 mM Mg^{2+}, 20 mM K^+ and 80 mM Na^+. All metallic salts were added as chlorides. The reaction was stopped by adding 0.2 ml of 50% trichloroacetic acid. The incubation mixtures were centrifuged and 1.0 ml of the supernatant solution was analyzed for inorganic phosphate. ATPase activity was determined by the increase in inorganic phosphate.

Oxygen uptake; P:O ratios

The effect of bile salts on oxygen uptake by the intestine was studied using a Warburg constant volume respirometer. Rings of rat jejunum approx. 3 mm in width, weighing a total of 50 to 100 mg, were suspended in 3.0 ml of Krebs–Ringer phosphate (pH 7.4) which was modified to contain only 1.3 mM Ca^{2+} (ref. 15) and 2.5 mg of glucose per ml. Rings were equilibrated with 100% O_2 for 10 min. Oxygen uptake was then measured at 15-min intervals for 60 min. Subsequently the tissue sections were gently blotted on a sheet of filter paper and weighed.

Mitochondrial P:O ratios were determined by the method of LEE AND WHITEHOUSE[23] with a modification in the method of phosphate determination. After oxygen uptake had been determined, 2.0 ml of the Warburg flask contents were immediately pipetted into 0.3 ml of 50% trichloroacetic acid in an ice-bath and mixed. After standing about 20 min, the protein was removed by centrifugation and 1.0 ml of the supernatant solution was analyzed immediately for inorganic phosphate.

Measurement of the incorporation of glucose into lipid

Washed jejunal segments were everted on a glass rod according to the technique of WILSON AND WISEMAN[40], and consecutive rings (about 40 mg each) were cut off and placed in groups of three rings each in a Petri dish containing Krebs–Ringer bicarbonate (pH 7.4) and 0.5 mg of glucose per ml. Each flask contained a group of intestinal rings randomly drawn from non-adjacent parts of the intestinal segment. The rings were blotted gently on filter paper, weighed and placed in 25-ml erlenmeyer flasks containing 4 ml of Krebs–Ringer bicarbonate (pH 7.4) with 0.5 mg of glucose per ml and $2 \cdot 10^6$ disintegrations/min of D-[$^{14}C_6$]glucose. The flasks were incubated at 37° for 30 min under an atmosphere of 95% O_2–5% CO_2 in a Dubnoff shaker.

Thereafter the reaction was stopped by the addition of 0.16 ml of 50% trichloroacetic acid. The rings were removed, blotted on filter paper and the lipid extracted from them by the technique of FOLCH, LEES AND SLOANE STANLEY[41]. The lipid extract was evaporated to dryness, the residue extracted twice with 5 ml of hexane and the hexane solution was washed twice with 10 ml of 0.9% NaCl. An aliquot of the hexane extract was transferred to a counting vial, evaporated to dryness and counted as given above.

Another aliquot of the washed hexane extract was chromatographed by thin-layer chromatography and the triglyceride spot was removed and counted as described above.

RESULTS

Perfusion experiments

Viability and integrity of the perfused intestine. Active preparations of perfused intestine exhibited strong peristalsis. After perfusion for 60 min in the presence or absence of pure 8 mM glycocholate, villi remained intact and mucosal sloughing was minimal, although some swelling of the submucosal spaces was apparent. Like observations have been reported by others[42,43]. Similarly, the control rate of glucose transport observed in our experiments agreed closely with that found by others[44-46]. Fig. 1 shows the change in the rate of glucose transport with time over a ten-fold range in the initial glucose concentration. At an initial glucose concentration of 2.5 mg/ml, the overall rate of transport was 1.8 mg/h per cm of intestine. From a plot of the reciprocal of the rate of glucose transport *versus* the reciprocal of the initial glucose concentration in the perfusion fluid, a half-saturation concentration (K_m) of 6 mM was obtained, which corresponds well with literature values of 7 mM (ref. 46) and 9 mM (ref. 45).

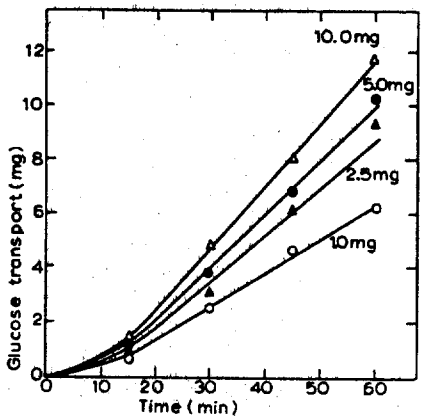

Fig. 1. Rate of glucose transport as a function of time at various initial glucose concentrations. Initial concentrations are expressed as mg of glucose per ml of perfusion fluid. Glucose transport is expressed as mg of glucose accumulated in the serosal fluid. Each curve represents the data from a single animal.

Metabolic inhibitors, such as 0.01 M iodoacetate, 10^{-5} M 2,4-dinitrophenol and 0.01 M fluoride, as well as lowering the temperature of the perfusion system to 4°, depressed glucose transport by 90–95 %. These observations confirm the requirements of an energy producing system for glucose transport in this preparation.

Effect of bile salts on the transport of glucose. Because of an initial 10–20-min lag in glucose transport, the steady state rate was calculated from the 30–60 min part of the perfusion period. As shown in Table I, the pure synthetic conjugated bile salts, glycocholate and taurocholate, had little, if any, effect on the transport of glucose. On the other hand, the unconjugated salts, cholate and deoxycholate, strongly inhibited glucose transport. A noticeable inhibition was present at a deoxycholate concentration of 0.2 mM and at a cholate concentration of 6 mM. The presence of 8 mM taurocholate in the perfusion fluid did not offset the inhibition of glucose transport by 0.4 mM deoxycholate. The tested commercial preparations of glycocholate and taurocholate, at

TABLE I

EFFECT OF BILE SALTS ON GLUCOSE TRANSPORT

Experimental conditions are given in the text. Reported values are the average of one to five replicate determinations. The average control value for glucose transport was 7.2 mg per h for the 6-cm jejunal segment.

Bile salt	*Glucose transport (% of control value)*
None (control)	100
8 mM glycocholate (synthetic)	100
8 mM glycocholate (Pfanstiehl)	2
8 mM taurocholate (synthetic)	124
8 mM taurocholate (Sigma)	24
1 mM cholate	97
2 mM cholate	114
4 mM cholate	99
6 mM cholate	24
8 mM cholate	1
0.05 mM deoxycholate	120
0.1 mM deoxycholate	99
0.2 mM deoxycholate	44
0.4 mM deoxycholate	15
0.8 mM deoxycholate	1

8 mM concentration, inhibited glucose transport in proportion to the relative amounts of deoxycholate in these preparations.

The inhibition of glucose transport caused by the free bile salts (8 mM cholate and 0.8 mM deoxycholate) was irreversible. When the bile salt-containing perfusion fluid was replaced after 45 min of perfusion with an equal volume of bile salt-free fluid, no change in the low, inhibited rates of glucose transport occurred. Apparently, free bile salts permanently damage the mucosa.

TABLE II

EFFECT OF BILE SALTS ON THE TRANSPORT OF VARIOUS SUBSTANCES

Experimental conditions are given in the text.

Substances transported	*Initial concentration in perfusion medium (mM)*	*Transport rate without bile salts (μmoles/h)*	*Per cent of normal transport rate*		
			*With 8 mM glycocholate**	*With 8 mM taurocholate**	*With 0.4 mM deoxycholate*
L-Lysine	2	2.0**	122	125	31
L-Leucine	2	8.0	95	98	16
L-Leucine	0.016	0.12	56	—	—
Acetate	1	0.63	135	124	65
Acetate	0.025	0.037	86	—	—
Urea	60	0.034	108	95	42
Sorbose	22	0.0017	114	100	175
Sodium	145	183	84	95	32

* Glycocholate and taurocholate are pure synthetic bile salts.
** Reported values are the average of one to four replicate determinations.

Effect of bile salts on the transport of various other water-soluble substances. The pure synthetic conjugated bile salts, glycocholate and taurocholate, had little, if any, effect on the transport of any substance tested, except for a possible slight acceleration of acetate transport (Table II).

When L-leucine was present at a concentration of $1.6 \cdot 10^{-5}$ M, a concentration used in previous studies[16], its transport was slightly inhibited by 8 mM glycocholate, whereas at higher concentrations no inhibition was observed. Deoxycholate, at a concentration of 0.4 mM, decreased the transport of all substances tested, except of sorbose, whose transport was increased.

Effect of bile salts on synthetic reactions of perfused jejunal segments. The effects of bile acids on the incorporation of lysine and leucine into protein, and on the incorporation of acetate into non-saponifiable lipid and long-chain fatty acid, are presented in Table III. The synthetic conjugated bile acids did not significantly affect any of these functions. Deoxycholate (0.4 mM) inhibited the incorporation of lysine and leucine into protein, but affected less the other functions tested.

TABLE III

EFFECT OF BILE SALTS ON VARIOUS METABOLIC FUNCTIONS OF PERFUSED JEJUNAL SEGMENTS

Experimental conditions are given in the text.

Metabolic function	*Control activity (no bile salts)*	*Per cent of control activity*		
		*With 8 mM glycocholate**	*With 8 mM taurocholate**	*With 0.4 mM deoxycholate*
Incorporation of lysine into protein	0.098**	90	106	66
Incorporation of leucine into protein	0.119	94	97	74
Incorporation of acetate into non-saponifiable lipid	0.019	99	116	79
Incorporation of acetate into the long-chain fatty acids fraction	0.074	108	109	85

* Glycocholate and taurocholate are pure synthetic bile salts.

** Protein and lipid formation are given in terms of μmoles of amino acids or acetate incorporated per h by the 6-cm jejunal segment. Entries are the average value of one to four replicate determinations.

ATPase activity of mucosal homogenates

In the absence of bile salts, mucosal homogenates liberated inorganic phosphate from ATP at an average rate of 55 μmoles of phosphate per min per g of tissue. In the presence of purified bile salts, ATPase activity was reduced to the following per cent of control values: 8 mM glycocholate, 63 %; 8 mM taurocholate, 66 %; and 1 mM deoxycholate, 52 %. Deoxycholate thus appeared to be at least eight times more inhibitory than taurocholate or glycocholate in this system.

When the mucosa was fractionated into brush border and supernatant fractions by the procedure of GALLO AND TREADWELL[47], 38 % of the ATPase activity was found in the brush borders and 48 % in the supernatant solution. 8 mM taurocholate inhibited the brush border ATPase 26 % and the supernatant ATPase 43 %, in comparison with a 32 % inhibition of the whole homogenate.

Oxygen uptake and phosphorylation studies

Oxygen uptake of jejunal rings. The average oxygen uptake in control flasks was 1.68 μl of O_2 per h per mg wet weight of tissue. With pure synthetic conjugated bile salts, the average oxygen uptake with 8 mM glycocholate was 112 % and with 8 mM taurocholate was 96 % of the control value. On the other hand, the unconjugated bile salt deoxycholate at a concentration of 0.4 mM reduced oxygen uptake to an average value which was 67 % of the control level.

TABLE IV

EFFECT OF BILE SALTS ON OXIDATIVE PHOSPHORYLATION BY LIVER MITOCHONDRIA

Experimental conditions are given in the text.

*Bile salt***	*Percentage activity*		
	*Oxygen uptake**	*Phosphorus incorporation*	*P:O*
None (control)	100	100	100
0.5 mM taurocholate	103	96	93
1 mM taurocholate	100	101	101
2 mM taurocholate	67	53	79
4 mM taurocholate	66	10	15
4 mM glycocholate	67	0	0
0.1 mM cholate	110	89	80
0.2 mM cholate	95	81	84
0.4 mM cholate	75	59	79
0.5 mM cholate	83	87	105
1 mM cholate	80	0	0
2 mM cholate	81	0	0
0.01 mM deoxycholate	91	97	107
0.02 mM deoxycholate	92	97	106
0.05 mM deoxycholate	133	0	0
0.1 mM deoxycholate	101	0	0
0.2 mM deoxycholate	112	0	0

* The average control oxygen uptake was 1.61 μatoms of oxygen per min per mg of mitochondrial protein and the average control phosphorus incorporation was 3.30 μmoles/min per mg of mitochondrial protein, yielding an average P:O = 2.05.

** Glycocholate and taurocholate are pure synthetic bile salts.

Liver mitochondrial P:O ratios. The results of these studies are given in Table IV. Noticeable lowering of oxygen uptake, phosphate incorporation and P:O ratios was caused by as little as 2 mM synthetic taurocholate. The unconjugated bile salts were much more efficient inhibitors, 0.2 mM cholate and 0.05 mM deoxycholate causing significant uncoupling. Thus deoxycholate was about forty times as inhibitory as taurocholate in this system.

Effect of bile salts on the incorporation of label from D-[$^{14}C_6$]*glucose into lipid by jejunal rings*

The effects of various bile salts on the synthesis of lipid from glucose are given in Table V. The synthetic conjugated acids, taurocholate and glycocholate, were without effect, even at fairly high concentrations. Deoxycholate exerted a slight inhibitory effect on the incorporation of labeled glucose into triglyceride, but more

TABLE V

EFFECT OF BILE SALTS ON THE INCORPORATION OF LABEL FROM D-[$^{14}C_6$]GLUCOSE INTO LIPID BY JEJUNAL RINGS

Experimental conditions are given in the text.

Bile salt added	*Percentage activity**	
	Incorporation into lipid	*Incorporation into triglyceride*
None (control)	100**	100
8 mM glycocholate (synthetic)	97	98
10 mM glycocholate (synthetic)	100	—
20 mM glycocholate (synthetic)	97	—
10 mM glycocholate (Pfanstiehl)	110	115
8 mM taurocholate (synthetic)	90	96
10 mM taurocholate (synthetic)	88	—
10 mM taurocholate (Sigma)	202	374
0.4 mM deoxycholate	50	74

* Average control incorporation values are 208 mμmoles of glucose into lipid and 70 mμmoles of glucose into triglyceride per h per g wet weight of tissue.

** Reported values are the average of one to five replicate determinations.

profoundly depressed its incorporation into the total lipid extract. In control runs approximately one-third of the radioactivity in the hexane extract was in the triglyceride fraction, and the remainder was in an unidentified polar fraction that migrated in the phospholipid region ($R_F < 0.1$) on thin-layer chromatography. Commercial taurocholate markedly increased the incorporation of radioactive glucose into triglyceride, with only a slight increase of label in the fraction having an $R_F < 0.1$. This increase occurred despite the fact that commercial taurocholate contained significant amounts of deoxycholate. Although commercial glycocholate contained considerably higher levels of deoxycholate than commercial taurocholate, the conversion of glucose to lipid in its presence was nonetheless not depressed.

The stimulatory effect of the commercial bile salt preparations was traced to the fatty acids present in the contaminating lipids. As indicated earlier, taurocholate (Sigma) contained 1.4% fatty acids by weight and glycocholate (Pfanstiehl) had 4.5% fatty acids. Upon extracting the fatty acids from the bile salt preparation, the stimulation was abolished as summarized in Table VI. The stimulation observed with fatty acids varied quantitatively with the length and unsaturation of the carbon chain. In order to duplicate this effect, various fatty acids were added to pure synthetic taurocholate at a level representing a 1% contamination by weight of the bile salt. In comparison to the incorporation of label from D-[$^{14}C_6$]glucose into triglyceride in the presence of 10 mM taurocholate alone, the incorporation in the presence of contaminating octanoic acid was 113%; myristic, 268%; palmitic, 150%; stearic, < 150%; oleic, 328%; and linoleic, 406%. In addition simulated "commercial" preparations of bile salts were prepared by mixing synthetic taurocholate or glycocholate with pure palmitic, stearic, oleic, and linoleic acids in the proportions found in the commercial taurocholate and glycocholate bile salt preparations. Compared to control incorporation values, the radioactivity in triglyceride in the presence of simulated commercial taurocholate was 340%, and of simulated commercial glyco-

TABLE VI

FRACTIONATION OF COMMERCIAL BILE SALTS WHICH STIMULATE THE INCORPORATION OF LABEL FROM D-[$^{14}C_6$]GLUCOSE INTO TRIGLYCERIDE

Experimental conditions are given in the text. The lipids were deposited in the dry flasks by evaporation of the hexane and were then dissolved, or in some cases well suspended, by brief gentle heating with the bile salt and a small amount of water. After cooling the calculated amount of a slightly concentrated Krebs–Ringer bicarbonate and the other constituents were added.

Fraction tested	*Percentage activity*	
	Taurocholate (Sigma)	*Glycocholate (Pfanstiehl)*
0.01 M taurocholate (synthetic) (control)	100	100
0.01 M bile salt (commercial)	361	115
0.01 M bile salt (commercial), lipid-extracted	81	71
0.01 M taurocholate (synthetic) *plus* total lipid extract of bile salt (commercial)	390	428
0.01 M taurocholate (synthetic) *plus* fatty acid fraction of lipid extract of bile salt (commercial)	422	512
0.01 M taurocholate (synthetic) *plus* non-fatty acid fraction of lipid extract of bile salt (commercial)	102	114

cholate was 333 %. These values are somewhat lower than those observed (422 % and 512 %, respectively) when the fatty acids extracted from the bile salts were added to synthetic taurocholate. Since the four acids added in the simulated mixtures constituted only 91 % of the fatty acid found in commercial taurocholate and 92 % of those present in commercial glycocholate, the remaining undetermined fatty acid components of the extract might well have stimulated glucose incorporation further.

DISCUSSION

In contrast to many prior reports[4,16–19,22], we have found that highly purified glycocholate and taurocholate at a 8 mM concentration have no effect on the following metabolic processes in the rat intestine: (1) the transport of glucose, leucine, lysine, acetate, sodium, sorbose and urea; (2) the synthesis of non-saponifiable lipid and fatty acids from acetate, of protein from leucine or lysine, and of triglyceride from glucose; and (3) the consumption of oxygen.

Past contradictory results seemingly were caused by the presence of two kinds of contaminants in preparations of conjugated bile salts: (1) free bile acids, particularly deoxycholate; and (2) free fatty acids, particularly long-chain unsaturated fatty acids. Deoxycholate at a concentration of 0.2 mM caused easily detectable inhibition of glucose transport by jejunal segments and of oxidative phosphorylation by liver mitochondria, and, at twice this concentration, inhibited almost all of the phenomena investigated here. This inhibition was not offset by 8 mM conjugated bile salts, and was essentially irreversible.

Since many studies have previously been conducted with approx. 0.01 M conjugated bile salts, the concentration found in rat bile[48], contamination of bile salt preparations with only 2 % deoxycholate, a level which might be overlooked in routine tests of purity, would give inhibitory effects. Free cholic acid, on the other hand, was

about one-twentieth as deleterious as deoxycholate, and only higher levels of contamination interfered with metabolic functions.

Free fatty acids, the second common contaminant of bile salt preparations, markedly stimulate the incorporation of D-[$^{14}C_6$]glucose into triglyceride-glycerol. When a 0.01 M conjugated bile salt solution is employed, a 1 % contamination with linoleic or oleic acid increased three- to four-fold the conversion of glucose into triglyceride. Saturated and shorter chain fatty acid contaminants have a less pronounced effect. That deoxycholate is toxic to intestinal cells and that fatty acids stimulate glucose utilization by the gut are not, of course, novel observations. The destructive effects of deoxycholate on gut epithelium have been well documented[4], and the inhibitory action of 0.5 mM deoxycholate on oxidative phosphorylation by rat-liver mitochondria has previously been reported[23]. Similarly glucose utilization by the gut has been shown to increase markedly when free fatty acids are present[4,49,50], an effect which may not be solely due to the additional requirements of the cell for L-α-glycerol phosphate[49]. But that extremely small amounts of these compounds in conjugated bile salt preparations yet exert their effects, and that these effects have often been mistakenly attributed to conjugated bile salts, are the new observations which justify this report.

Only a few findings are seemingly at odds with the generalization that conjugated bile acids influence neither the active or passive transport of water-soluble nutrients by the upper gut nor the intracellular metabolism of these nutrients. For example, the transport across perfused gut of L-leucine, when present at $1.6 \cdot 10^{-5}$ M as previously used[16], was inhibited 44 % by 8 mM glycocholate, but when present at 2 mM, was unaffected by glycocholate. Possibly the observed inhibition at the lower concentration was caused by binding of appreciable amounts of this lipophilic amino acid to the bile salt micelle, which thereby reduced its effective concentration in the perfusate. By comparison, the transport of $2.5 \cdot 10^{-5}$ M acetate, which should not interact appreciably with bile salt micelles, was unaffected by 8 mM glycocholate. More difficult to rationalize is the observation of SAUNDERS AND DAWSON[5] that absorbed oleic acid suspended in taurocholate appears in a more fully esterified state in the lymph than oleate given as a sonicated emulsion. Although taurocholate may have a direct intracellular effect in this case, other explanations based on differences in the dispersion of oleate between micelle and emulsion are possible[5].

The mechanism by which deoxycholate at concentrations well below its critical micelle concentration affects membrane activity is unknown. In the present study 0.4 mM deoxycholate inhibited the transport of all substances tested except sorbose, whose transport it increased. This increased sorbose transport accords with the observations of ROSENBERG AND HARDIN[51] that deoxycholate and cholate increase the permeability of the intestinal cell to passively transported substances as well as inhibit active transport systems. Surprisingly, the transport of urea, which presumably occurs by diffusion, is depressed by deoxycholate rather than augmented, an observation which is not readily explained in terms of a general increase in permeability. Further studies on the interaction of deoxycholate with lipoprotein membranes would be most welcome.

In an earlier study we suggested that the apparent inhibitory effects of conjugated bile salts on the transport of water-soluble nutrients and their apparent stimulatory effects on glucose utilization could be rationalized in terms of an initial

depression in the activity of membrane-bound, $Na^+ + K^+$-dependent ATPase[19]. In view of the general inactivity of highly purified conjugated bile salts in our present transport studies, their possible effects on intestinal ATPase were reexamined. In keeping with previous results[19], pure 8 mM glycocholate and taurocholate and 1 mM deoxycholate inhibited ATPase in intestinal homogenates by 40–50%. Since glycocholate and taurocholate have no apparent intracellular effects, however, we are at present reluctant to attribute physiological significance to the ATPase inhibition. In all probability transport-linked ATPase is oriented within the microvilli or terminal web and may not react freely with bile salt micelles in the lumen. Furthermore, FAUST AND WU[18] have reported that ATP levels of rat and hamster jejunum are depressed by conjugated bile salts, a finding which superficially is at odds with any hypothesis based on ATPase inhibition.

Since reduced ATP levels might result from an uncoupling of oxidative phosphorylation, the effects of pure conjugated and free bile salts on liver mitochondria were investigated. Results quantitatively similar to earlier reported values[23] were again obtained. By analogy to the ATPase studies, however, we are uncertain whether such inhibitory effects on isolated mitochondria have any meaningful relationship to physiological events within the intact cell.

The present studies, in addition to exposing some artifacts, point up some interesting physiological relationships. Conjugated trihydroxycholanic acid derivatives are the major end products of sterol degradation in the liver of many species[52]. By bacterial action in the gut, however, these compounds may be hydrolyzed to the free acids[52,53], deoxygenated and possibly modified in other ways[52] to form derivatives which are highly toxic to the upper gut, but apparently are better tolerated by epithelial cells of the ileum and colon. Upon transport to the liver, these derivatives are again hydroxylated and conjugated by the microsomes, processes which must be highly efficient to prevent toxic levels of free bile acids from appearing in the bile. Impairment of this liver microsomal system, even in small part, might give rise to steatorrhea of a previously unexpected type[51].

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Bile Acid-Lipoprotein Relationships Using Cholic Acid-24-C^{14} [1]

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ABSTRACT

PORTMAN, OSCAR W. AND PAUL MURPHY. *Bile acid-lipoprotein relationships using cholic acid-24-C^{14}.* Am. J. Physiol. 195(1): 189–193. 1958.—The sera of rats with biliary obstruction of short duration and of rats fed diets containing cholesterol and cholic acid were analyzed for lipoprotein concentrations and lipoprotein composition by ultracentrifugal techniques. Both rats with biliary obstruction and those that were fed cholesterol and cholic acid had beta lipoproteins with increased cholesterol composition (relative to total beta lipoproteins and to protein content of the lipoproteins). When cholate-24-C^{14} was injected intraperitoneally into rats with biliary obstruction, a considerable portion of the radioactivity remained in the sera as taurocholate 6 hours after administration. Isolation and purification of the alpha and beta lipoproteins from such rats indicated the association of little or no radioactivity. The radioactivity in whole sera from these rats and in human sera incubated with radiocholate was largely nondialyzable and had electrophoretic mobility similar to that of serum albumin. The incubation of human sera with different bile salts had no effect on concentrations or mobility of beta lipoproteins (ultracentrifuge) or proteins (electrophoresis).

THE ELEVATION of serum cholesterol values in patients with diseases involving obstruction of the biliary tract is a venerable observation (1). Several recent studies (2–5) indicated that not only were lipoprotein concentrations, as separated by physical means, different in patients suffering from obstructive biliary tract disease, but that also lipoproteins of similar physical properties from such patients and from controls had very different chemical compositions.

It now seems clearly established (6) that in patients or animals with biliary obstruction there is a measurable quantity of bile salts in the blood (probably bound to albumin (7)) The relationship of cholatemia, per se, to the presence of abnormal quantities of cholesterol and of abnormal quantities and types of lipoproteins in such subjects has not been demonstrated.

Many laboratories include cholate and cholesterol in the diets of experimental animals to produce elevations of serum lipids and lipid infiltration of aortae. In the course of such studies the unusual distribution and composition of the serum lipoproteins has been noted (8, 9).

This paper reports findings relative to the chemical and physical properties of lipoproteins in rats with acute biliary obstruction (in which hepatocellular damage is at a minimum and in which hypercholatemia exists in a less complicated form) and in rats fed diets including cholesterol and cholic acid.

Cholate-24-C^{14} was also used to elaborate the relationship of bile acids, per se, to the changes in the serum cholesterol and lipoprotein levels.

EXPERIMENTAL

Preparation of Animals. Male albino rats weighing between 300 and 350 gm were used for those studies involving animals. Some rats were fed Purina laboratory chow, and others were fed a synthetic diet including 5% cholesterol and 1.5% cholic acid. The bile ducts of a third group of animals fed Purina chow were

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doubly ligated through a small right subcostal incision 4 days prior to study.

Analytical Determinations. Total cholesterol determinations were performed on sera and on preparations from the ultracentrifuge by the method of Abell *et al.* (10). Protein determinations were made by a biuret method (11). The beta lipoproteins and alpha lipoproteins were separated by ultracentrifugal techniques as outlined by Gofman *et al.* (12). In some instances quantitation of the various flotational classes of beta lipoproteins was performed by the analytical technique outlined by Gofman *et al.* (12). Paper electrophoretic patterns of serum proteins were determined by running at pH 8.65 for 16 hours at a strip voltage of 5.1 v/cm. Dialyses of samples containing labeled bile acids were performed against distilled water at 5° for 48 hours.

Bile Acids. The samples of bile acids were the same as those used for an associated study (13). Cholic acid-24-C^{14} was synthesized by the method of Bergström *et al.* (14) and had a specific activity of 2.2 μc/mg. Analyses by paper and column chromatography indicated that all of the radioactivity behaved like cholic acid. When the bile acids were injected or were added to sera, they were dissolved in pH 7.4 phosphate buffer. Bile acids in biological materials were in some cases separated by the paper chromatographic techniques outlined by Sjövall *et al.* (15). Radiochromatography was performed by repeatedly eluting the increments of paper with hot ethanol or by radioassaying the increments of paper directly. Similar procedures were followed on the paper electrophoretic separations of sera in which labelled bile acids were present.

TABLE 1. CHOLESTEROL AND BETA LIPOPROTEIN CONCENTRATIONS AND COMPOSITION OF ALPHA AND BETA LIPOPROTEINS (ULTRACENTRIFUGE)*

Measurement	Group		
	Control (n = 5)	Cholate fed (n = 15)	Bile duct ligation (n = 5)
Serum cholesterol, mg%	66	395	220
Beta lipoproteins, mg%			
S_f 0–11	35	20	306
12–20	0	84	101
20–100	14	480	87
100–400	19	56	1
Beta cholesterol, mg%	13	367	154
Beta cholesterol ÷ beta lipoprotein	0.19	0.58	0.29
Beta cholesterol ÷ protein of beta lipoprotein	0.363	6.10	2.45
Alpha cholesterol	40	32	48
Alpha cholesterol ÷ protein of alpha lipoprotein	0.54	0.47	0.44

* Normal albino rats (fed Purina laboratory chow), rats maintained for 30 days on diets including 5% cholesterol and 1.5% cholic acid, and rats in which bile duct had been ligated 4 days previously (also fed Purina chow).

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN URINE, SERA AND LIVER ½, 1, 3 AND 6 HOURS AFTER THE INTRAPERITONEAL ADMINISTRATION OF 2 MG (4.4 μC) OF CHOLATE-24-C^{14} TO RATS IN WHICH THE BILE DUCTS WERE TIED 4 DAYS PREVIOUSLY

Time After Injection	% Injected Activity* in Sera	% in Liver	% in Urine
½ hour	14.2	29.5	
1 hour	24.1	13.3	2.1
3 hours	19.6	17.0	20.4
6 hours	9.6	27.6	6.8

* Based on sera equal to 5% of body weight.

RESULTS

Lipoprotein-Cholesterol Relationships in Rats. The analyses of sera from the three groups of rats described above are shown in table 1. Serum cholesterol and beta lipoprotein levels were elevated in both the rats fed cholesterol-cholate and in those in which the bile duct was acutely obstructed. The relative distribution of the various ultracentrifugal classes of beta lipoproteins were also very different for the three groups of rats. The rats with bile duct ligation had a high concentration in the S_f0–11 class and progressively lesser concentrations in the 12–20 and 20–100 classes. The rats fed cholesterol and cholate had very low S_f0–11 concentrations and very high S_f20–100 levels. The mean beta cholesterol levels (cholesterol in the material floating at density 1.063) were also very different for the three groups of rats, varying from 13 mg% (expressed as mg/100 ml of sera) for the control group to 367 mg% for the rats fed cholesterol and cholate. The ratios of serum beta cholesterol to beta lipoproteins (0.19 in control group and 0.58 in the group fed cholesterol and cholate) and the ratios of beta cholesterol to the globulin of beta lipoproteins were al-

different for the three groups of rats. The absolute amounts of protein in beta lipoproteins were only slightly elevated in the two hypercholesteremic groups. It is interesting to note that the main S_f peak in the group of rats fed cholesterol and cholate was around 35 which would not normally be a cholesterol rich species of lipoproteins in man (16); nevertheless, 58% of the apparent weight of beta lipoproteins from this group was cholesterol. The alpha lipoprotein composition (based on cholesterol and protein concentrations) were not different for the three groups of rats. Chylomicrons were not previously separated in these analyses; however, rats had been fasted for 12 hours prior to obtaining blood samples and chylomicrons were presumably absent.

Distribution of Radioactivity After Intraperitoneal Administration of Cholate-24-C^{14}. Since it was possible that the changes in serum lipids and lipoproteins associated with biliary tract obstruction or cholate feeding were due to the presence of bile salts in sera, the disposition of labeled cholate injected intraperitoneally was investigated. The sera of normal rats similarly injected contained extremely low amounts of radioactivity at the periods under consideration (no greater than 1% of portal sera radioactivity). Table 2 illustrates the quantities of radioactivity found in sera, urine, and liver of surgically jaundiced rats at ½, 1, 3 and 6 hours after the administration of 2 mg (4.4 μc) of radiocholate. A very considerable quantity of the injected radioactivity was present in all three fractions. The level of activity was still high in the sera 6 hours after radiocholate administration. When ethanol soluble extracts of tissues from rats killed 3 hours after cholate administration were chromatographed on paper according to the method of Sjövall (15) (fig. 1), it appeared that most of the activity in the liver and sera behaved like taurocholic acid while most of the radioactivity in the urine migrated like cholic acid. Six hours after injection of radiocholate all of the activity in both liver and sera was present as taurocholate.

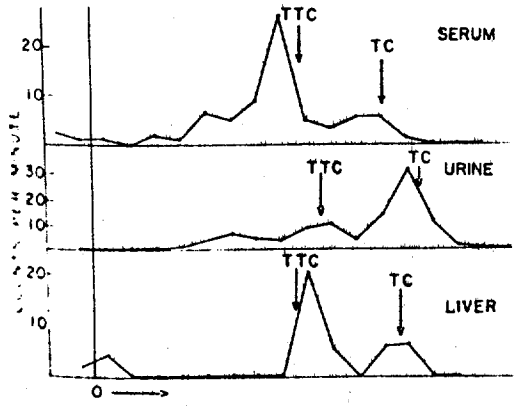

FIG. 1. Paper chromatographic separation (15) of radioactive materials from serum, urine and liver of a surgically jaundiced rat 3 hr. after the intraperitoneal administration of cholate-24-C^{14}. Stationary phase was glacial acetic acid; moving phase was isopropyl n-butanol (9:1 v/v). TTC indicates the taurotrihydroxycholanic acid locus and TC the trihydroxycholanic acid locus.

TABLE 3. ULTRACENTRIFUGAL DISTRIBUTION OF RADIOACTIVITY IN SERA OF SURGICALLY JAUNDICED RATS AFTER THE INTRAPERITONEAL ADMINISTRATION OF CHOLATE-24-C^{14} (SEE TABLE 2)

Time After Injection	Total Radioactivity in Sera, %		
	Beta prep.	Alpha prep.	Subnatant
½ hour	6.8*	9.5	80.8
1 hour	8.8	7.4	80.9
3 hours	15.0	9.7	78.0
6 hours	4.3*	7.9	85.2

* Beta lipoprotein preparations were diluted, again made up to a density of 1.063 and centrifuged for reisolation of the beta lipoprotein. Less than 1% of the total radioactivity in the sera was present in the reisolated beta preparation. The ratio of the volumes of the first beta preparation to the volume of solution for the reisolation was 1:18.

Ultracentrifugal Distribution of Radioactivity in Sera. Sera of the jaundiced rats which had been injected with radiocholate were fractionated by ultracentrifugation at different densities according to the method of Gofman *et al.* (12). Materials floating at densities of 1.063 (16 hr.) and 1.21 (40 hr.) (1 ml from 9 ml in the centrifuge tube was taken) were designated beta and alpha lipoproteins, respectively. Table 3 shows the percentages of labeled material in sera that were in the beta, alpha and subnatant fractions. A significant portion was found in the beta (4.3–15.0%) and in the alpha fractions (7.4–9.7%). However, when portions of the beta preparation from two of the samples were diluted and rerun at density 1.063, most of the initial

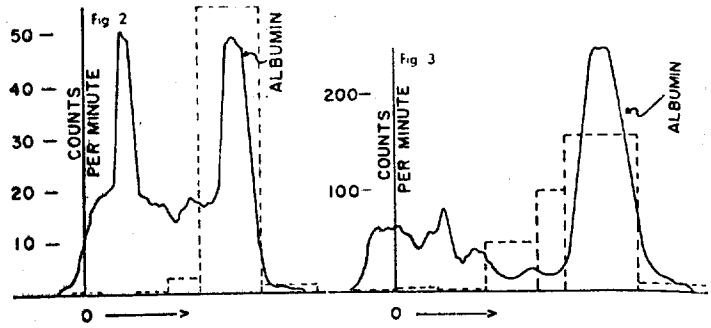

FIG. 2. Paper electrophoretic separation of serum proteins and radioactivity from a surgically jaundiced rat injected with cholate 24-C^{14} 3 hr. previously (see fig. 1). —— intensity protein stains; - - - registered counts per min.

FIG. 3. Paper electrophoretic separation of serum proteins and radioactivity from human sera which had been incubated with cholate-24-C^{14} (pH 7.4) for 3 hr. Concentration of labeled and carrier cholate was 15 mg%. —— intensity protein stain; - - - registered counts per min.

activity was absent from the beta preparation. Thus, the activity present in the preparations from the first runs appeared to be the result of contamination of the samples with the subnatant material or of failure of the cholate to concentrate in any portion of the centrifuge tube.

Dialysis of Samples Containing Radiocholate. A preliminary comparison of the binding of radiocholate in various biological materials was made by dialysis. These studies indicated that the radioactivity in the following materials was less freely dialyzable than cholate-24-C^{14} (dissolved in pH 7.4 phosphate buffer): bile and portal sera from rats injected with cholate-24-C^{14}, peripheral sera from bile duct-ligated rats injected intraperitoneally with cholate-24-C^{14}, alpha and beta lipoproteins isolated (ultracentrifuge) from the same rats and normal human sera incubated with cholate-24-C^{14}. A more precise evaluation of the degree of binding of bile acids in biological experiments is being undertaken with dialysis procedures.

Paper Electrophoresis of Material Containing Cholate-24-C^{14}. The correlation of the movement in an electric field of the radioactivity and of proteins in the sera of rats injected with radiocholate was studied. Figure 2 illustrates the patterns for the jaundiced rat injected with radiocholate 3 hours prior to collection of the sera. Figure 3 shows a similar pattern for human sera to which radiocholate had been added (final concentration of cholate = 15 mg%). Under the conditions used, the radioactivity peaks approximated the areas of albumin staining. However, free cholate analyzed alone ran only slightly behind the albumin peak, and taurocholate analyzed alone was only slightly ahead of the albumin peak.

Effect of Addition of Various Bile Salts to Human Sera on Ultracentrifugally Separated Lipoproteins and Electrophoretically Separated Proteins. Three large samples of freshly separated human sera were subdivided into 4 ml aliquots and 0.15 ml of 7.4 phosphate buffer or 0.15 ml phosphate buffer containing various bile acids were added to the sera aliquots. After the samples had stood for

TABLE 4. EFFECT OF THE ADDITION OF BILE SALTS TO HUMAN SERA ON THE ULTRACENTRIFUGAL PATTERN OF BETA LIPOPROTEINS AND ON BETA LIPOPROTEIN COMPOSITION

Sample	Serum Cholesterol	Beta Lipoproteins					Beta Cholesterol	Beta Lipoprotein
		0–11	12–20	21–35	35–100	100–400		
	mg %	mg %					mg %	mg
A	222	189	10	9	27	5	113	15
A		191	13	10	33	4	115	15
A + 5 mg% cholate		189	10	9	27	5	122	15
A + 15 mg% cholate		225	12	10	26	6	106	15
A + 15 mg% taurocholate		232	12	10	30	5	112	15
F	267	366	53	22	100	51	172	24
F		388	53	26	108	62	178	25
F + 15 mg% taurocholate		388	46	23	86	38	178	22
F + 30 mg% taurocholate		369	51	19	118	62	187	26
F + 15 mg% chenodeoxycholate		403	61	23	112	56	184	27
P	194	273	13	10	5	0	100	[illegible]
P		284	13	8	3	0	106	[illegible]
P + 30 mg% taurocholate		242	7	9	3	0	103	
P + 15 mg% chenodeoxycholate		237	10	8	3	0	100	[illegible]

hours, the density was adjusted and the preparatory ultracentrifugation performed (12). Part of the supernatant was taken for beta cholesterol and beta lipoprotein protein determinations, and part was taken for analytical ultracentrifugation. As the results in table 4 indicate, there was no consistent change in the quantities of lipoproteins floating at the various rate increments, in the quantities of beta lipoprotein cholesterol, or in those of protein in beta lipoprotein. Furthermore, when sera and sera with added bile salts were subjected to paper electrophoresis, the relative quantities of protein staining sub-fractions were almost identical.

DISCUSSION

It seems clear that either the feeding of cholic acid and cholesterol or the acute obstruction of the bile ducts of rats results in markedly elevated beta lipoprotein levels (ultracentrifugation) and in beta lipoproteins of an apparently unusual composition. The levels of beta cholesterol were greatly elevated, while the quantity of protein in the beta preparations were elevated only slightly above the values for untreated control rats.

It is not clear (17) whether the abnormal beta lipoproteins seen in humans with primary biliary cirrhosis result from the elevations of bile acids in the sera or from secondary hepatocellular changes. The rapidity with which the lipoprotein concentration and composition changed in rats after 4 days bile duct obstruction suggested the former alternative. There was no evidence from ultracentrifugal or electrophoretic analyses that any of the labelled taurocholate in sera of rats treated with cholate-24-C^{14} was found in either alpha or beta lipoprotein or alpha or beta globulins, respectively. Although the electrophoretic analyses were not such as to afford certain proof of taurocholate-albumin binding, the findings do support those of Rudman and Kendall (7) and others in this respect.

The complete inability to modify the ultracentrifugal analyses of lipoprotein concentration and composition or electrophoretic mobility and distribution of serum proteins by the addition of various bile salts to sera strongly suggests that cholatemia, per se, does not account for the lipoprotein abnormalities seen in bile duct obstruction or cholesterol-cholate feeding.

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Various Natural and Modified Bile Acids in Cholesterol Metabolism[1]

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It has been observed consistently that rats fed diets containing cholesterol or cholic acid have slightly higher serum cholesterol levels than those fed unsupplemented diets. When cholesterol and cholic acid are fed together, the serum cholesterol levels are dramatically increased, the values being proportional to the logarithm of dietary cholic acid concentration over a fairly wide range (Hegsted et al., '57; Portman, '60).

In an attempt to elaborate the mechanism of the cholic acid-induced hypercholesterolemia, a number of studies comparing cholic acid with other bile acids have been carried out (namely, Member et al., '44; Swell et al., '53). These reports indicated that taurocholic and glycocholic acids are more active than cholic acid in rats. Another study (Portman, '60) indicated that certain reported bacterial conversion products of cholic acid, namely, deoxycholic, 7-ketodeoxycholic, 12-ketolithocholic, and dehydrocholic acids, were not as active as cholic acid in elevating the serum cholesterol level of rats. On the other hand, Howe et al. ('60) observed that taurocholic acid was less active than cholic acid in elevating the serum cholesterol level of mice. Beher and co-workers ('60) have studied the effect of several bile acids and have emphasized the low serum cholesterol values in mice fed hyodeoxycholic acid. In addition, Howe et al. ('60) have emphasized the effectiveness of hyodeoxycholic, chenodeoxycholic, and lithocholic acids in counteracting the hypercholesterolemic effect of cholic acid in mice.

In view of the demonstration by Bergström ('59) that the conversion of cholesterol to bile salts in the liver is dependent on the level of a bile acid "feedback" via the portal vein, it is possible that the effects of the various antagonistic bile salts may be mediated through an interference with the feedback signal, perhaps by interfering with the absorption of endogenous taurocholate (in the rat).

The present report involves the evaluation in rats of a number of naturally occurring and substituted bile acids for their effects on serum and liver cholesterol concentrations, serum β-lipoprotein concentrations, and weight gains. The metabolism of 3α-, 7α-, 12α-triformoxycholanic acid-24-C^{14}, which was completely inactive in the feeding trials in elevating the serum cholesterol level, was studied. An evaluation of the effect of feeding diets containing triformoxycholanic acid to Cebus monkeys was also made.

EXPERIMENTAL

Male albino rats weighing 200 ± 10 gm were selected. They were caged individually and given water ad libitum. The animals were control-fed; this was essentially ad libitum feeding, since there was no difference in food intake with the relatively low levels of dietary bile acids which were used. The percentage composition of the basal diet was as follows: casein, 12.0; sucrose, 73.0; hydrogenated vegetable fat,[3] 10.0; salts (Hegsted et al., '41), 4.0; inositol, 0.1; choline, 0.2; *p*-aminobenzoic

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[2] This study was made during the tenure of an Established Investigatorship of the American Heart Association.

[3] Spry, Lever Bros. Company, New York.

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TABLE 1
Bile acids evaluated in this study

Trivial name	Chemical name	Source
Cholic acid	3α, 7α, 12α-trihydroxycholanic	Eastman Organic Chemicals, Rochester, New York
Taurocholic acid	Taurine conjugate (a)	Cortese and Bashour ('37)
Cholic acid + taurine (2%)		Eastman Organic Chemicals: Nutritional Biochemicals Corporation
Deoxycholic acid	3α, 12α-dihydroxycholanic	General Biochemicals, Inc. Chagrin Falls, Ohio
Lithocholic acid	3α-hydroxycholanic	Mann Research Laboratories, Inc. New York
Apocholic acid	3α, 12α, $\Delta^{8(14)}$-dihydroxycholenic	Boedecker ('20)
Triformoxycholanic acid	3α, 7α, 12α-triformoxycholanic	Cortese and Bashour ('37)
Diformoxycholanic acid	3α, 12α-diformoxycholanic	Modification of Cortese and Bashour ('37)
Triformoxycholanyl chloride	3α, 7α, 12α-triformoxycholanyl chloride	Cortese and Bashour ('37)
Triacetoxycholanic acid	3α, 7α, 12α-triacetoxycholanic	Bergstrom et al. ('53)
Very unsaturated acid	Unknown—apocholic preparation	Boedecker ('20)
Cholanic acid	Same	Mann Research Laboratories, Inc.
3 β-Hydroxy bisnor Δ^5 cholenic acid	Same	Mann Research Laboratories, Inc.
Methyl cholate	Methyl-3α, 7α, 12α-trihydroxycholanate	Transmethylation with H_2SO_4-MeOH

acid, 0.1; cholesterol, 0.45; and standard supplements (Portman and Mann, '55) of fat and water-soluble vitamins. Cholesterol was excluded from some of the diets.

Bile acids were included at 0.0098 moles per kg of diet (equivalent to 0.40% of cholic acid). The trivial names, chemical names, and the source or method of synthesis of the bile acids which were evaluated are listed in table 1. The structural formula for the parent bile acid, cholanic acid, is illustrated in figure 1. Rats were fed the experimental diets for 21 days. They were weighed twice weekly and at the termination of experiments.

The rats were decapitated and samples of serum and liver were obtained. Aliquots of both fractions were taken, homogenized in chloroform:methanol (2:1), incubated at 40°C for 30 minutes, brought to the mark and filtered through fast filter paper into culture tubes with teflon lined caps. Liver and serum lipids were chromatographed on silicic acid columns, by a

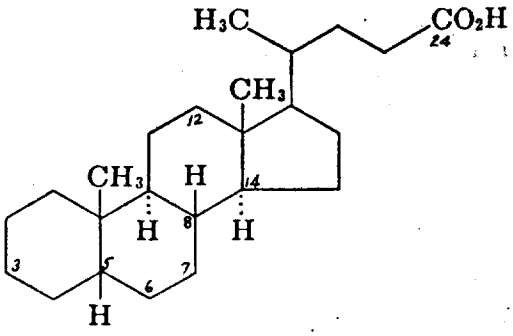

Fig. 1 The structural formula of cholanic acid, the parent compound to those bile acids evaluated in this study. Positions 3, 5, 6, 7, 8, 12, 14 and 24 which were involved in the various substituted compounds considered, are indicated.

method previously described (Portman et al., '59). Free and ester cholesterol determinations were performed on the appropriate fractions by a modification of the method of Abell et al. ('52). Serum β-lipoproteins were separated and analyzed

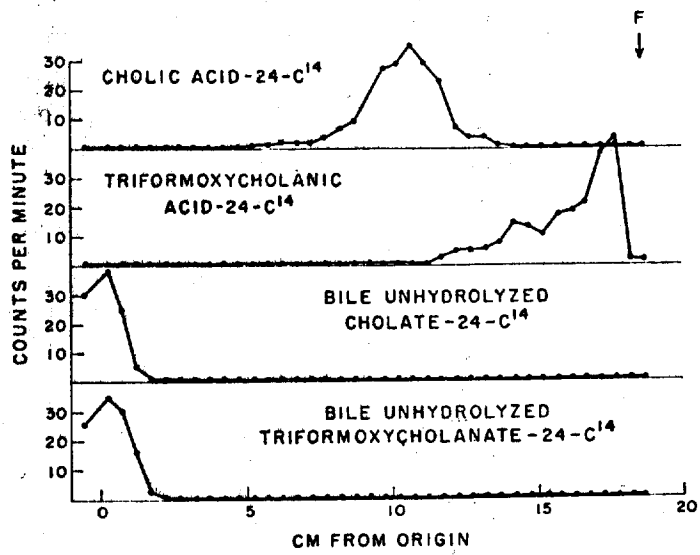

Fig. 2 Paper radiochromatographs of cholic acid-24-C^{14}, triformoxycholanic acid-24-C^{14}, unhydrolyzed bile from a rat given cholate-24-C^{14} and bile from a rat given triformoxycholanate-24-C^{14}. The chromatographic system was that of Sjövall ('54). The moving phase was isopropyl ether: *n*-heptane, 80:20 (v/v).

in certain cases by the ultracentrifugal method of Gofman et al. ('49). Bile acids were separated in certain instances by paper chromatography using the procedures of Sjövall ('54).

Four Cebus monkeys were also used to evaluate the effect of one of the bile acids, 3α-, 7α-, 12α-triformoxycholanic acid, which gave interesting results in the rat experiments. The serum cholesterol levels of these monkeys varied between 120 and 600 mg per 100 ml at the time of initiation of bile acid feeding, depending on the previous diet. Serum cholesterol determinations were measured weekly.

RESULTS

Effect of bile acids in diets containing cholesterol. In table 2 are shown mean values for total serum cholesterol, serum β-lipoproteins, total and free liver cholesterol, liver weight, and 21-day weight gain for the first experiment in which 0.45% of cholesterol and a variety of bile salts were included in the diet.

Three of the bile acid supplements resulted in mean serum cholesterol values which were not significantly elevated above those observed in the rats fed the basal (cholesterol-containing) diet without bile acids added. These were lithocholic and triformoxycholanic acids, and triformoxycholanyl chloride. Diformoxycholanic, triacetoxycholanic, and the highly unsaturated bile acids produced serum cholesterol values which were slightly higher than those of the first group ($P \leq 0.1$). At the other end of the spectrum, cholic acid, taurocholic acid, methyl cholate, and cholic acid fed in a diet containing 2% of taurine produce very high mean terminal serum cholesterol levels which statistically were indistinguishable. Intermediate mean serum cholesterol values were observed in the rats fed deoxycholic and "apocholic" (3α-, 12α-dihydroxy $\Delta^{8\,(14)}$-cholenic) acid.

Mean total liver cholesterol concentrations ranged from 7.5 mg per gm of wet tissue for the group fed triformoxycholanic acid to 50.6 mg per gm for the group fed cholic acid. The values for the other groups were distributed in the same general order as were the serum values. The liver free-cholesterol values of the various groups were not strikingly different, since most of the elevations in the liver cholesterol concentrations were in the ester fractions.

Effect of dietary bile acids and mixtures of bile acids. Since triformoxycholanic

TABLE 2

The effect of feeding a variety of bile acids at 0.0098 moles/kg of diet (equivalent to 0.4% of cholic acid) on the serum cholesterol and β-lipoprotein concentrations, liver weight, liver free and ester cholesterol concentrations and 21-day weight gain[1]

Supplement	Serum total cholesterol	β-Lipoproteins[2]					Liver weight	Liver cholesterol		Weight gain
								Total	Free	
	mg/100 ml	*mg/100 ml*					*gm*	*mg/gm*		*gm*
None[3]	110 ± 13.6[4]	0	0	0	0	24	11.88	7.6	2.2	67
Triformoxy-cholanic	113 ± 5.5	0	0	0	0	119	14.63	7.5	2.4	75
Triformoxy-cholanyl-Cl	118 ± 10.6	0	0	0	0	100	12.66	8.5	2.3	55
Lithocholic	118 ± 9.4	0	0	0	0	119	13.35	10.1	2.6	71
Diformoxy-cholanic	139 ± 7.2	0	0	10	100	124	13.20	13.9	2.8	67
Triacetoxy-cholanic	145 ± 16.5	0	0	2	52	119	14.75	11.2	2.7	62
Very unsaturated acid	166 ± 16.5	0	0	17	119	48	15.55	27.4	3.2	63
Apocholic	236 ± 11.8	0	0	14	157	171	15.76	36.4	3.6	82
Deoxycholic	262 ± 27.2	0	0	28	188	112	15.03	30.7	3.2	62
Taurocholic	325 ± 17.9	0	0	31	262	176	15.61	46.7	3.7	63
Cholic	334 ± 19.6	0	0	40	252	169	16.39	50.6	3.8	79
Cholic + 2% taurine	336 ± 31.2	0	0	31	236	131	15.38	43.9	3.8	66
Methyl cholate	377 ± 27.3	0	0	28	242	248	15.39	46.0	3.8	60

[1] All diets contained 0.45% of cholesterol.
[2] β-Lipoprotein concentrations list, respectively, the concentrations (mg/100 ml of serum) of the Sf 0–12, 12–20, 21–35, 35–100, and 100–400 fractions. The concentrations are based on refractive indices determined for human β-lipoproteins.
[3] Five rats/group.
[4] Values are means ± standard errors of the means.

acid seemed to be devoid of the serum- and liver-cholesterol-elevating properties characteristic of cholic acid in diets containing cholesterol, this compound was further evaluated in a second experiment. Triformoxycholanic acid was fed in cholesterol-containing and cholesterol-free diets, and was also fed in combination with cholic acid. In this trial two other bile acids, cholanic acid and 3 β-hydroxy bisnor Δ^5–cholenic acid were evaluated in diets containing cholesterol. The results are indicated in table 3.

The rats fed cholesterol-free diets with either cholic acid or cholic acid plus triformoxycholanic acid had higher serum and liver cholesterol values, whereas those fed diets containing triformoxycholanic acid were not different in these respects from the unsupplemented controls.

Among the rats fed cholesterol, the group fed cholic acid again had the highest concentrations of serum and liver cholesterol. The serum concentrations of the groups fed triformoxycholanic or cholanic acids were not statistically different from the values for the controls not fed bile acids. The group fed 3 β-hydroxy bisnor Δ^5–cholenic acid had a lower mean serum cholesterol value than the control group ($P = 0.10$). The group fed equal molar concentrations of cholic and triformoxycholanic acids (two times the molar concentration of bile salts fed to the other groups) had a lower mean serum cholesterol concentration than the group fed

TABLE 3

The effect of feeding different bile acids at 0.0098 moles/kg of diet (equivalent to 0.4% of cholic acid) in cholesterol-free and cholesterol-containing diets[1,2]

Bile acid supplement	Cholesterol supplement	Serum total cholesterol	β-Lipoproteins					Liver weight	Total liver cholesterol	Weight gain
		mg/100 ml	*mg/100 ml*					*gm*	*mg/gm*	*gm*
None	0	80 ± 1.8[3]	0	0	0	0	296	11.20	2.1	70
Cholic	0	111 ± 5.9			—			11.90	5.0	55
Triformoxycholanic	0	91 ± 8.9			—			12.60	2.3	80
Cholic + triformoxycholanic	0	107 ± 7.6			—			13.60	3.8	59
Cholic	+	499 ± 41.0	0	0	88	536	224	14.30	39.0	63
Triformoxycholanic	+	127 ± 11.4	0	0	31	0	228	13.70	5.5	78
Cholic + triformoxycholanic	+	328 ± 15.9	0	0	38	266	133	14.00	23.9	56
Cholanic	+	142 ± 14.1			—			14.20	10.4	19
3 β-OH Bisnor Δ^5-cholenic	+	108 ± 11.2			—			10.80	7.1	51
None	+	134 ± 10.2	0	0	0	0	228	11.90	12.2	59

[1] In those cases where two bile acids were fed in the same diet, 0.0098 moles of each acid were included/kg of diet.
[2] Footnotes of table 2 also apply to this table.
[3] Values are means ± standard errors of the mean.

cholic acid alone (P = 0.01). The liver cholesterol values formed a distribution similar to that observed for the serum cholesterol. The liver cholesterol values of the triformoxycholanic groups, however, were lower than those seen in the unsupplemented control group.

The rats fed cholanic acid gained less weight than those in the other groups. There was no significant difference between the mean weight gains in any of the other groups. The sera of all of the rats in the cholanic acid group were of a yellow-green color in contrast with the appearance of all other sera.

Triformoxycholanic acid in Cebus monkeys. Since triformoxycholanic acid appeared to be, of the bile acids tested, one of the least active in elevating the serum and liver cholesterol concentrations of rats, and, in fact, inhibited the effect of cholic acid, further evaluation of this acid was made in Cebus monkeys. Triformoxycholanic acid was fed to three monkeys at 0.0049 moles per kg of diet and to one monkey at 0.0098 moles per kg (the concentration used in the rat diets). The mean pretreatment serum cholesterol values for the monkeys administered the lower concentration of bile acid were 356, 271, and 116 mg per 100 ml; the mean treatment values were 259, 241, and 117 mg per 100 ml. The mean pretreatment and treatment values for the monkey given the higher concentration of dietary triformoxycholanic acid were 670 and 636 mg per 100 ml, respectively. Although a further evaluation of this substituted acid at higher concentrations seemed to be justified, there was no clear-cut effect on Cebus monkeys at the concentrations used.

Absorption and biliary excretion of triformoxycholanic acid-24-C^{14}. Two rats weighing about 350 gm whose bile ducts were cannulated, each received into the upper duodenum one milligram of labeled triformoxycholanic acid (neutralized with NaOH) in 0.6 ml of H_2O. One rat was similarly treated with cholic acid-24-C^{14}.

The cumulative excretion of labeled material via biliary fistulas at 5, 24 and 48 hours for each of the experimental animals is shown in table 4. These data indicate that after the administration of C^{14}-labeled triformoxycholanic acid, large amounts of labeled material are rapidly excreted in the bile. These results indicate that the acid is absorbed from the gastrointestinal tract and then excreted into the bile, although they do not indicate whether there was prior deformoxylation in the intestine or liver. There also is no indication as to whether the labeled acid was conjugated.

Nature of the labeled biliary products. Figure 2 illustrates the paper radiochromatographs (procedure according to Sjövall et al. ['54]) obtained for cholic acid-24-C^{14}, triformoxycholanic acid-24-C^{14}, bile from the radiocholate-treated rat, and bile from a radiotriformoxycholanate - treated rat. The triformoxy acid is markedly less polar than cholic acid. Radioactivity in the two rat biles remained at the origin as is consistent with conjugated acids in this system. Unfortunately, alkaline hydrolysis used for hydrolyzing conjugated bile salts will also hydrolyze the formoxy groups. Therefore, definite identification of the conjugated bile salt in the bile of the rats treated with triformoxycholanic acid-24-C^{14} must await the characterization of taurocholic and synthetic taurotriformoxycholanic acids in other chromatographic systems.

DISCUSSION

Very little definitive information is available about the mechanisms involved in the induction of a pronounced hypercholesterolemia by feeding cholesterol and cholic acid to rats. When either component is fed alone the serum cholesterol level is increased about 25% above the level of the unsupplemented controls. The liver cholesterol ester fraction is increased one-hundred-fold or more when both components are fed together, whereas the feeding of cholesterol without cholic acid results in a smaller and slower accumulation of liver cholesterol ester. The feeding of cholic acid alone causes a relatively small increase in liver cholesterol concentrations.

Clearly, cholic acid is not completely specific in the above respect. Taurocholic and glycocholic acids, as well as methyl cholate, are at least as active as cholic acid. This study, and others (Howe et al., '60), however, indicate that there is a wide range of serum- and liver-cholesterol- potentiating activities of various other natural and substituted bile acids.

The activity of the bile acids is not a simple function of polarity. Apocholic acid and the very unsaturated preparation obtained in the synthesis of apocholic acid should have polarities similar to that of cholic acid, but they possess only intermediate serum- and liver-cholesterol-potentiating activity. One property characteristic of those bile acids which had minimum serum- and liver-cholesterol-elevating properties was the absence of or esterification of the 12 α-hydroxy grouping. The acids in this study which fulfill this criterion were cholanic, lithocholic, 3 β-hydroxy bisnor Δ^5-cholenic, triformoxycholanic, diformoxycholanic and triacetoxycholanic acids. All of the acids which contained a 12 α-hydroxy grouping had some cholesterol-elevating activity. It was also shown in a previous study (Portman, '60) that dehydrocholic and 12-ketolithocholic acids, which have a carbonyl group

TABLE 4

Excretion of labeled material into the bile after the administration of one milligram of triformoxycholanic acid-24-C^{14} or one milligram of cholic acid-24-C^{14} (both neutralized with NaOH) in 0.6 ml of H_2O[1]

Exp. no.	Acid used	Cumulative excretion		
		0–5 hours	0–24 hours	0–48 hours
		%	%	%
1	TFC[1]-24-C^{14}	41.7	85.3	85.3
2	TFC-24-C^{14}	29.8	61.4	78.6
3	Cholic-24-C^{14}	19.2	57.5	69.0

[1] TFC(3 α-, 7 α-, 12 α-triformoxycholanic acid-24-C^{14}) was prepared from cholic acid-24-C^{14} by a modification of the method of Cortese and Bashour ('37).

at position 12, also have some serum-cholesterol-elevating activity.

The studies of Beher et al. ('60) and of Howe et al. ('60) lend weight to the apparent importance of position 12 in the structure of bile acids which elevate the serum cholesterol. Their studies have shown that hyodeoxycholic (3α-, 6α-dihydroxy) and chenodeoxycholic (3α-, 7α-dihydroxy) did not elevate the serum cholesterol level of mice. On the other hand, Howe et al. ('60) reported that the acetylation of the 3- and 7-hydroxy groups of methyl cholate, leaving the 12-hydroxy group unsubstituted, resulted in an inactive compound. It, therefore, remains to be demonstrated whether the other hydroxyl groups contribute to the activity related to the critical 12 position.

The present study agrees with that of Howe et al. ('60), using mice, that methyl cholate is as effective as cholic acid in promoting increased serum cholesterol levels. Since hydrolysis of the methyl ester bond in the gut is, at least, a possibility, it will be necessary to test other substituents in the side chain before concluding that the nature of the side chain does not influence the serum-cholesterol-elevating properties of cholic acid.

This study indicates that the inactivity of triformoxycholanic acid is apparently not related to decreased absorption of the compound. Similarly, cholanic acid, which is also inactive, has been shown by Sjövall and Åkesson ('55) to be absorbed from the gut. Also, Lindstedt and Norman ('56) have not observed striking differences in the turnover rates of a variety of labeled bile acids.

A number of other substituted bile acids are being prepared for dietary evaluation in order to gain a more complete picture of those components of the cholic acid molecule which influence the serum cholesterol level. These include a variety of 12-hydroxy acids, compounds with side chain substitutions, and compounds with ring hydroxy substitutions (namely, amino for hydroxy substituted compounds). If the most active groupings can be established, it will then be possible to investigate the most appropriate substitutions for the development of an active serum-cholesterol-depressing agent.

SUMMARY

A variety of bile acids was included in the diet of rats at 0.0098 moles per kg diet to evaluate their effect on the serum and liver cholesterol concentrations and on serum β-lipoprotein concentrations. A wide range of values was observed. Triformoxycholanic acid, triformoxycholanyl chloride, lithocholic acid, cholanic acid and 3 β-hydroxy Δ^5-cholenic acid did not elevate the serum cholesterol level. The remaining 9 regimens resulted in varying degrees of elevation of serum and liver cholesterol values, but none was more active than cholic acid. Triformoxycholanic acid partially inhibited the action of cholic acid when both compounds were fed together. Triformoxycholanic acid was without significant effect on the serum cholesterol level of Cebus monkeys. Three α-, 7α-, 12α-triformoxycholanate-24-C^{14} was rapidly absorbed from the intestine and excreted into the bile as a conjugated (but uncharacterized) bile acid. The evidence suggesting that the configuration at position 12 is crucial to the serum- and liver-cholesterol-elevating properties of certain bile acids was discussed.

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Hypertension, Thrombosis, and Atherosclerosis in the Rat

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ABSTRACT

A simple high fat diet containing cholic acid has been devised for producing hyperlipemia and an increased incidence of thrombosis in the small coronary vessels of the rat, but without producing significant atherosclerotic lesions. The influence on this syndrome induced by six weeks of desoxycorticosterone administration, 2 mg. daily, and 10 weeks of oral saline (1%) ingestion was investigated in 30 115-g. male rats. Marked hypertension developed only when it was induced prior to beginning the dietary feeding. In comparison to the control groups, the group that was both hyperlipemic and hypertensive had severer hypertension, severer hyperlipemia, double the mortality due to thrombosis and fatty streaks in the aorta but very few lesions of periarteritis nodosa. However, the early atherosclerotic lesions did not seem to be responsible for the increased production of thrombosis. It is therefore probable that under these experimental conditions hypertension has a more direct action on the production of thrombotic effect than that of worsening the atherosclerotic lesions.

A LARGE number of observations in man as well as in experimental animals suggest that intravascular pressure is of paramount importance in the formation of arteriosclerotic lesions.[1] In man, systolic and diastolic blood pressures have been reported to be positively correlated with the degree of coronary sclerosis.[2, 3] Experimentally, in the rabbit fed cholesterol, the intimal lesions develop more quickly and tend to be larger and more widely distributed when the blood pressure is raised by different means.[4, 5] In the rat on certain high fat diets,[6, 7] it has been demonstrated that hypertension increases the sudanophilia in the coronary vessels and the aorta, although no atheromatous plaques have been reported.

The higher incidence of myocardial infarction in hypertensive patients[8, 9] could be explained by the increased severity of the atherosclerotic lesions in this group. However, it is also known that the production of thrombosis does not seem to be always correlated with the degree of atherosclerosis found at autopsy.[10] It is therefore possible that hypertension affects the induction of thrombosis more directly than by merely aggravating the atherosclerotic lesions. In order to verify such a hypothesis, the influence of hypertension on the production of thrombosis in vessels not presenting intimal lesions should be studied. Experimentally, a thrombotic thrombocytopenia has recently been induced in the rat by using a simple diet containing butter and cholic acid.[11, 12] With this diet, the thrombi are preceded by marked serum lipidic changes[11] and by some intimal lipidic infiltration of the large arteries, but not by atherosclerotic plaques or even fatty streaks. The thrombi, composed of platelets and fibrin, occur in capillaries and small vessels, mostly in the heart; there is no apparent intimal lesion of the vessel involved. This experimental condition seems to be ideal for testing the direct role, if any, played by hypertension in the production of thrombosis. It is the purpose of this communication to report the influence of metacorticoid-induced hypertension[13, 14] on some pathologic and biochemical changes caused by the hyperlipemic and thrombogenic diet already described.[11, 12]

MATERIAL AND METHODS

For this experiment 30 male albino rats from the Quebec Breeding Farm, with an initial body weight of 115 g. (range 108-124 g.), were divided equally into three groups. The rats were housed five per cage and were given food and drinking fluid *ad libitum*, 750 g. of Liquamycin per gallon being added to the latter to prevent infections.

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Systolic blood pressure was regularly taken under ether anesthesia by the microphonic cuff method.[15]

Techniques for collecting blood and determining serum total cholesterol and total lipids were those previously described.[11] However, these determinations were made only on the eighth and the 22nd week of the experiment on the pooled serum of five animals: the results expressed in Table I are, therefore, the means of two determinations per group.

For the first seven weeks of the experiment all of the groups were fed Purina Laboratory Chow. From the beginning of the eighth week until the end of the experiment, this was replaced by the following diet in Groups I and III: Purina 53%, butter 40%, cholesterol 5% and cholic acid (Na-salt) 2%. Groups I and II were also given 2 mg. of desoxycorticosterone acetate (DCA) daily by subcutaneous injection, from the beginning of the experiment. After six weeks, hypertension being well developed, this treatment was discontinued. Throughout the entire experiment, Group II received the normal diet of Purina Laboratory Chow and Group III were given tap water to drink.

The study was terminated on the 35th week when all the rats of Group I had died. The survivors in the two other groups were killed with chloro-

TABLE I.—INFLUENCE OF HYPERTENSION ON THE PRODUCTION OF SERUM LIPIDIC CHANGES, AORTIC FATTY STREAKS AND CORONARY THROMBOSIS IN THE RAT

Group	Treatment	Total cholesterol (mg.%) week 12th	Total cholesterol (mg.%) week 20th	Total lipids (mg.%) week 12th	Total lipids (mg.%) week 20th	Incidence of coronary thrombosis (%)	Incidence of aortic fatty streaks (%)	Mortality (%)
I	Purina + cholesterol + butter + cholic acid + (DCA + NaCl)	480	551	943	869	90	70	100
II	Purina + (DCA + NaCl)	113	130 (range 81-248)	308	288	0	0	20
III	Purina + cholesterol + butter + cholic acid	328	438	583	650	30	10	40

Number of animals per group = 10. Total duration of the experiment = 35 weeks.

form, and most of the organs, especially the aorta and the heart, were examined under a stereoscopic microscope and then subjected to histological study. After fixation in Bouin's fluid and embedding in paraffin, the organs were stained by the periodic acid-Schiff (PAS) and in certain cases the phosphotungstic acid-hematoxylin techniques.

RESULTS

In agreement with the work of others,[13,14] DCA administration, NaCl in the drinking fluid, and a Purina diet induced, in six to eight weeks, marked hypertension (Groups I and II, Fig. 1) that was self-sustained even after withdrawal of DCA and NaCl (Group II). Under these conditions, substitution for Purina by a hyperlipemic diet resulted in a further increase in the hypertension, the blood pressure being significantly higher in Group I than in Group II from the eighth to the 24th week. In fact, even the rats of Group III, in which the hyperlipemic diet was the only treatment given, manifested a certain degree of hypertension for several weeks (Fig. 1).

As indicated in Table I, 20% of the animals with hypertension alone (Group II) died in the course

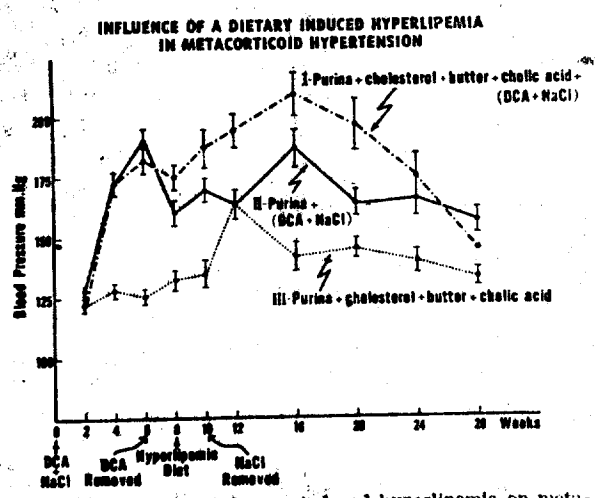

Fig. 1.—Influence of dietary-induced hyperlipemia on metacorticoid hypertension. Results are expressed as mean blood pressure, with standard error, of 10 rats per group.

of the experiment, and did not present more severe lesions than did some of those killed at the end of the study. The mesenteric arteries of 50% of the entire group presented grossly visible nodes ascribed to periarteritis nodosa.[16] Present also in about half of the animals of this group was a single salient fibrous plaque, located in the aortic arch, in front of the openings of the carotid arteries.

In Group I, the combination of hypertension and hyperlipemia resulted in a 100% mortality rate (Table I). All these animals presented small or large yellowish patches on the surface of the heart, particularly visible under stereoscopic microscope examination and similar to those described in previous experiments.[11] On the inner surface of the aortic arch, most of the rats of this group exhibited the peculiar fibrous plaque seen in Group II; but there were also fine white streaks or longitudinal lines slightly raised above the inner surface of the aorta, mostly in its abdominal part but sometimes along its entire length. There was complete absence of grossly visible lesions of periarteritis nodosa in the mesenteric arteries of this group.

In Group III, given the hyperlipemic diet as sole treatment, only 40% of the animals died during the 35 weeks of the experiment, 30% of which (Table I) presented small yellowish patches on the heart surface. White streaks or lines were seen in only one animal of this group, and the fibrous plaque was seen in one other.

As reported in Table I, serum total cholesterol as well as total lipids was constantly higher in hypertensive animals on the hyperlipemic diet (Group I) than in the animals given the hyperlipemic diet only (Group III). In some of the hypertensive rats on Purina diet (Group II), a definite hypercholesterolemia was seen in the 20th week. Even when the mean serum cholesterol concentration was moderate (130 mg.), individual determinations showed a range of 81 mg. (a value essentially normal for a rat on Purina) to 248 mg. (a value reflecting a marked hypercholesterolemia). In the other rats of this group, the serum cholesterol distribution was within these limits.

HISTOPATHOLOGY

Heart.—Histologically, all of the hearts in the Group I animals except one presented some thrombi in capillaries or small vessels, consisting mainly of strongly PAS-positive granular material (Fig. 2) and similar to those described in previously reported experiments.[11, 12] In addition, in the larger vessels of some animals, thrombi responsible for large infarctions were present. Finally, two rats presented voluminous thrombi in the cardiac cavities. Some degree of arterial thickening and hyalinization was also present, with few or no intimal changes. However, in two animals there was a marked subendothelial accumulation of fat (Fig. 3), resembling that seen under different experimental conditions.[12]

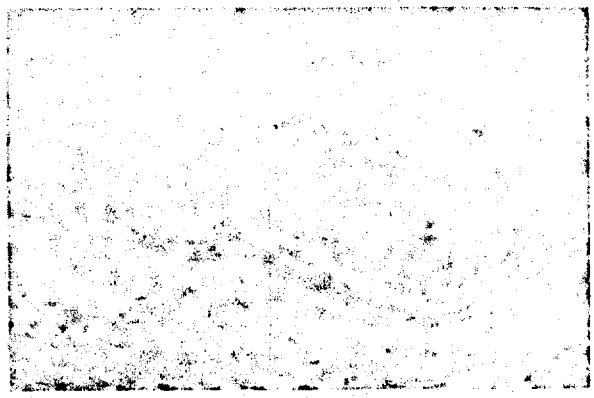

Fig. 2.—A thrombus consisting mostly of strongly PAS-positive granular material in the lumen of a dilated coronary arteriole of a hypertensive-hyperlipemic rat (Group I). (PAS, × 250.)

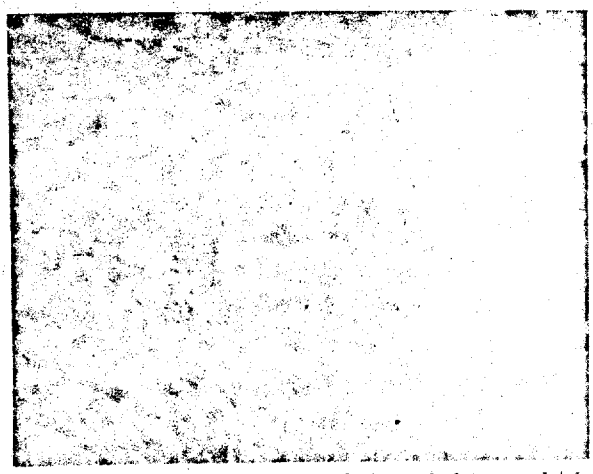

Fig. 3.—Subendothelial accumulation of fat completely obliterating the lumen of a small coronary artery of a rat of Group I. (PAS, × 250.)

The only lesion seen in the hearts of Group II was a mild degree of thickening and hyalinization of the arteries, usually more severe than in Group I; but neither extensive hyalinization of cardiac fibres nor the presence of Aschoff bodies, observed in certain types of experimental hypertension,[16] was noted in this experiment.

The only evident changes in Group III were multiple thrombi in the small vessels in 30% of the animals. These thrombi were responsible for small disseminated patches of cardiac necrosis.

Kidneys.—The kidneys of the animals of Group II presented different degrees of nephrosclerosis, characterized by thickening of basement membranes, hyalinization of the walls of the arteries and of some glomerular tufts, interstitial fibrosis and hyaline casts, as already described for acute experimental hypertension.[16]

The hypertensive-hyperlipemic animals of Group I presented, in addition to the preceding lesions, the nephrotic changes characteristic of the hyperlipemic diet alone.[11]

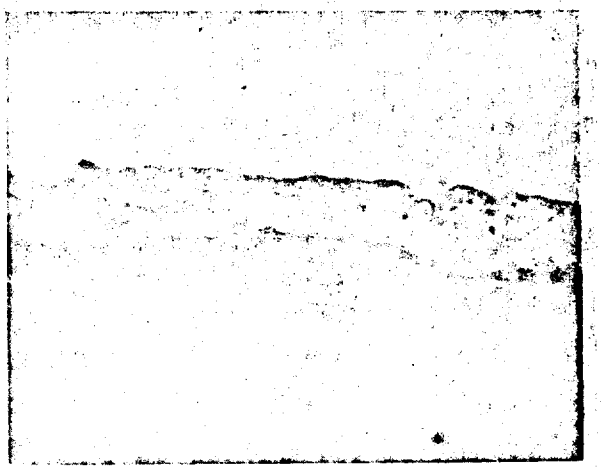

Fig. 4.—A fatty streak in the aorta of a hypertensive-hyperlipemic animal (Group I). Note the lipid-laden macrophages lying on the duplicated internal elastic membrane and covered by endothelial cells. (PAS, × 250.)

In Group III, only lesions of nephrosis were present, with desquamation of the tubular cells and bile pigment in the epithelial cells and the tubular lumen. However, a certain degree of thickening of basement membranes and some hyaline casts were also seen in this group.

Arteries.—In the aortas of the rats of Group I, the streaks or lines grossly visible were found to consist of one or several layers of foam cells on the intimal surface and were covered by a thin endothelium (Fig. 4). Duplication of the internal elastic membrane frequently accompanied these streaks (Fig. 4). In what seemed to be the more advanced stage, fibrosis occurred underneath the endothelium, with proliferation of subendothelial cells, while degenerated lipophages were localized in the area near the internal elastic membrane (Fig. 5). In addition, vegetation-like lesions consisting of layers of foam cells were frequently seen on the semilunar valves of this group (Fig. 6).

In Group III, only one animal presented lipid-laden macrophages in the aortic intima. Even the valves, which in the previous group were a site of predilection for the accumulation of foam cells, were rarely involved in Group III.

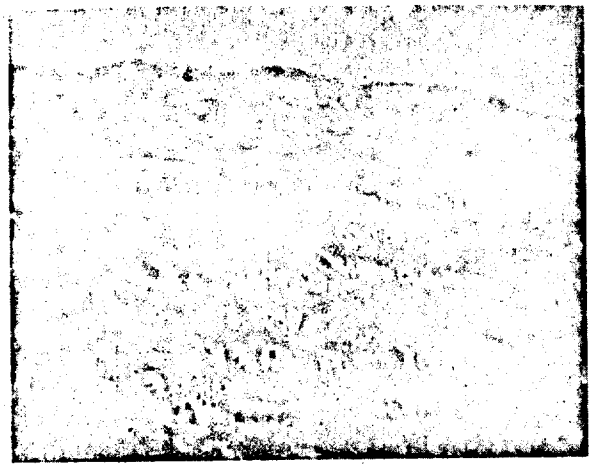

Fig. 5.—A more advanced stage of atherosclerosis than that illustrated in Fig. 4, and characterized by fibrosis and subendothelial cellular proliferation. Only a few degenerated foam cells are still present in the deeper part of the thickened intima. (PAS, × 250.)

Fig. 7.—A fibrous plaque in the aortic arch of a hypertensive rat (Group II). Note the marked thickening of the intima, with duplication of the internal elastic membrane. Some cartilage cells are seen in the deeper part of the intima surrounded by spots of basophilia with a considerable increase in strongly PAS-positive ground substance in the entire plaque. (PAS, × 250.)

The sole marked aortic lesion of Group II was the voluminous plaque seen macroscopically in the aortic arch, involving mainly the intima. This plaque was characterized by a strong increase in PAS-positive ground substance by rupture and duplication of the internal elastic membrane, and in its deeper portion there were cartilage cells surrounded by spots of basophilia (Fig. 7). In Group I, the plaques had essentially the same structure and neither in Group I nor in Group II did they appear to contain lipidic material.

Considerable changes were seen in the mesenteric, splenic and pancreatic arteries of Group II. The walls of these vessels were markedly thickened, with intimal hyalinization, the lumen sometimes being almost completely obliterated. The nodules seen macroscopically in the mesenteric arteries were due to an enormous dilatation of the vessel wall, the inner part being occupied by a voluminous thrombus, organized and recanalized. In the animals of Group I, these vessels presented decidedly less severe lesions, the only evident change being manifested as a certain degree of thickening of their walls.

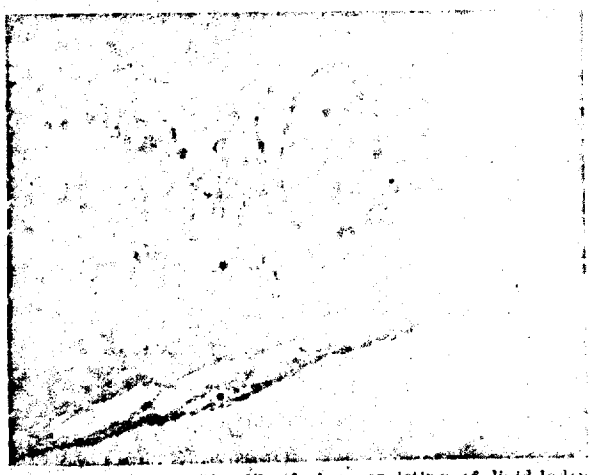

Fig. 6.—A vegetation-like lesion consisting of lipid-laden macrophages on a semilunar valve near its margin attached to the aorta (Group I). (PAS, × 250.)

DISCUSSION

In preliminary experiments devised to induce hypertension in rats already on a hyperlipemic diet, either with or without nephrectomy, marked hypertension failed to develop. It has been reported that a moderate degree of liver damage is sufficient to prevent hypertension.[18] Since the hyperlipemic diet administered caused a rapid lipidic infiltration of the liver, it was concluded that such an investigation was not feasible under the conditions used. For this reason, hypertension was induced first on Purina diet and then, the hypertension being self-sustained, the rats were fed the hyperlipemic diet. This time the hypertension not only persisted but even increased in severity and had the advantage of being almost pure in form, there being no necessity to administer further substances or to induce permanent lesions.

The present experiment would seem to demonstrate the marked potentiating role of hypertension on the production of thrombosis. In fact, for individuals of the same age, the rate of mortality due to thrombosis was more than doubled if, in addition to being hyperlipemic, they were also hypertensive. Of course, the disease produced here is not the experimental counterpart of the human coronary disease. At least, thrombi in which blood platelets and fibrin are involved[12] are significantly induced by more or less physiologic dietary means.

The increase in mortality rate that followed the combination of hypertension and hyperlipemia could have been due to either the increased hyperlipemia or the pre-treatment by DCA. However, previous experiments have shown that, even if continuous DCA administration increases the hyperlipemia produced by the diet used in this

study, the mortality rate is not significantly changed.[19] Nor can the increase be due to the stressful effect of hypertension: various stressor agents applied to rats on this hyperlipemic diet resulted in a decrease of the lipemia and of the mortality rate.[19]

In confirmation of several other observations,[4-7] hypertension seems to potentiate markedly the production of early lesions of the atherosclerosis by cholesterol feeding. In our previous experiments, even when intense lipidic infiltration of the intima and the media of some arteries could be detected,[11] unquestionable early lesions of atherosclerosis, such as fatty streaks, were not evident. In the present experiments, at the time the animals received the hyperlipemic diet they were older, a factor that could explain the changes recorded here. At any rate, at the end of the experiment, only one animal, after 27 weeks on the diet and having received no other treatment, exhibited some deposition of foam cells on the intimal surface of the aorta and semilunar valves that could be interpreted as fatty streaks. On the other hand, all of the animals with the combination of hypertension and hyperlipemia exhibited lipid-laden macrophages in the superficial part or in subendothelial layers of the thickened aortic intima, fibrosis, and also constant duplication of the internal elastic membrane: these lesions seem to be an experimental counterpart of early human atheromatous processes.[20, 21]

The plaques found in the aortic arch, with intimal fibrosis and even with the presence of cartilage, are probably due to hypertension, since these lesions do not correspond to those of ageing breeders[22] fed Purina and since they were mainly present in Group I, in which, during most of the experiment, the blood pressure was highest.

The present experiments seem to confirm the findings that a hyperlipemic diet by itself produces a certain degree of hypertension[23] and that the hypertension is accompanied by a certain increase in blood cholesterol.[24] As a result, a marked potentiating effect on the production of fatty streaks and thrombosis ensued when hypertension was accompanied by hyperlipemia. But in the present experiment the increased incidence of thrombosis in the hypertensive group does not seem to be due to the more severe atherosclerotic lesions. Here the thrombi occur mostly in the small coronary vessels or capillaries not presenting any other evident changes. Hypertension could then have a more direct effect on the production of thrombosis: in fact, thrombi are frequently found in the pure hypertensive state, as was seen in this study in the mesenteric arteries of Group II animals.

A surprising finding was the absence of severe lesions of periarteritis nodosa in the mesenteric or pancreatic arteries of the hypertensive-hyperlipemic animals of Group I, although during most of the experiment the blood pressure in this group was higher than in Group II, in which the animals were not hyperlipemic. We can offer no definite explanation as yet for this phenomenon, since pre-existing hypertension is the most evident etiologic factor in the production of periarteritis nodosa.[25]

SUMMARY

Male rats kept on a simple high-fat diet containing cholic acid presented, within seven months, multiple thrombi in the small vessels of the heart without marked atherosclerotic changes. If hypertension was induced in such animals before the dietary feeding, a marked potentiating effect ensued: blood pressure, serum lipidic changes, occurrence of thrombosis and mortality rate were considerably increased in these animals in comparison with their controls. In addition, consistent aortic lesions of an early atheromatous process were demonstrable in these rats. Nevertheless, the atherosclerotic lesions do not seem to be responsible for the increased incidence of thrombosis.

Although the blood pressure was significantly higher in the hypertensive-hyperlipemic rats than in those which were hypertensive only, lesions of periarteritis nodosa were decidedly less severe.

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COMPARATIVE STUDY OF SOME BILE ACIDS

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Helv. Physiol. Acta 2 (1944)

The preparation of bile acids in the pure form made possible the clean experimental analysis of these important components of bile. H. Wieland /1/ was the first to test the hemolytic effect, the effect on cardiac activity and the toxicity of cholic, desoxycholic and choleinic acis. In 1922, Neubauer /2/ published a report concerning the choleritic effect of bile acid salts and presented comparative data with respect to their surface activity. In 1923, Willstatter /3/ observed the activating effect of bile acids, especially for lipase. These activities created the basis of experimental physiology and pharmacology. The development of this field of research in its final results lead to practical applications in clinical and technical use. Let us mention only the therapeutical utilization of bile acids, as cholereticum and in fat embolism and their technical application as dissolving intermediaries, The very extensive literature concerning the physiological and pharmacological effects of bile acids has been listed monographically on several occasions, e.g. by Bariety /4/ 1927, Shimizu /5/ 1935, Lettre & Inhoffen /6/ 1936, Sobotka /7/ 1937. A review of these works reveals especially the lack of comparative investigations with quantitative and qualitative differentiation of different bile acids from the same source of investigation. The results of the present study primarily represent the satisfaction of this requirement. It extends to the determination of the surface activity, the activation of pancreas lipase, choleretic and hemolytic effects and toxicity investigations. Sodium salts of the most important physiological bile acids: cholic acid, glyco-tauro-and desozycholic acids, and the non-biogenic desoxycholic acid in their chemically pure form. Analysis of the results leads to very substantial quantitative, but also qualitative differences. They permit the establishment of effective series, which differ with respect to biological criteria but still unmistakably reveal certain rules of definite effectiveness.

1. Surface Activity (S.A.)

Neubauer /2/ determined the S.A. of sodium salts of several bile acids for 7 different concentrations by the stalagmometric method. Adler /8/ investigated by the breaking method of Brinkmann & Van Dam /9/ the S.A. of sodium cholate dissolved in serum, bile and urine. Gillert /10/ also reported stalagmometrically obtained results for cholic, desozy- apo-, dehydro-, glyco- and taurocholic acids. Finally, some data were reported by Adlersberg & Lustig /11/ concerning apocholic and dehydroapocholic acid.

The author's determinations were executed according to the recommendations of Lecomte de Nouy /12/ with the breaking method on a torsion balance. Aqueous solutions of the sodium salts of the bile acids listed were used in concentrations between maximum and zero S. A. Each concentration was determined at least four times and the mean value used. The method required rigourously clean work and extensive experience in order to obtain true final values by uniform breaking. The values are set relative to distilled water. The series determination performed are graphically presented in Fig. 1.

FIGURE 1

Relative Surface Tension of Aqueous Solutions of Different Sodium Salts of Bile Acids (Method of Lecomte de Nuys)

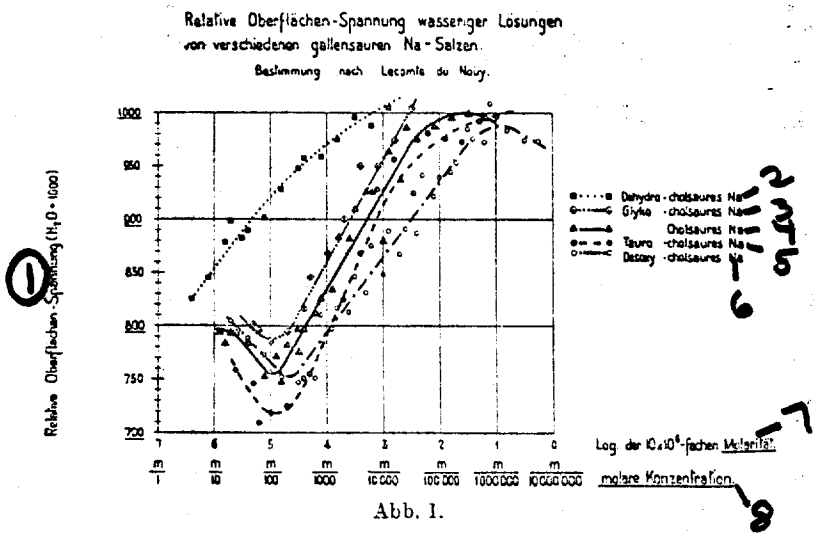


Abb. I.

Key: 1. Relative surface tension (H_2O = 1000); 2. Sodium dehydrocholate; 3. Sodium glycocholate; 4. Sodium cholate; 5. Sodium Taurocholate; 6. Sodium Desoxycholate; 7. Log of molarity x 10 x 10^6; 8. Molar concentration.

Figure 1 presents the variation of the relative S.S. of the five sodium salts of bile acids tested within the concentration range of interest physiologically and pathologically. The reproduction of all of the mean values reveals the substantial scatter of the highly delicate method for all of the concentrations. This scatter renders the image of the family of curves somewhat uneven, but improves its validity. A comparision of the five curves with respect to shape and position yields two fundamental types of bile acids.

Type 1: this includes the 4 natural bile acids: cholic, glyco-, tauro-, and desoxycholic acids. They exhibit an S shape with slight and substantial characteristics. The curves identify three effective ranges: a) the initial S curve in the range of high concentrations, the left leg of which indicates maximum S. A. to a maximum

(point of infection) with decreasing concentrations, followed by the right leg of decreasing S. A. ; the width and depth of the initial S curve varies for the four acids; they are slight for glycocholic acid, wider and deeper for the other three; b) the connective line of the two S curves with S. A. and concentration nearly propostional (linear function); c) the terminal S curve in the range of low concentration. The latter is pronounced with cholic and desoxycholic acid, less characteristic for taurocholic acid and is nearly absent for glycocholic acid. The latter represents a connection between Type 1 and Type 2.

Type 2: the only acid of this type is the non-biogenic dehydrocholic acid. Its variation is almost linear in the concentration range investigated. It exhibits a slight variation from linear behavior in the range of slight concentrations only.

The position of the curves in the coordinate systemcharacterizes the relative S. A. in the concentration range investigated. Dehydrocholic acid has the lowest S. A., followed by glyco-, cholic- tauro- and desoxycholic acids; in conformity with this order, the maxima are at 825; 785; 750; 715. The most important numerical values are given in Table 1, for the range of maximum effect, i. e. for the concentrations which cause a decline of the S. A. of water by more than 20%, also for the limit of effectiveness, i. e. a lowering of the S. A. of water by 2%. The values are given in g% and 10^{-6} mole. Bile acids are listed from the top to the bottom in order of effectiveness. Dehydrocholic acid does not produce a 20% depression of the S. A. of water even in the 10% concentration. Glyco-, cholic-, tauro- and desoxycholic acids follow. The same order is found with respect to the limiting values, i. e. the depression of the S. A. of water by 2%; here, the relative order, assigning the value of 1 to dehydrocholic acid, is such that glycocholic acid is 7 times, cholic acid 18 times, taurocholic acid 82 times and desoxyxholic acid 235 times more effective; mole values are used as the basis for comparision. Relative values for maximum S. A. are dehydrocholic acid 1, clyco- and cholic acid 20, taurocholic acid 30 times and desoxycholic acid 160.

TABLE 1. Surface Activity of Different Sodium Salts of Bile Acids in Aqueous Solution

Key: 1. Sodium salts of . . . ; 2. Dehydrocholic acid; 3. Glycholic acid; 4. Cholic acid; 5. Taurocholic acid; 6. Desoxycholic acid; 7. Range of maximally effective concentrations reducing surface tension more than 20%; 8. Minima, i. e. concentration reducing surface tension of water by 2%; 9. Relative values of effectiveness of dehydrocholic acid = 1; 10. Comparision of planimetrically determined curve areas; 11. Reduces not more than 17%.

SEE NEXT PAGE FOR TABLE 1 ---------

Tabelle 1

Oberflächenaktivität verschiedener gallensaurer Na-Salze in wäßriger Lösung

1 Na-Salze der	Zone maximaler Wirkung Konzentrationen für Erniedrigung der O.F.Sp. über 20% 7		Minima, d. h. Konzentration für Erniedrigung der O.F.Sp. des H_2O um 2% 8		Rel. Wirkungswerte Dehydrocholsäure = 1 9		Vergleich der planimetrisch bestimmten Kurvenflächen 10	
	g/100	10^{-6} Mol	g/100	10^{-6} Mol	Maxima	Minima	absol. in cm^2	rel.
2 *Dehydro-*cholsäure M.G. = 403	10 erniedrigt nicht mehr als 17% 11	248130	0,018	443,1	1	1	60,0	1,00
3 *Glyko-*cholsäure M.G. = 465	2 bis 0,21	43010 bis 4537	0,0028	60,58	20	7,30	93,4	1,55
4 *Chol*säure M.G. = 408	3 bis 0,088	74270 bis 2149	0,001	24,51	20	18	152,6	2,54
5 *Tauro-*cholsäure M.G. = 515	2 bis 0,033	38830 bis 647	0,00028	5,39	30	82	171,5	2,85
6 *Desoxy-*cholsäure M.G. = 392	4 bis 0,036	102040 bis 911	0,000074	1,89	160	234	189,5	3,15

Planimetric measurements of the area limited by the curves and the coordinates yields a measure of the effectiveness of the entire concentration range investigated. In the last column of Table 1 these areas are given in cm^2 (for the arbitrary scale of the original figure) and their relative values. The order is the same as for maximum and minimum effedtiveness, except that differences are smaller because the difference in concentrations has been neglected. The action mechanism of bile acids, especially in the range of high, i. e. 1/10 and 1/1000 molar concentrations, is described here for the first time. It states that bile acids, especially cholic, glucholic, taurocholic and desoxycholic acids which strongly affect the surface tension, display maxima in the range mentioned. Gillerr /10/ actually observed a break at concentrations of n/10 and n/100, but did not interpret it. The order of effectiveness given by the present author does not agree with the findings of Gillert. His tabulated values yield the following order for maximum effect at a concentration of n/10: tauro- glyco-, desoxy- cholic and dehydrocholic acid; the relative series of minimum effect or limiting value is: dehydro- 1. cholic 12. 5, glyco- and desoxy- 30, taurocholic acid 100. Comparision with the authors values yields contradictions primarily with respect to the maximum and minimum surface tension of tauro- and desoxycholic acids.

2. Lipase Activation

In the years 1900 - 1902, Pfluger /13/ described the dissolving effect of bile with respect to fatty acids in the presence of alkali in detail. His findings serve as the basis of the study of the role of bile in the digestion of fats. While no chemically pure bile acids were available and ferment research lacked the necessary foundations, no explanation was possible. Willstatter /3/ succeeded in 1923 in proving through the development of refined fermentation methods in his investigations of activators that sodium cholate has an activating effect on pancreas lipase. Shoda /14/ then examined certain bile acids with respect to their lipase activating ability and compared them qualitatively.

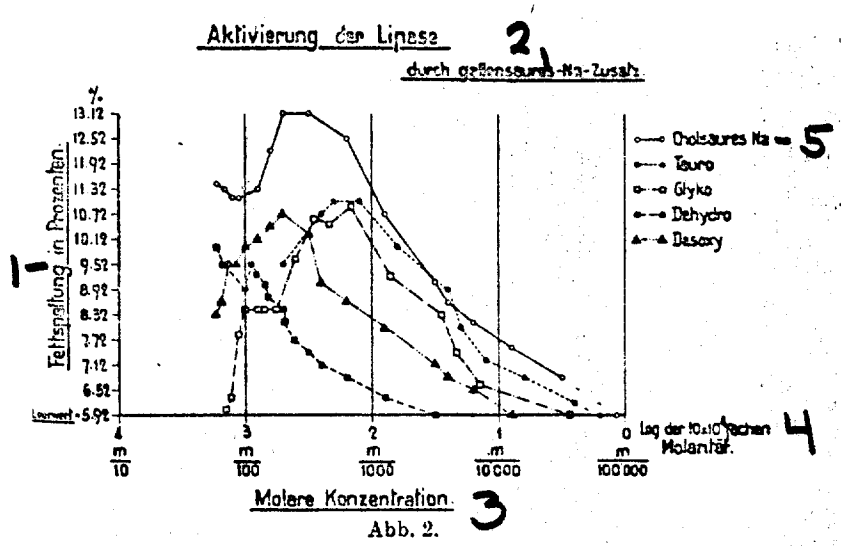
The authors investigation was conducted in accordance with the methodology of Willstatter, but with the elimination of albumin in the initial material. This proved to be necessary, because Willstatter's material creates optimum experimental conditions and reveals only weak bile acid effects on lipase. In the adsence of albumin, different activators including the bile acids. achieve a much greater cleavage of fats. The pancreas lipases were obtained by glycerine extraction. The cleavage values determined are presented in the form of curves in Fig. 2. They refer to concentrations of 1:150-1:75000 or mole/80-60000. Minimum values for tauro- and cholic acids are undetermined. The curves, from right to left, display a rather uniform rise. The initial values of effective limiting concentrations are substantially different (Table 2). The curves show that the lower the limiting value, the higher the maximum. The following order of decreasing effect exists: cholic acid, taurocholic acid, glycocholic acid, desoxycholic acid, dehydrocholid acid. The variation of effect following the maximum is remarkable. As in the case of surface tension, a break occurrs and with increasing concentration the effect becomes weaker. The maximum of breaking point occurs earlier for tauro- and glycocholic acids than for cholic and glycocholic acids. The leg of the curve which declines with increasing concentrations, declines for glycocholic acid to the zero value, which does not occur in the case of tauro- and desoxycholic acid. At the highest concentration investigated, cholic acid exhibits stronger lipase activation than the maximum of the other bile acids. The weakened effect associated with rising concentrations following the turning point (maximum effect), is related to the differential Ca precipitating effect of different bile acids. Dehydrocholic acid here again behaves singularly by not having a leg which declines to the left. It could be assumed that this may take place at higher concentrations, but subsequent tests did not support the assumption.

FIGURE 2

Lipase Activation

Key: 1. Cleavage of fats, %; 2. By a sodium salt of bile acids; 3. Molar concentration; 4. log of 10×10^{6} molarity; 5. --ic acid.

SEE NEXT PAGE FOR FIGURE 2 ---------



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The range of maximum activation and that of limiting values is presented numerically in Table 2. Bile acids are listed in the sequence of their effect on lipase activity. The numerical values show unambigously that in the case of maximum activation of lipase, dehydrocholic acid has the lowest and cholic acid the highest effect, while the three other representatives, desoxycholic, glycocholic and taurocholic acid are roughly equivalent and occupy a center position. However, if one rearranges the sequence with respect to the maximum concentration necessary for maximum effect, the order changes substantially: here tauro- and glycocholic acids with very similar and low concentrations are in first place with 0.76 and 0.81%, followed by cholic acid with twice the concentration at 1.6%, then desoxycholic acid with 2%, and finally the least effective dehydrocholic acid with 3.6%. Relative values calculated with respect to dehydrocholic acid express this shift in order. With respect to mole values, the following order is established for concentrations of maximum effect: tauro- and glycocholic acid, cholic acid, desoxycholic acid, dehydrocholic acid.

TABLE 2. Experiments in Lipase Activation by Different Bile Acids

Key: 1. Sodium salts of . . . ; 2. . . . -ic acid; 3. Maximum activation of fat cleavage in comparision with control without bile acid addition; dehydrocholic acid = 1; 4. Concentration at maximum activation = optimum concentration; 5. Limiting concentration, i.e. zero value; 6. Planimetrically calculated areas of effective curves, dehydrocholic acid = 1.

SEE NEXT PAGE FOR TABLE 2 ---------

Tabelle 2

Versuche der Lipase-Aktivierung durch verschiedene Gallensäuren

Na-Salze der	Maximale Aktivierung der Fettspaltung im Vergleich zur Kontrolle ohne Gallensäurezusatz Dehydrocholsäure = 1		Konzentration bei maximaler Aktivierung = optimale Konzentration				Grenz-Konzentration bzw. Leerwert		Flächen aus den Wirkungskurven planimetrisch berechnet Dehydrocholsäure =	
	in %	rel.	‰	rel.	10^{-6} Mol.	rel.	‰	10^{-6} Mol.	absol. in cm²	rel.
Dehydrocholsäure M.G. = 403	59	1,00	3,64	1	9030	1	0,13	330,8	15,5	1,00
Desoxycholsäure M.G. = 392	79	1,34	2,00	1,8	5100	1,77	0,03	85,03	41,0	2,65
Glykocholsäure M.G. = 465	80	1,36	0,76	4,8	1630	5,55	0,013	28,67	51,2	3,30
Taurocholsäure M.G. = 515	84	1,42	0,81	4,5	1570	5,75	0,0082	15,9	63,5	4,09
Cholsäure M.G. = 408	119	2,02	1,60	2,30	3920	2,30	0,0047	11,4	89,0	5,74

The same order as for the lowest effective concentration is obtained for maximum activation. Finally, in analogy to the procedure followed in the determination of surface tension, the effect of bile acids in the entire rnage investigated, was measured planimetrically. Here again the order found for maximum activation and limiting concentration was reestablished. Dehydrocholic acid proved to be the weakest, cholic acid the strongest among the bile acids tested.

3. The Choleretic Effect of Bile Acids

Neubauer /2a/ and Pohl /15/ in 1922 investigated and compared the choleretic effect of pure bile acids in rabbits. Additional investigations by Neubauer /2b/, 1923/24, Brugsch /16/, Stransky /17/, Neubauer & Adlersberg /18/ were concentrated mainly on dehydrocholic acid which came into clinical use in 1925, while Adlersberg & Lustig /11/ in 1936 supplemented these investigations by testing the choleretic effect of dehydroapocholic acid.

The investigations of the above authors were performed mainly on rabbits, less frequently on rats or dogs and even more seldom on cats. Although the flow of bile of rabbits normally is rather substantial under narcosis, the choleretic function has a very high stimulation threshold and, above all, numerous experiments indicate that the flow of bile drops rapidly under the initial value and that after repeated stimulation complete exhaustion of secretion may occur. Since it was the authors' intention to compare the effect of the different bile acids quantitatively, this fatigue phenomenon in the bile secretion of rabbits represented an obstacle. Stransky therefore recommended the reinjection of bile secreted and obtained better results. In order to obtain uniform results, this procedure should be continuous. All of these difficulties can be avoided, as shown by extensive experience, by selecting another experimental animal, the cat. Although the bile secretion of narcotized cats (urethane 5 ccm/kg of a 25% solution, subcutaneous) is slow compared to rabbits and dogs, with a droplet of liver bile per 1-3 minutes (the ductus cysticus is tied off), the secretion is uniform to the extent that the droplets appear punctually almost by the second and there is practically no fatigue, even with repeated stimulation; the latter is of special importance. This constant behavior of secretion permits the quantitative evaluation of different bile acids in the same experimental animal and all of the experiments described in this paper were performed on cats. The latter also offer the advantage that the threshold values of choleretically effective substances such as bile acid, Atophan, etc. are lower by several magnitudes than in rabbits. The choleretic effect of the five pure bile acids was investigated in the form of their sodium salt with intravenous and duodenal (with a canulla bound into the duodenum) application, cholic acid serving as the standard. All reported values are verified by at least 4-6 experiments. The progress of the effect after i. v. administration of an effective dose, approx. 4 mg/kg cholic acid, is the following: latent time 2-4 min., maximum effect after 10-15 min.; duration 40-70 min; after the duodenal administration of equally effective doses: latent time 10-15 min., maximum effect after 35-40 min., duration $1\frac{1}{2}$-$2\frac{1}{2}$ hours. As expected, the progress of the i. v. effect is more rapid, steeper and shorter than the duodenal stimulation. Table 3 presents information concerning the absolute effectiveness after intravenous and intraduodenal application, the proportion of the two methods of administration and relative effectiveness. With respect to effectiveness, average doses with the same cholertic effect are compared. The order with intravenous administration with increasing effectiveness is the following: desoxycholic acid has the least effect (1. 0) taurocholic acid is close with 1. 13, followed by the equally effective glycocholic and cholic acids with 1. 35 and, finally, the most effective dehydrocholic acid with three times the effect of desoxycholic acid. .The order of effectiveness with intraduodenal administration shows certain shifts: taurocholic acid is weakest, followed by glycocholic acid with 1. 2; desoxycholic acid is twice as effective, cholic acid 2. 21 times and dehydrocholic acid 5 times stronger than taurocholic acid. A comparision of effectiveness with i. v. and intraduodenal administration is of particular interest: the effect of cholic and desoxycholic acid is equally strong with both methods of application, desoxycholic acid is somewhat stronger intraduodenally than intravenously, glyco- and taurocholic acids, on the other hand,

are only half as strong intraduodenally than intravenously. This comparison includes the entire incremental secretion over the entire duration of the experiment, i.e. total secretion minum initial or basis secretion. It has been mentioned earlier that the increment in bile for i. v. application is attained within 40-70 minutes and with intraduodenal application within $1\frac{1}{2}$-$2\frac{1}{2}$ hours.

TABLE 3. Comparision of the Choleretic Effect of Different Bile Acids Experiments on cats, Intravenous and Intraduodenal Application

Tabelle 3

Vergleich der choleretischen Wirkung verschiedener G[illegible]säuren

Versuche an Katzen, Verabreichung intravenös und intraduodenal

Na-Salze der	Wirksamkeit mittlerer Dosen mit gleichem choleretischem Effekt				Verhältnis der Wirksamkeit	Relative Wirksamkeit, berechnet aus den Mol.-Werten	
	bei i.v. Injektion		bei i.d. Verabreichung		Mol.-Werte i.v. zu i.d.	intravenös Desoxy = 1,00	intraduodenal Tauro = 1,00
	mg/kg	10^{-6} Mol./kg	mg/kg	10^{-6} Mol./kg			
Cholsäure M.G. = 408	4,0	9,804	4,0	9,804	100 : 100 = 1	1,35	2,21
Glykocholsäure M.G. = 465	4,6	9,89	7,9	16,99	100 : 172 = 0,58	1,34	1,28
Taurocholsäure M.G. = 515	6,04	11,73	11,17	21,69	100 : 185 = 0,54	1,13	1,00
Desoxycholsäure M.G. = 392	5,2	13,26	4,16	10,61	100 : 80 = 1,25	1,00	2,04
Dehydrocholsäure M.G. = 403	1,74	4,32	1,74	4,32	100 : 100 = 1	3,07	5,02

Key: 1. Sodium salts of ...; 2. ...-ic acid; 3. Effectiveness of average doses with equal choleretic effect; 4. With i.v. injection; 5. With i.d. application; 6. Ratio of effectiveness, molar values, i.v./i.d.; 7. Molar values; 8. Relative effectiveness calculated from molar values.

In the experiments, the sodium salts of bile salts were used always. The problem was whether in intraduodenal applications there is a difference between the effect of free acids insoluble in water and their soluble sodium salts. The result is absolutely unambiguous in the sense that bile acids have the same effect in both forms. This is true for all five acids investigated.

4. The Hemolytic Effect of Bile Acids

Patzschke /19/ was the first in 1919 to describe hemolysis experiments with Na glycocholate. He was followed in 1920 by H. Wieland who found that Na desoxycholate was 9 times stronger hemolytically with respect to the erythrocytes of cattle than Na cholate. Next, the investigations of Neubauer /1922-1924/ must be mentioned in particular; he demonstrated the extraordinarily weak hemolytic effect of dehydrocholic acid. Additional experiments were performed by Kaziro & Tsuji /20/, 1930, Adlersberg & Lustig /11/, 1936.

The authors'experiments were conducted in the following manner: the experimental arrangement was identical with the one used in the testing of erythrocytes for osmotic resistance against NaCl. The erythrocytes obtained from humans, cats and rabbits by repeated washing with 0.9% NaCl and centrifuging were maintained in a 0.9% NaCl solution and suitable series of concentrations of the five bile acids at room temperature and values read in intervals of 10, 20 and 30 minutes and 1, 2, 3, 4, 6 and 24 hours. The hemolytic effect after 60 minutes served as the reference value. Since the three types of erythrocytes behave differently, relevant values are presented in the tabular summary of Table 4 and both concentrations and their molar values are considered. Relative values are with reference to mole values.

FIGURE 3

Hemolytic Effect of Different Bile Acids (Sodium Salts) Representation of the Range from Start to Complete Hemolysis Humans: Erythrocytes, Cats: Erythrocytes Rabbits: Erythrocytes

Key: 1. log of 10×10^6 molarity, 2. Molar concentration, 3. Order of effectiveness.

SEE NEXT PAGE FOR FIGURE 3 ---------

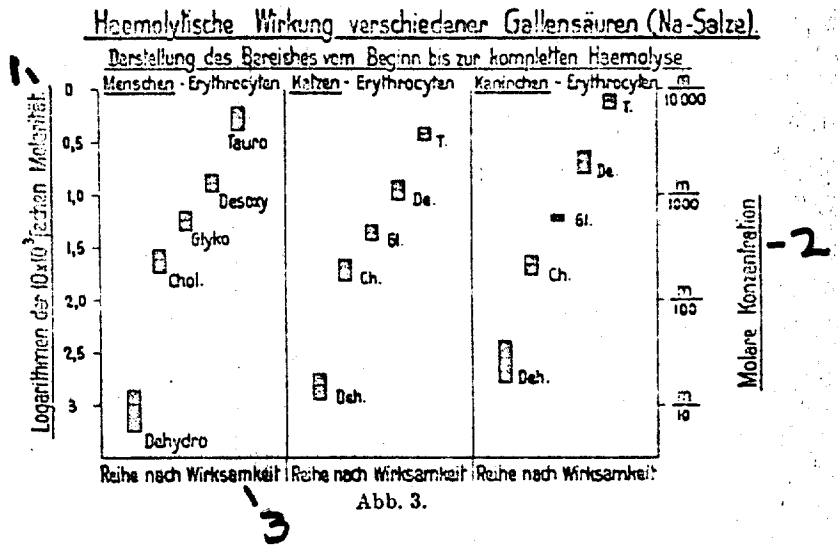


Abb. 3.

It is seen from the values presented that the erythrocytes of cats are more resistant against all bile acids -- with the exception of dehydrocholic acid -- than those of humans; the erythrocytes of rabbits were always the most sensitive. This behavior of the 3 types of erythrocytes is valid not only for hemolysis but also for osmotic resistance. Quantitative differences between the types of erythrocytes are different in magnitude with reference to different bile acids; they are pronounced for dehydrocholic acid, small for desoxy-, tauro- and glycholic acid, and very small for cholic acid. The compilation of relative effectiveness with respect to molar values yields very great differences inthe hemolytic effects of the 5 bile acids. For all 3 types of erythrocytes, the following order exists according to rising effectiveness: dehydro- (1), cholic (24) glycocholic (50), desoxycholic (135) and taurocholic acid (420). The figure in parentheses refer to erythrocytes of humans, for which the greatest differences exist in ehmolytic effectiveness.

TABLE 4. Hemolytic Effect of Different Bile Acids

Key: 1. Sodium salts of ...; 2. -ic acid; 3. Average hemolysis concentration; 4. Of humans; 5. Of cats; 6. Of rabbits; 7. Relative effectiveness with respect to molar values, dehydrocholic acid = 1.

SEE NEXT PAGE FOR TABLE 4. ---------

Tabelle 4

Hämolytische Wirkung verschiedener Gallensäuren

Na-Salze der	Mittlere Hämolyse-Konzentrationen						Relative Wirksamkeit auf Mol.-Werte bezogen Dehydrocholsäure = 1		
	Erythrozyten von Mensch		Erythrozyten von Katze		Erythrozyten v. Kaninchen				
	‰	10^{-4} Mol.	‰	10^{-4} Mol.	‰	10^{-4} Mol.	Mensch	Katze	Kaninchen
Dehydrocholsäure M.G. = 403	4,0	992,5	26	645	14,3	355	1	1	1
Cholsäure M.G. = 408	1,66	40,8	2,0	49,0	1,9	46,6	24	13,2	7,6
Glykocholsäure M.G. = 465	0,80	17,2	1,05	22,6	0,77	16,6	58	28,6	21,4
Desoxycholsäure M.G. = 392	0,30	7,65	0,35	8,95	0,186	4,75	130	72,0	75,0
Taurocholsäure M.G. = 515	0,1	1,94	0,13	[illegible]	0,066	1,30	511	256	273

In Fig. 3, the hemolytic capability of the 5 bile acids investigated is presented. It demonstrates not only the order of effectiveness for the three types of erythrocytes, but also reproduced the range of concentration from incipient to complete hemolysis; the latter is expressed by the height of the column. The line in each column corresponds to the mean hemolysis concentration at 20°C within 60 minutes. The scatter of the other bile acids are rather regular for humans and cats, but are irregular for rabbits. The scatter of dehyrdocholic acid is particularly great for the erythrocytes of humans and rabbits.

5. Toxicity

Lethality experiments on guinea pigs with pure bile acids were performed by Wieland /1/ for cholic and desoxycholic acid, Neubauer /3c/ for dehydrocholic acid on rabbits and guinea pigs -- he found the acid of little toxicity even with i. v. injection, Gillert /10/ for glyco-, tauro-, and apocholic acid on rabbits, by Williams /21/ for taurocholic acid with i. p. injection and, finally, by Aldersberg & Lustig /11/ for apo- and dehydro-apocholic acid in rats.

The author's experiments were conducted on white mice with i. v. administration and a constant injection duration of 30 sec. No clear differences in toxic phenomena are apparent either in toxic or lethal doses of the 5 bile acids. Immediately following the injection, strong motor agitation appears; it is manifested mainly by scratching; the motor agitation increases with rising dosage to clonic-tonic convulsions with atexia and confusion rising in the interval, then,

after a phase of extreme convulsions, complete exhaustion dominates the toxic syndrome in which exitus occurs. In most cases, the animals succumb within 5-10 minutes after the injection; they last for 1-2 hours in rare cases only; surviving animals display the same symptoms in a more or less pronouned degree; they recover slowly, usually within 5-8 hours, completely.

After bracketing the upper and lower limiting doses in preliminary experiments, the principal experiment was performed with 36-74 animals for each bile acid. In Table 5, the results are calculated for the mortality range, for mean toxicity L. D. 50% by the method of Karber; the relative toxicity thus determined is listed and arranged for the 5 bile acids in order of increasing effectiveness; the order is: dehydro-, glyco-, cholic, tauro- and desoxycholic acid. The calculated relative toxicity indicates that desoxycholic acid is 8 times, cholic, glyco- and taurocholic acids 3.5-4.7 times more toxic than the least toxic dehydrocholic acid. The toxicity of the different bile acids thus displays very great differences. The values for the range of mortality further indicate that it is very broad for cholic and desoxycholic acid and very narrow for tauro-, glyco- and dehydrocholic acid. The values are in good agreement with those of Gillert /10/ found with i. v. injection in rabbits.

TABLE 5. Toxicity of Different Bile Acids in White Mice I. v. administration; 30 second injection

Tabelle 5

Toxizität verschiedener Gallensäuren bei der weißen Maus

Verabreichung intravenös: Injektionsdauer 30 Sekunden

Na-Salze der (1)	Anzahl der Versuche (3)	Bereich der Mortalität L.D. 0% bis L.D. 100% mg/g (4)	L.D. 50% nach *Kärber* (5) mg/g	L.D. 50% nach *Kärber* (5) 10^{-6} Mol./g	Relative Toxizität (L.D. 50%) Dehydrocholsäure = 1,00 (6) Gew. Menge mg/g (7)	Relative Toxizität (L.D. 50%) Dehydrocholsäure = 1,00 (6) 10^{-6} Mol./g
Dehydrocholsäure (2) M.G. = 403	36	1,1 –1,4	1,23	3,05	1,00	1,00
Glykocholsäure M.G. = 465	54	0,24–0,40	0,37	0,8	3,30	3,85
Cholsäure M.G. = 408	74	0,20–0,50	0,35	0,86	3,50	3,55
Taurocholsäure M.G. = 515	56	0,20–0,36	0,33	0,64	3,70	4,76
Desoxycholsäure M.G. = 392	60	0,10–0,20	0,15	0,38	8,20	8,00

Key: 1. Na salts of ...; 2. -ic acid; 3. Number of experiments; 4. Mortality range, L. D. 0% to L. D. 100%; 5. L. D. 50% by Karber's method; 6. Relative toxicity (L. D. 50%); dehydrocholic acid = 1; 7. Weight.

For the purpose of discussion, experimental results of 5 bile acids were summarized in relative effectiveness series calculated from molar values in Table 6. The weakest member was set equal to 1.0 and the effect of the other bile acids was related to it. The weakest member is at left, the strongest at the right of the table, the rest is arranged between them. 1, 2, 3, 4, 5 indicate relative degrees of effectiveness. Essential criteria of each principal effect of the bile acids investigated are considered. With a single exception, concentration of maximum lipase activation, the effect always rises from left to right. A glance at the sequence of bile acids tested, shows that they differ for all of the criteria. Analysis of the relationship between the individual criteria for the different bile acids yields highly interesting results.

TABLE 6. Summary of the Relative Effective Series of Bile Acids Investigated

Key: 1. Surface tension in water: maximum effect, minimum effect, planimetric values; 2. Activation of pancreas lipase of swine: maximum clevage, concentration for maximum cleavage, planimetric values; 3. Choleretic effect in cats: i.v. injection, intraduodenal administration; 4. Hemolytic effect on erythrocytes: humans, cats, rabbits; 5. Toxicity in white mice; 6. i.v. injection.

Tabelle 6

Zusammenfassung der relativen Wirkungsreihen der untersuchten Gallensäuren

	1	2	3	4	5
Oberflächen-Spannung in Wasser:	Dehydro	Glyko	Chol	Tauro	Desoxy
1- Maximale Wirkung	1,00	20,00	20,00	30,00	160,00
Minimale Wirkung	1,00	7,30	18,00	82,00	234,00
Planimetrie-Werte	1,00	1,55	2,54	2,85	3,15
Aktivierung der Pankreas-Lipasen des Schweins:	Dehydro	Desoxy	Glyko	Tauro	Chol
Maximale Spaltung	1,00	1,34	1,36	1,42	2,02
2- Konzentration für maximale Spaltung	1,00	1,77	5,50	5,75	2,30
Konzentration für minimale Spaltung	1,00	3,9	11,5	26,00	30,00
Planimetrie-Werte	1,00	2,65	3,30	4,09	5,74
Choleretische Wirkung bei der Katze:	Desoxy	Tauro	Glyko	Chol	Dehydro
3- *Intravenöse* Injektion	1,00	1,13	1,34	1,35	3,07
	Tauro	Desoxy	Glyko	Chol	Dehydro
Intraduodenale Verabreichung	1,00	2,04	1,28	2,21	5,02
Hämolytische Wirkung auf Erythrozyten	Dehydro	Chol	Glyko	Desoxy	Tauro
4- Mensch	1,00	24,00	58,00	130,00	511
Katze	1,00	13,00	28,6	72,00	255
Kaninchen	1,00	7,6	21,4	75,00	273
5- *Toxizität* bei der weißen Maus:	Dehydro	Chol	Glyko	Tauro	Desoxy
6- Intravenöse Injektion	1,00	3,55	3,85	4,76	8,00

a) Surface Tension to Lipase Activity (LA)

There is no parallel in this relationship, only dehydro- and taurocholic acid with a rang of 4 occupy the same place for both criteria, the other three are found in different places. Desoxycholic acid is 5th for surface tension and 2nd for LA; positions of cholic acid are almost as disparate. It must be assumed that surface tension and LA are two independent important biological properties.

b) Surface tension and Choleresis

Here, positions are entirely analogous to those for surface tension and LA. No bile acid occupies the same place for both criteria. Greatest discrepancies exist for dehydro-, tauro- and desoxycholic acids. It is concluded that two entirely independent effect grades exist. This is more remarkable because these are two of the biologically most important properties of bile acids.

c) Surface Tension and Hemolysis

The two series are nearly indentical and only adjacent poistions are interchanged. This indicates a close relationship between the two effects.

d) Surface Tension and Toxicity

Both series are nearly identical so that very close relationships must exist between the two effects.

e) For L. A. and the choleretic effect, agreement exists for glychocholic acid 3, near agreement for desoxycholic acid 2 and for cholic acid 5:4. On the other hand, for dehydro-, 1:5 and taurocholic acid 4:2 or 1; great discrepancies are present. It is thus likely that the effects are related.

f) L. A. and Hemolysis

Agreement is found for dehydro- 1, for glyco- 3 and near agreement for taurocholic acid 4 or 5, but positions are reversed for cholic and desoxycholic acids.

g) L. A. and Toxicity

Positions are analogous to e) and f). It is concluded from the existing great discrepancies that no regular relationships exist between L. A. and the other criteria.

h) Choleresis and Hemolysis or Toxicity

These are evidently entirely different effects, because the sequences are opposed in both cases.

i) Hemolysis and toxicity, on the other hand, again display practically the same order, these effects of bile acids apparently have the same cause.

It is seen clearly that the effects of bile acids with respect to L. A., hemolysis and toxicity are closely interrelated; it is evident that the causation of hemolysis and toxicity are based on the physicol-chemical properties of surface tension, while the two physiological properties of bile acids, i. e. lipase activation and choleresis, are independent.

Bile acids thus have 3 physiologically interesting fundamental properties:

1. Surface activity
2. Lipase activation
3. Choleretic effect

These three properties proved to be indepdent of each other and should be considered the three specific effective characteristics of bile acids.

TABLE 7. Table of Point Evaluation of the General and Special Relationships of Bile Acids and Their Physiological-Therapeutic and Toxic Effect.

Tabelle 7

Tabelle nach Punktbewertung für die allgemeinen und besonderen Beziehungen zwischen Gallensäure und physiologisch-therapeutischer bzw. toxischer Wirkung

Na-Salze der	O.Fl.A.	L.A.	Cholerese	Physiolog.-therapeut. Punkte	Hämolyse	Toxizität	Toxische Punkte
Dehydrocholsäure	1	1	5	7	[illegible]	1	2
Glykocholsäure .	2	3	3	8	3	3	6
Desoxycholsäure .	5	2	1,5	8,5	4	5	9
Taurocholsäure .	3	4	1,5	9,5	5	4	9
Cholsäure	4	5	4	12	2	2	4

Key: 1. Na salts of . . . ; 2. -ic acid; 3. Surface tension; 4. Lipase activation; 5. Choleresis; 6. Physiological-therapeutical points; 7. Hemolysis; 8. Toxicity; 9. Toxic points.

The effectiveness of bile acids has been rated with points in this summary for the sake of a better overview: 1 means weakest, 5 the strongest effect for the effect involved. Physiological and therapeutical effects are treated separately from toxicity. The order of the bile acids is based on the physiologic-therapeutic effectiveness.

A better overview of the general and special relationships of bile acids with the criteria investigated is found in Table 7. Here, each degree of effect is evaluated in accordance with the effect sequence of Table 6, by a 1-5 point system; the weakest effect associated with 1, the strongest with 5. The evaluation is arranged for each bile acid in accordance with therapeutic and toxic effects. The acids are ranked according to their physiologic-therapeutic evaluation; the acid with lowest total is the first, the one with the highest the last. The table shows the relationship between surface tension, hemolysis and toxicity. These three sequences, viewed in vertical direction, are practically identical for all of the bile acids. The close relationships between surface tension on the one hand and hemolysis and toxicity on the other, are emphasized by connecting brackets for the three effects. Lipase activation and choleretic effect, on the other hand, exhibit no solid relationships, either among themselves or with other effects. The above characterizes the interrelationships between the five bile acids in their physiologic-therapeutic and toxic aspects.

Individual analysis shows the unique position of dehydrocholic acid. Its strongest effect is choleretic, affecting lipase and surface activity least; the latter explains its lower hemolytic and toxic activity. This characteristic of the acid immediately renders its special suitability for i. v. injection understandable. Glycocholic acid occupies an intermediate position, both in physiological and therapeutical and in toxic sense. Although its physiological effect is unambigous, it is not as pronounced as that of dehydrocholic acid. Desoxycholic acid has the best effect on surface tension, however, its effect on lipase activation is slight and particularly so on choleresis; the strong surface activity is responsible for the strong hemolysis and toxicity. Taurocholic acid competes with desoxycholic acid, its physiologic-therapeutic effects are pronounced on surface tension and lipase activation, very weak on choleresis; with respect to toxicity, the poistions of both acids are the same. Cholic acid is in first place with respect toall of the physiological effects with the point values of 3, 4, 5 and, with respect to toxicity, it is only slightly toxic. In an overall evaluation of the physiologic-therspeutic effects investigated, cholic acid has the best and most varied effect among all the bile acids investigated, with a relatively low toxicity. Its conjugates, glyco- and taurocholic acid behave differently. The first is less effective physiologically and somewhat more toxic, the latter has a lesser choleretic effect with substantially greater toxicity.

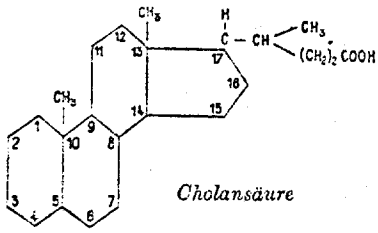
The difference in the quantitative and qualitative effect of natural bile acids apparently corresponds to their different physiological tasks to be per-

formed by these "hormones" of the liver in the metabolic processes of the organism. It should be mentioned finally that the bile acids exercise their maximum effect with respect to surface tension and L. A. at concentrations which are well within the concentrations found under the physiological conditions of the body. The range of maximum effect is between 0.8-2% for L. A. tension and 0.36-40% for surface tension.

Based on their analyses, the authors postulated three independent principal effects of bile acids. A fourth independent property, i. e. the dissolving power for fatty acids, the so-called choleinic acid principle, was not included. According to H. Wieland, these are addition compounds. As seen from the literature, desoxycholic acid is in first place in this respect. Since desoxycholic acid also has the highest surface activity, it is likely that these two physico-chemical properties are based on the same chemical cause.

In the following, a brief attempt will be made to discover relationships between differentiating effects and the structure of the bile acids investigated. A chclopentane-perhydrophenantren structure (see structural formula) is common to all five bile acids, also the side chain attached to C_{17}, $CH\text{-}(CH_2)_2COOH$ (CH_3). The conjugated glyco-and taurocholic acids exhibit in CO-NH bonding glycocoll $H_2N \cdot CH_2 \cdot COOH$ or taurin $H_2NCN_2\text{-}CH_2 \cdot SO_3H$. This differentiates them from their mother substance, the cholic acid. It is seen that the substitutions in peptide bonding (-CO-NH-) do not change the character of a bile acid proper, but rather the intensity of the effects. Compared with cholic acid, glycocholic acid is physiologically somewhat less effective, throughout, but its toxicity is slightly higher. Taurocholic acid exhibits stronger surface activity and lipase activation than cholic acid, but its choleretic effect is strongly reduced and its toxicity increased. The conjugation with glycoll and taurin thus effects both the physiologic-therapeutic and the toxic effects of cholic acid unfavorably.

STRUCTURAL DIAGRAM



Structural formula of cholic acid; if the side chain at C_{17} is removed, the cyclopentane-perhydrophenanthrene central structure of cholesterin, the bile acids, sexual and the adrenal cortex hormones, the cardiacally effective glycosides, of ergosterin, the initial substance of Vitamin D, is present.

Structural differences on the core structure are as follows: all of the bile acids can be derived from cholic acid (see formula):

a) Desoxycholic acid is a 3, 12-dioxycholic acid,

b) Cholic acid and their conjugated derivatives are 3, 7, 12-trioxycholicanic acids,

c) Dehydrocholic acid is a cholic acid less 3 H_2, 3, 7, 12-triketocholanic acid.

Thus, only slight structural differences exist. They are related to the number of OH groups: desoxycholic acid has 2 in the C_3 C_{12} position, cholic acid 3 in the C_3, C_7 and C_{12} positions; also three keto groups of dehydrocholic acid at the atoms of the 3 OH groups of cholic acid. The absence of the OH group of desocycholic acid in the C_7 position results in the increase of surface activity and the decrease of L. A. and especially of choleresis, but in a strong rise in toxicity; groups leads to a strong decline in surface tension and L. A. and a remarkable increase choleresis and a decrease in toxicity. The most important change in biological properties thus is due to the dehydration of cholic to dehydrocholic acid. But the removal of a single OH group: cholic acid desoxycholic acid results in a strong but opposite shift in effects; there is a strong rise in surface activity, hemolysis and toxicity and a significant decrease in the choleretic effect. The effect of the alteration of cholic acid through conjugation with glycocoll and taurin leads to an unfavorable effect in physiological and toxic respects; in the first case on all properties, in the second mainly on choleresis and toxicity.

Summary

1. A comparative investigation of five bile acids: cholic acid, glyco-, tauro-, desoxy- and dehydrocholic acids and their effects on surface activity, lipase activation, choleresis, hemolysis and toxicity, was conducted.

2. The difference in effects is demonstrated by the following effectiveness sequence in increasing order:

a) for surface activity: dehydro-, gluco-, cholic, tauro- and desoxycholic acid;

b) for lipase activation: dehydro-, desoxy-, glyco-, tauro- and cholic acid;

c) for choleresis: desoxy-, tauro-, glyco cholic and dehydrocholic acid;

d) for hemolysis: dehydro-, cholic, glyco-, desoxy- and taurocholic acid;

e) for toxicity: dehydro-, cholic, glyco-, tauro-, and desoxycholic acid.

Dehydrocholic acid is found exclusively in terminal positions of the series. It has the strongest choleretic but the weakest surface and lipase activation effects, also the lowest effect. Cholic acid gives the best average with respect to physiological properties with low toxicity; glycocholic acid is close to the latter, is less effective physiologocially, but with more toxicity; tauro- and desoxy- cholic acids are close to each other; they are special in surface activity, the first also in lipase activation, both have the weakest choleretic effect and are more toxic than any other bile acid. Numerical values are given in the tables.

3. The experimental series indicates further that practically the same order exists for surface activity, hemolysis and toxicity, i. e. these properties are casually linked to the extent that the first is responsible for the two others. L. A. and choleresis should be considered effects which are independent of each other and the others.

Bile acids thus have three principal effects:

1. The physio-chemical properties of surface activity (and fat solutility),

2. Lipase activation;

3. Enhancement of choleresis.

With respect to choleresis, it should be mentioned that the intraduodenal administeration of cholic acid and dehydrocholic acid is as effective as the i. v injection of the same dose. Glyco- and taurocholic acid, on the other hand, proved to be weaker by a factor of 2 with intraduodenal application and the less effective desoxycholic acid is rather stronger intraduodenally than with i. v. application; it should also be mentioned that the increased choleresis lasts for 40-70 minutes with i. v. administration and $1\frac{1}{2}$-$2\frac{1}{2}$ hours with intraduodenal application.

4. Consideration of the chamical constitution and biological effects of the bile acids investigated yields the conclusion that not only the conjugation of cholic acid with glycocoll or taurin, i. e. substitution at the side chain, but also alterations of the structure of the cyclopentanoperhydrophenanthren core in the sense of more or fewer OH groups or dehydration are causing similar shifts in active effects.

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Role of Cholate in Dietary-Induced Hypercholesteremia of Rats and Rabbits[1]

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It has long been known that dietary-induced hypercholesteremia may be augmented by the simultaneous ingestion of cholic acid (1). The mechanism of this phenomenon has been thought to be due to increased intestinal absorption of cholesterol effected by administration of excess cholic acid. Indeed, recent measurements of the intestinal absorption of cholesterol in the rat have shown quite clearly that cholate is capable of increasing the fraction of absorbed cholesterol (2). However, studies from this laboratory (3, 4) also have indicated that cholate itself, when present in excess in the plasma, is capable of inducing hypercholesteremia. In view of these two distinct effects of cholate upon cholesterol metabolism, it appeared important to us to determine whether this second or direct effect of cholate upon cholesterol might also underlie the hypercholesteremia produced by feeding of both cholate and cholesterol. The results of this study suggest that the augmentative effect of ingested cholate upon dietary induced hypercholestermia is one limited to its intestinal action and not due to its direct hypercholesteremic action.

METHODS

Two species of experimental animals were employed in this study, the rat and the rabbit.

Rats. Adult male rats (Long-Evans) were divided into four groups and were fed powdered stock ration. Eleven controls rats comprised *group I*. Powdered cholesterol (2% of the diet) was added to the diet of 8 rats of *group II*, powdered cholic acid (1% of the diet) was added to the ration of 10 rats of *group III*, and the diet of 8 rats of *group IV* contained both cholesterol (2%) and cholic acid (1%). After an 8-week interval, the rats were killed, the aorta of each was examined for evidence of atherosclerosis, and blood was obtained for determination of plasma cholesterol, cholic acid and phospholipids (5, 6).

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Rabbits. Six-week old male rabbits were divided into three groups and were fed stock pellet ration. *Group I* consisted of nine control animals. Cholesterol (2% of the diet) was added to the ration of five rabbits of *group II* and cholic acid (1% of the diet) was added to that of five rabbits of *group III*. The cholesterol and the cholic acid were dissolved in heated vegetable oil (5% of the diet) and then thoroughly mixed with their respective rations. After an 8-week interval each animal was bled and the plasma cholesterol and cholate levels were determined, and plasma phospholipids then determined in a pooled sample from each group of rabbits.

RESULTS

The data obtained from the study utilizing rats are presented in table 1 and show that a slight rise of plasma cholate occurred in all rats fed excess cholesterol, cholic acid or both. A slight rise of plasma cholesterol was observed in the rats fed excess cholate (*group III*) but marked hypercholesteremia developed only when cholate and cholesterol were fed simultaneously (*group IV*). No correlation was found between the degree of rise of cholesterol and cholate in the various groups of rats, and it appears significant that a lesser rise of cholate occurred in the rats with a marked hypercholesteremia (*group IV*) than that which developed in the rats with no rise or only a slight rise of plasma cholesterol (*groups II, III*). A marked rise of plasma phospholipids occurred in the rats fed both cholesterol and cholate (*group IV*). No gross evidence of atherosclerosis was observed in the aortas of any of the rats in this study.

The data obtained from rabbits are shown in table 2. Moderate hypercholesteremia occurred in the animals fed cholate (*group III*) and a marked rise in those fed cholesterol (*group II*). A slight rise of plasma cholate occurred in both groups of animals and again could not be correlated with the degree of hypercholesteremia. The plasma phospholipids were considerably elevated in the rabbits fed excess cholesterol and to a somewhat lesser extent in those fed cholate. Extensive athero-

sclerosis was observed in the aortas of the animals fed cholesterol, while the aortas of those fed cholate displayed only a few scattered atherosclerotic plaques.

DISCUSSION

As Page and Brown (7) recently reported, our rats rapidly became hypercholesteremic without developing atherosclerosis, when simultaneously fed cholesterol and cholic acid. It perhaps is significant in the latter regard that a marked rise of plasma phospholipids also developed in these rats.

Previous studies of this laboratory have indicated that *a*) when cholate is present in excess in the intestinal contents, it increases the intestinal absorption of cholesterol (2), and *b*) when cholate is present in excess in the plasma, it directly induces hypercholesteremia (3) by interfering with the normal rate of disappearance of cholesterol from the blood (4, 8). In the present study only a slight increase of plasma cholate occurred in the rats which developed marked hypercholesteremia when simultaneously fed cholate and cholesterol and in rabbits which became markedly hypercholesteremic when fed cholesterol. No correlation, however, was obtained between the variable rise of cholate and of cholesterol in either series of animals. It would appear that the augmentative effect of cholate upon dietary induced hypercholesteremia was due to its intraintestinal and not to its 'intravascular' properties.

TABLE 1. PLASMA LIPID CONCENTRATIONS IN DIETARY-INDUCED HYPERCHOLESTEREMIA IN RATS

Group	Diet Supplement	No. Rats	Av. Wt., gm	Av. Plasma Conc., mg/100 cc		
				Cholesterol	Cholic acid	Phospholipids
I	None	11	351	53 (43–60)*	1.1 (0.3–1.6)	131 (69–160)
II	Cholesterol (2%)	8	351	83 (66–92)	5.3 (3.2–6.1)	184 (127–292)
III	Cholic acid (1%)	10	322	82 (69–101)	6.5 (5.1–8.0)	151 (102–194)
IV	Cholesterol (2%) Cholic acid (1%)	8	304	631 (435–1050)	3.7 (2.5–6.0)	714 (470–1450)

* Range of values.

TABLE 2. PLASMA LIPIDS IN RABBITS WITH DIETARY-INDUCED HYPERCHOLESTEREMIA

Group	Diet Supplement	No. Rabbits	Av. Wt., gm	Plasma Conc., mg/100 cc		
				Cholesterol	Cholic acid	Phospholipids
I	None	9	2592	36 (18–48)*	1.3 (0.5–2.0)	144
II	Cholesterol (2%) Vegetable oil (5%)	5	2560	2832 (1380–4170)	4.6 (4.3–4.9)	865
III	Cholic acid (1%) Vegetable oil (5%)	5	1936	310 (269–348)	3.0 (2.6–3.4)	531

* Range of values.

SUMMARY

The present studies have confirmed the finding that marked chronic hypercholesteremia without atherosclerosis occurs in rats simultaneously fed cholesterol and cholate. The hypercholesteremia produced in rats and rabbits fed cholesterol, cholate or both together is independent of the plasma cholate concentration. It was concluded that dietary induced hypercholesteremia as well as the augmentative effect of feeding cholate are independent of the known 'intravascular' ability of cholate to induce hypercholesteremia.

We wish to express our thanks to Dr. Edwin E. Hays of The Armour Laboratories, Chicago, for the cholesterol used in these studies.

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DEPENDENCE OF CHOLESTEROL LEVEL IN BLOOD SERUM, LIVER AND ADRENALS IN RATS ON FEED CONSISTING OF NATURAL AND PARTLY HYDROGENATED RAPE OIL OR LOW FAT DIET*

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ROŻYNKOWA D., KOZIOŁOWNA W., HORST A.: *Dependence of cholesterol level in blood serum, liver and adrenals in rats on feed consisting of natural and partly hydrogenated rape oil or low fat diet.* Acta Physiol. Polon. 13 (5) : 591—607, 1962. — Much attention is given in the world literature to the problem of the fats consumed influence on the transformation of cholesterol. The aim of this work was to elucidate the dependence of cholesterol level in blood serum, liver and adrenals in rats on feed, consisting of natural and partly hydrogenated rape oil or low fat diet. The total number of 60 Wistar rats were fed diets containing 15% by weight of rape-seed oil, iodine number 111, the same oil hydrogenated to iodine number 68 and a low-fat diet containing 2% of non-vegetable fat. The other animals were kept on the same diets, but with addition of cholesterol (1%), and cholic acid (0·4%). The levels of free and total cholesterol in blood serum, liver and adrenals were determined according to the method of *Zak et al.* In the result of experiments it was established that the rape oil when added to the diet of rats does not cause any significant deviations from the normal cholesterol concentration in the blood serum, liver and adrenals. Moreover, the cholesterol level is significantly lower in the blood serum of rats receiving hydrogenated oil. The hypercholesterolemic effect of hydrogenated fat appears in the rat only after cholesterol and cholic acid addition to the diet. The mechanism of hypercholesterolemia is probably connected with the deficiency of poly-unsaturated fatty acids.

Much space is given in the ample world literature to the problem of the influence of the fats consumed on the transformation of cholesterol. It has been established that fat consumed in food is the source of fatty acids occurring in cholesterol esters. It is also known that the esterification of cholesterol occurs in the first place with unsaturated fatty acids and particularly linoleic acid. Hence one group of investigators claimed

* A grant for this work was received from the Committee on Pathogenesis of Atherosclerosis, Polish Academy of Sciences and from the Oil Industry Concern, Warsaw.

that the indispensable poly-unsaturated fatty acids play an important part in the transport of cholesterol [19], while on the contrary other authors ascribe the function of transporter of unsaturated fatty acids to cholesterol [13].

Changes in the fatty acids composition in food correspond to the composition of cholesterol esters in the blood. This fact renders possible changes in the rate of the transport of cholesterol and in the distribution of the „cholesterol pool" among organs and tissues. In other words, the transformation of the ester form of cholesterol may depend on the kind and degree of saturation of the esterifying fatty acid. On the basis of these considerations *Sinclair* and *Kinsell* advanced the hypothesis, that cholesterol esterified with saturated fatty acids is more difficult to eliminate from blood for further use, it therefore is deposited in undesirable places e. g. arterial walls [11].

Taking this into consideration the authors applied three experimental diets in the present work: two containing vegetable fat of the same origin (rape seed), in one case in natural form and in the second in partly hydrogenated form. The third diet had a low content of nonvegetable fat.

Rape oil was chosen for experiments owing to its wide use as the basic vegetable fat in this country. Experiments with animals on the influence of hydrogenation of vegetable oils mainly performed in American centers pertain to other oils as cotton, maize, soya etc.

In this work the authors mainly considered the behavior of cholesterol in blood serum, liver and suprarenal bodies.

The liver is the main organ of synthesis, esterification and indirect transformation of cholesterol and moreover and also the place of its storage under conditions of excessive consumption [15]. These functions of the hepatic cells secure the control of esterified cholesterol concentration in blood serum and are the main regulating mechanism of the total cholesterol amount in the liver-blood serum system [7].

Studies of cholesterol in the adrenals were undertaken for two reasons. In the first place, since cholesterol is regarded as the precursor of steroid hormones, the adrenals may take part in the catabolism of cholesterol particularly under certain definite experimental conditions e. g. stress [8, 9]. It is not known, however, whether adrenal cholesterol is influenced by varying degree of fatty acids saturation in the diet, although this seems hardly probable. The second cause for undertaking the experiments with the adrenals is of a specific nature. Namely, some authors [3] observed a several-fold increase of cholesterol concentration in the adrenals in the rat strain Sprague Dawley, if rape oil was added to their nutrition. The mechanism of this phenomenon is completely obscure, though probably it is due to the presence of erucic acid in this oil. The investigations

undertaken were to solve the question whether this phenomenon can be regularly reproduced in rats of other strains.

All the dietetic experiments were performed in two variants: in the first the influence of the content and degree of saturation of fat in the diet on the level of endogenous cholesterol was studied; in the second series the authors applied suralimentation with exogenous cholesterol with cholic acid. The procedure is described below in detail.

METHODS

Wistar rats 9-months-old (males weighing 320—330 g) were used for the experiments. The total of 60 rats was divided into the following dietetic groups, consisting of 20 individuals each:

— group A — received in their food 15 percent (weight) of natural rape oil with iodine number 111;
— group B — received in their food 15 percent (weight) of hydrogenated rape oil the iodine number of which reached up to 68 and m. p. 34°C;
— group C — received a low fat diet about 2 percent.

Table 1. Composition of experimental diets

Components	A Natural rape oil group iodine value 111 g	B Hydrogenated rape oil group iodine value 67 g	C Low fat group g
Basic mixture:			
casein 60 g, powdered milk 90 g, dry powdered alfalfa 33 g, dried brewer's yeast 90 g, cod-liver oil 9 g, ground wheat 100 g, NaCl 3 g, $CaCO_3$ 15 g	400	400	400
Starch	—	—	600
Natural rape oil	220	—	—
Hydrogenated rape oil	—	220	—
2·5 agar solution	880	880	500

All the diets composed after *Fabry* were isocaloric. Their composition is summarized in Table 1. The weight and caloric value of the main nutritive components is given in Table 2.

The fluid and partly hydrogenated rape oil was obtained from the Oil Mill in Gdansk. The percentual content of fatty acids in these fats is summarized in Table 3.

The rats received water and food in illimited amounts, the mean daily consumption was 30—35 g per 1 animal.

Table 2. Comparison of basic composition and caloric value of the diets

Diet	In 100 g of diet			Calories accounted for in 100 g by			Caloric value of diets in cal/g
	protein %	fat %	carbohydrates %	protein %	fat %	carbohydrates %	
A — Natural rape oil	8·2	17·2	8·3	14·7	69·8	15·5	2·27
B — Hydrogenated rape oil	8·2	17·2	8·3	14·7	69·8	15·5	2·27
C — Low fat	8·6	2·6	41·9	15·2	10·5	74·3	2·31

Table 3. Percentual content of fatty acids in the fats fed *)

Fatty acid	Number of carbon atoms	Number of double bonds	Rape oil	
			natural i. v. 111	hydrogenated up to i. v. 68
Lauric	12	0	—	0·7
Myristic	14	0	—	1·5
Palmitic	16	0	3·4	6·9
Stearic	18	0	—	7·7
Arachidic	20	0	—	1·6
Behenic	22	0	—	8·0
Oleic	18	1	11·5	32·4
Gadoleic (eicosanic)	20	1	11·3	2·8
Erucic	22	1	52·5	29·6
Linoleic	18	2	14·5	6·6
Linolenic	18	3	6·9	2·3

*) On the basis of gas chromatography analysis performed by the Institute of Oil Industry in Warsaw.

Analyses of the cholesterol level in blood serum, liver and adrenals were performed after three different time intervals which will be further referred to as "experimental periods".

- period I — rats fed diets A, B and C in the course of 18 weeks, then 27 animals were sacrificed for analyses;
- period II — cholesterol (1 percent) and cholic acid (0·4 percent) were added to the diet of the remaining rats. After 5 weeks analyses were performed in 14 animals;

— period III — involved investigations of the remaining rats which were fed the diets with cholesterol and cholic acid addition for further 6 weeks. Thus they received the normal diets for 18 weeks and the additional load of cholesterol and cholic acid for 11 weeks.

The method of *Zak et al.* was employed for determination of total and free cholesterol. Blood was collected from fasting rats by puncturing the heart under ether anesthesia. Subsequently the liver and both adrenals were removed. A liver section (about 1 g) and the adrenals deprived of the fatty tissue were weighed and thoroughly ground in a mortar and then extracted with a 1 : 1 alcohol-acetone solution. Then determinations were performed as in blood serum.

RESULTS

Weight and appearance of animals. The mean weights of rats within the individual dietetic groups are illustrated in Fig. 1. The animals fed with natural and hydrogenated rape oil groups A and B gained weight in the

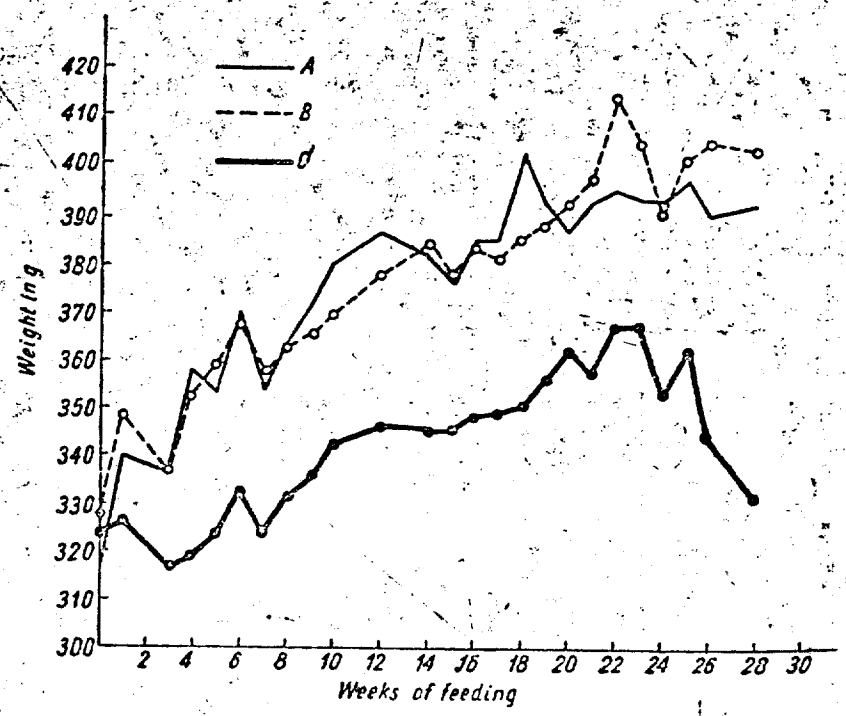

Fig. 1. Mean weights of rats in individual diet groups.

course of the first 18 weeks. The mean increments amounted to 27 and 19 percent of the original weight. A lower weight increment amounting on the average to 8 percent was observed in the group of rats fed the low fat diet. After the addition of cholesterol and cholic acid to the diet the

rats fed the „fatty" diets gained no more weight while those on the low fat diet returned to their original weight.

A thinned and hirsute pelage was the only change in appearance observed in the rats fed the low fat diet. These changes appeared only in the third experimental period.

Blood serum. It may be seen in Table 4 and the graph in Fig. 2 that in the first period the influence of diets consisted in a significant though

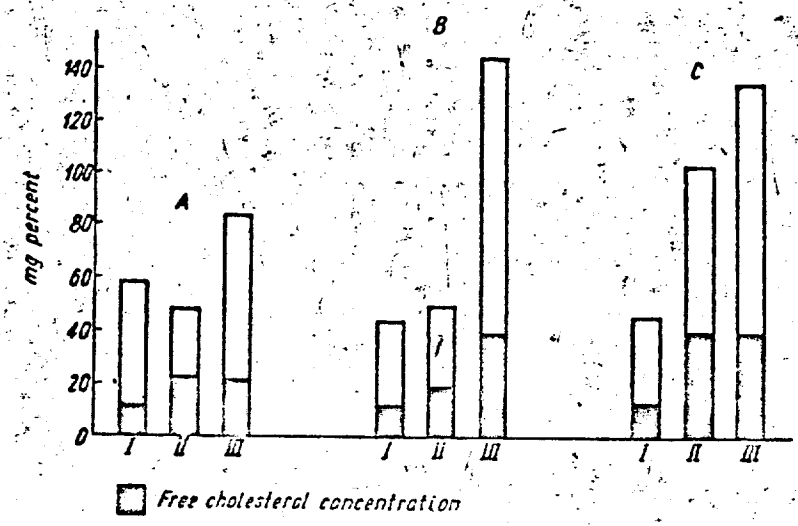

Fig. 2. Mean blood cholesterol level. Shaded segments indicate free cholesterol concentration.

slight difference in cholesterol concentrations. The value of total cholesterol was higher in rats fed with natural rape oil than in those receiving partly hydrogenated oil. On the other hand, the second group (B) exhibited a higher percentage of free cholesterol. The cholesterol level in the group fed the low fat diet was similar to that in group B.

When the diets were enriched with cholesterol and cholic acid periods II and III an increase of cholesterol concentration in the blood serum of the natural oil group was observed only in period III, while a temporary percentual increase of free cholesterol content occurred in period II. The same successive changes occurred in the hydrogenated oil group, however, the increment of the cholesterol concentration at the conclusion of experiments was very high in this case, since it exceeded the threefold original value. The cholesterol concentration in blood serum of the low fat group exhibited the earliest increase occurring in period II and exceeding the double original value. A threefold increment was observed in period III. The unchanged percentage of free cholesterol indicates a uniform increase of esters and free cholesterol.

Liver. It is visible in Table 5 that the mean weights of liver in rats of various groups did not vary in the first experimental period, when diets without cholesterol and cholic acid additions were administered. The hepatic parenchyma in rats of groups A and B at this period had a slightly

Table 4. Mean values of total and free cholesterol in blood serum

Determination	Diet group	Experimental period									
		I			II				III		
		N	$\bar{x}$	$\pm S\bar{x}$	N	$\bar{x}$	$\pm S\bar{x}$		N	$\bar{x}$	$\pm S\bar{x}$
Total cholesterol mg%	A	9	58·2	3·6	5	48·4	8·4	P>0·2	4	84.2	5·2 P<0·01
	B	10	44·2 P<0·01	2·2	5	49·4 P<0·9	16.3	P>0·4	4	144·7 P<0·02	15·6 P<0·001
	C	8	44 6 P<0·01	2·6	4	103·5 P<0·01	13·8	P>0·001	4	133.7 P>0·05	17·6 P<0·001
Free cholesterol mg%	A	9	12.0	2·4	5	22·8	6·9	P>0·1	4	21·7	11·8 P>0·05
	B	10	11·6 P>0·8	1·8	5	18·4 P<0·6	2·2	P>0·05	4	38·4 P>0·1	4·2 P<0·02
	C	8	11·6 P>0·9	3·1	4	39·7 P>0·01	8·3	P<0·001	4	38·4 P>0·05	10·4 P<0·001
Percent of free cholesterol	A	9	20·1	3·6	5	44·5	5·0	P>0·001	4	24·5	5·1 P>0·1
	B	10	26·4 P>0·05	2·7	5	43·9 P>0·9	8·1	P>0·01	4	25·8 P>0·8	3·8 P>0·9
	C	8	24·5 P>0·4	5·5	4	37·6 P>0·4	4·5	P<0·1	4	35·9 P>0·1	2·4 P>0·1

Legend:

I, II, III — experimental periods; I after 18 weeks of feeding the fat (A, B) and low fat diet (C). II — after 23 weeks of feeding the diets, to which 1 percent of cholesterol and 0·4 percent of cholic acid were added for the last 5 weeks, III after 29 weeks of feeding the diets including 11 weeks with cholesterol and cholic acid addition. A, B, C — diet groups; A — diet containing natural rape oil, B — diet containing partly hydrogenated rape oil, C — low fat diet, N — number of animals.

$\bar{x}$, $\pm S\bar{x}$ — arithmetical mean and standard error of the mean. Comparative estimation of the significance of differences (P) between individual groups was performed in reference to the mean value in group A in each period. Changes withing each group were estimated by the comparison of values in periods II and III with those of period I.

yellowish tint while in the group receiving the diet rich in carbohydrates it had a dark cherry-red color. After the end of cholesterol and cholic acid administration i. e. in experimental period III the mean weight of the livers was higher in all the rats. A most striking increase of liver weight

Table 5. Liver: Mean weights of the organ and mean values of cholesterol concentration

	Diet group	Experimental period										
		I			II				III			
		N	$\bar{x}$	$\pm S\bar{x}$	N	$\bar{x}$	$\pm S\bar{x}$		N	$\bar{x}$	$\pm S\bar{x}$	
Mean weight of liver g	A	9	12·7	1·1	5	14·0	1·5		7	16·8	2·4	
	B	10	11·4	1.7	5	15·3	1·4		6	17·5	0·8	
	C	9	11·6	1·0	4	15·0	1·0		4	13.7	1·9	
Total cholesterol mg/100 g	A	9	711·7	69·9	5	3329·2	695·6	P<0·001	7	4088·0	616·0	P<0·001
	B	10	735·2 P>0.8	41·3	5	1914·9 P<0·001	121·4	P<0·001	3	4420·0 P=0·7	173·5	P<0·001
	C	8	638·1 P<0·3	43·9	4	1707·5 P>0·1	627·6	P<0·05	4	2215·6 P>0·1	473·0	P<0·001
Free cholesterol mg/100 g	A	9	320·3	120·4	5	773·9	65·6	P>0·4	7	941·1	201·3	P<0·02
	B	10	306·8 P>0·9	24·5	5	692·3 P>0·9	78·8	P<0·001	3	664·5 P>0·4	26·8	P<0·001
	C	8	249·4 P=0·6	16·0	4	512·5 P>0·7	31·2	P<0·001	4	379·3 P>0·05	28·4	P<0·001
Percentage of free cholesterol	A	9	44·9	2·7	5	27·1	5·6	P<0·01	7	22·7	2·8	P<0·001
	B	10	42·3 P>0·5	3·7	4	32·1 P>0·5	6·6	P>0·1	3	15·0 P>0·1	0·7	P<0·01
	C	8	39·6 P>0·1	2·2	4	32·7 P>0·5	7·1	P>0·2	4	16·2 P>0·1	2·2	P<0·001

Legend as in Table 4

was observed in rats fed the hydrogenated oil. At this period examination of liver sections demonstrated that the liver of all the rats receiving fat and cholesterol (A and B) had a clayey-yellow color and a friable parenchyma. The livers in rats fed the low fat diet also underwent marked fatty degeneration.

The liver cholesterol level depended neither on the amount of fat in the diet nor on the degree of fatty acids saturation (period I). The differences,

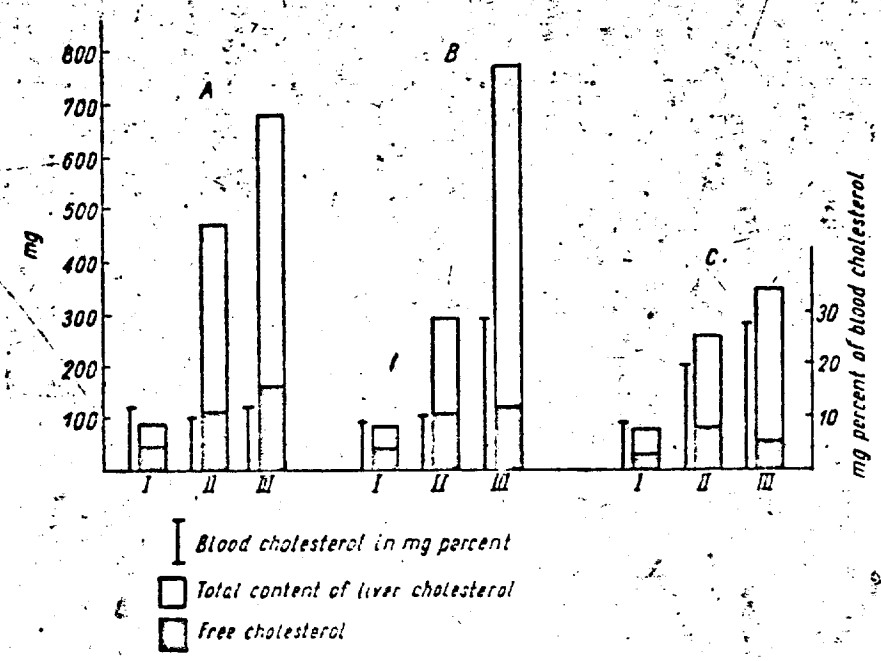

Fig. 3. Mean cholesterol content in liver in mg. Blood cholesterol level in mg percent. Total cholesterol content in liver. Shaded segments — free cholesterol.

however, became prounced when the cholesterol and cholic acid charge was applied for 5 weeks (period II). A considerable increase of both cholesterol and its esterified form was observed in all groups. This increase however, was far higher (double) in the natural oil group than in the others.

After further 6 weeks (period III) the increase of cholesterol concentration in the livers of rats of group B equalled that of group A. The mean values of total cholesterol concentration in the livers of the low fat group were lower than those obtained for group A and B. Nevertheless this difference is not statistically significant.

Therefore the mean amounts of cholesterol in mg in the entire organ, computed on the basis of the mean cholesterol concentration and mean

liver weight in the given period were represented graphically in Fig. 3. Simultaneously the mean cholesterol levels in blood serum were given for comparative purposes.

We seee that the „cholesterol pool" in A and B rats increased 5 and 3 times in period II and in period III even 9 times in group B. At the same time rats of the low fat group exhibited a relatively low cholesterol storage.

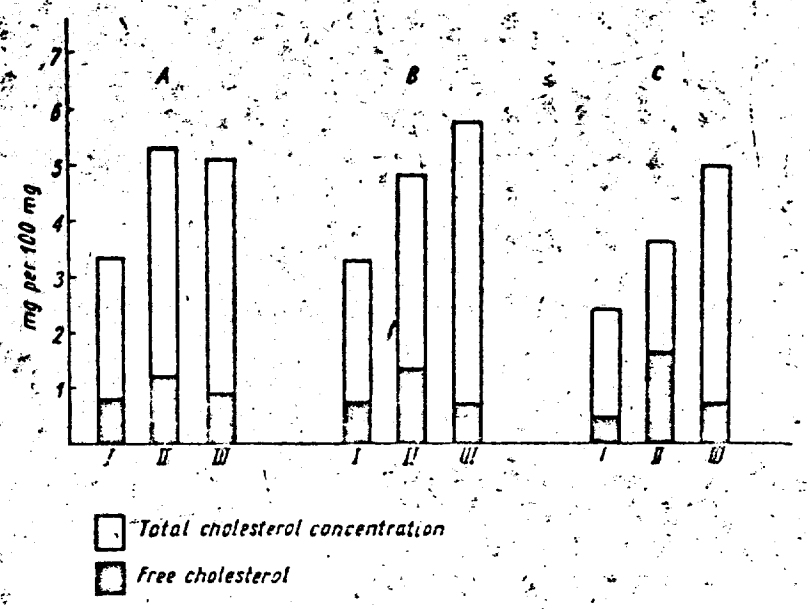

Fig. 4. Mean values of cholesterol concentration in adrenals. Total cholesterol concentration. Shaded segments — free cholesterol.

Adrenals. In the first experimental period no statistically significant differences were observed in the weight of the adrenals between individual rat groups. When cholesterol and cholic acid were added to the diet only a temporary increase of the adrenals weight was observed in the hydrogenated oil group after five weeks of the diet.

Table 6 indicates that the adrenals of rats fed rape oil, notwithstanding whether in the natural or partly hydrogenated form, exhibited a cholesterol level by 30 percent higher than those fed the low fat diet. This value, however, does not exceed 4 percent of the organ's weight. On application of the additional cholesterol the level of this substance in the adrenals of all groups increased considerably. After five weeks of cholesterol addition to the diets (period II), rats fed rape oil (A and B) still exhibited a cholesterol level only by 30 percent higher than in the adrenals of the

Table 6. **Adrenals: Mean weight of pair and mean cholesterol levels**

	Diet group	Experimental period										
		I			II				III			
		N	$\bar{x}$	$\pm S\bar{x}$	N	$\bar{x}$	$\pm S\bar{x}$		N	$\bar{x}$	$\pm S\bar{x}$	
Mean weight of adrenals mg	A	8	59·7	8·1	5	74·0	7·7		7	57·3	4·3	
	B	10	52·1	9·5	5	61·4	6·5		4	58·8	5·3	
	C	7	54·7	8·4	4	50·9	2·9		4	51·3	0·7	
Total cholesterol mg/100 mg	A	8	3·36	0·25	5	5·29	0·66	$P<0·01$	7	5·06	0·22	$P>0·1$
	B	10	3·31 $P>0·8$	0·30	5	4·84 $P>0·7$	1·20	$P>0·1$	4	5·80 $P>0·5$	0·36	$P<0·01$
	C	7	2·48 $P<0·05$	0·29	4	3·65 $P>0·05$	0·26	$P<0·02$	4	4·93 $P>0·8$	0·11	$P<0·1$
Free cholesterol mg/100 mg	A	8	0·80	0·22	5	1·18	0·21	$P>0·2$	7	0·92	0·12	$P>0·1$
	B	9	0·72 $P>0·7$	0·17	5	1·36 $P>0·5$	0·12	$P<0·05$	4	0·74 $P>0·4$	0·12	$P>0·1$
	C	5	0·47 $P>0·2$	0·08	4	1·63 $P>0·1$	0·23	$P<0.01$	4	0·72 $P>0·3$	0·19	$P>0·2$
Percentage of free cholesterol	A	8	23·8	6·1	5	23·2	4·0	$P>0·9$	7	17·4	3·3	$P>0·3$
	B	9	20·3 $P>0·6$	3·4	5	34·5 $P>0·2$	6·4	$P>0·05$	4	12·7 $P>0·0$	2·0	$P>0·1$
	C	5	17·0 $P>0·4$	2·0	4	44·5 $P<0·01$	4·5	$P>0·001$	4	14·3 $P>0·5$	3·7	$P>0·3$

Legend as in Table 4

[illegible] the end of the experiments i. e. after fourteen days [illegible] did not observe any difference between the cholesterol level in the adrenals of rats fed natural rape oil and that of rats fed the low fat diet. The considerable increase of cholesterol in the adrenals in period III in first place concerned its esterified form. The mean values of cholesterol concentration in the adrenals are illustrated in Fig. 4.

DISCUSSION

On the basis of the results described we shall attempt to give answers to several questions:

1) Did the degree of fatty acids saturation in fats added to the diet exert any influence on the cholesterol level in blood serum and liver?

— The authors claim, that, the higher content of unsaturated acids in the fat added to the diet (A) the higher is the cholesterol level in the blood serum. (Reference is made here to the diet without addition of cholesterol and cholic acid in period I). This concentration, however, does not exceed the normal value. It was observed that the trend of changes in cholesterol concentration in rats is opposite to that in humans. It is known, that in humans, fats rich in unsaturated fatty acids decrease the cholesterol level in the blood. This different reaction, probably due to specific conditions, was earlier observed by *Klein* as well as *Swell et al.*

The authors found no significant differences in the cholesterol levels in the livers of rats, what would indirectly indicate some dependence of the quantity of endogenous cholesterol synthesised on the kind and amount of fat applied.

2) Did the varying degree of fat saturation in the diet significantly influence the extent of hypercholesterolemia under the conditions of suralimentation with cholesterol with an addition of cholic acid?

— The authors confirm this dependence in view of the fact, that the increment of cholesterol concentration in blood serum is higher in the group of animals fed with the partly hydrogenated rape oil with iodine number 68 than in animals receiving natural rape oil with iodine number 111. The same hypercholesterolemic effect was observed by *Sasking et al.* in their experiments on rats in which partly hydrogenated cottonseed oil and cholesterol were applied.

3) The authors arrived at yet another conclusion in the dietetic groups observed. They namely established that not only cholesterol + saturated fat but also cholesterol + low fat diet lead to hypercholesterolemia, the latter diet giving this effect even more rapidly.

[illegible] experiments. The [illegible] the liver was [illegible] for this purpose with the blood [illegible]

— No correlation could be established between the blood cholesterol level and its content in the liver. For instance in the case of a fivefold increase of cholesterol in the liver no increase of cholesterol occurred in the blood of one group (A in period II) while in another (group C in period III) the increment was even threefold.

Also there is no proof that larger amounts of cholesterol stored in the liver are associated with a lowered increment of cholesterol concentration in the serum. Such a dependence is excluded by a comparison of the groups in period III, when the blood cholesterol level of these groups varied very widely, while the large amount of cholesterol stored in the liver was almost equal (group A and B).

5) Thus the alternative should be accepted that the cholesterol level in the blood serum depends on the degree of saturation of the fatty acids forming the cholesterol esters.

This assumption may be proved only indirectly by the following facts:

a) hypercholesterolemia was most rapidly induced by the diet containing the smallest amounts of unsaturated fatty acids (low fat). Simultaneously the lowest cholesterol content in the liver of the rats in this group indicates that probably even a smaller amount of exogenous cholesterol was absorbed from the alimentary tract of these animals. In view of the known influence of nutritive fat on cholesterol absorption [14] this seems evident;

b) only a moderate hypercholesterolemia was observed in rats fed the diet containing the largest amount of unsaturated fatty acids;

c) in the course of their investigations [17] the authors established, that the low fat diet applied lowers the iodine number of esterified fatty acids in the blood serum of the rats. Similarly, the addition of cholesterol and cholic acid to diets rich in fats and low fat diets also lowers the iodine value of esterified fatty acids in the blood serum, i. e. increases the degree of their saturation. The iodine number of the fatty acids esterifying cholesterol was not determined in the present investigations. It is known, however, for instance from Portmann's investigations on Cebus monkeys that the application of the low fat led to a drop of the percentage of unsaturated fatty acids in the cholesterol esters from 70 to 5 percent after seven months. The normal composition of cholesterol esters was already restored 24 hours after the addition of maize oil to the diet [16].

On the basis of this [illegible] we arrive at the conclusion that the mechanism of hypercholesterolemia in rats, owing to the administration of hydrogenated fats and cholesterol, is connected with a certain [illegible]

condition or may be also with a deficit of unsaturated fatty acids. The degree of this hypercholesterolemia is independent of the cholesterol storage in the liver.

6) The problem whether rape oil, in contrast to other fats, caused particular changes in the cholesterol transformation in the investigated rats should be separately discussed.

This question arises owing to the results summarized in Table 5, period II, where group A (natural rape oil — more erucic acid) exhibited a higher accumulation of cholesterol in the liver than the group fed the partly hydrogenated oil. The same differences were, however, observed when using other vegetable oils as soya, maize, sunflower and cotton seed oils [10], [14], [19], [21]. These differences are attributable to the different degree of saturation of fatty acids in both the natural and hydrogenated fats investigated.

The influence of administration of rape oil in the diet on the cholesterol level in the adrenals is suggested by Table 6. It is seen, that in period I the cholesterol level in rats of groups A and B, i. e. those which received rape oil and erucic acid in it, was by 30 percent higher than in the low fat group. It cannot in the present state of investigations be established whether the same increment would occur in the case of every fat added to the diet. Nevertheless the increase of the cholesterol level in the adrenals of Wistar rats on the diet with rape oil is considerably lower than that observed by *Carroll*. This author obtained a fivefold increase of the cholesterol level in the adrenals of the Spraque Dawley strain rats fed rape oil without cholesterol. Since the present authors were unable to verify this phenomenon, they support *Beare's* conclusion that the influence of rape oil on the adrenal cholesterol in specific and characteristic only for Spraque Dawley rats.

CONCLUSIONS

1. Rape oil in its natural form with iodine number 111 or in the partly hydrogenated form with iodine value up to 68 when added in the amount of 15 percent (weight) to the diet of rats does not cause any significant deviations from the normal cholesterol concentration in the blood serum, liver and adrenals.

Moreover the cholesterol level is significantly lower in the blood serum of rats receiving hydrogenated oil. This result confirms the fact, that the trend of changes under the influence of a higher degree of saturation of fatty acids in the diet is opposite in the rat to that in humans.

2. The hypercholesterolemic effect of hydrogenated fat appears in the rat only after cholesterol and cholic acid addition to the diet.

3. Hypercholesterolemia occurs also under the influence of low fat [illegible] (2 percent of fat) with the same cholesterol and cholic acid addition.

4. In both cases the mechanism of hypercholesterolemia is [illegible] connected with the deficiency of polyunsaturated fatty acids.

5. The influence of rape oil on the cholesterol level in rat adrenals [illegible] pends on the strain of rats used for experiments. The several-fold [illegible] observed by other authors in the cholesterol concentration in the adren[illegible] of rats of the Sprague Dawley strain under the influence of rape oil, [illegible] not occur in Wistar rats.

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Absorption of Bile Acids from the Large Bowel in Man

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ABSTRACT The absorption of bile acids from the human large bowel was studied in eight patients. All patients had cholecystitis and cholelithiasis and had to undergo cholecystectomy. Cholic acid-^{14}C was injected during surgery into the lumen of the cecum, hepatic flexure of the colon, or transverse colon in six patients, under the visual control of the surgeon. Common duct bile was collected by T tube daily for 5 days, and bile acids were extracted. Significant amounts of radioactivity appeared in T tube bile in each patient. T tube bile acids contained a total of 43.6–84.6% of the administered radioactivity; the average for the six patients was 58.9%. The majority of the tracer was excreted during the first 24 hr. In an additional patient cholic acid-^{14}C was given in the form of an enema 5 days postoperatively. In this subject 30.8% of the retained radioactivity was excreted through the T-tube in 48 hr. The labeled cholic acid was recovered as both cholic and deoxycholic acid from T tube bile. Thin-layer chromatographic analysis of the bile acid samples indicated that the fraction of radioactivity recovered as deoxycholate increased with time during the postoperative period. Gas-liquid chromatographic analysis showed that the daily total quantity of excreted bile acids increased significantly from the 1st–5th days of the experiment. The amount of cholate excreted in T tube bile increased markedly with time, that of chenodeoxycholate increased moderately, and that of deoxycholate decreased sharply during the 5 days of the experiment. In three patients, injection of radiopaque material mixed with the tracer showed no evidence of regurgitation into the small bowel by serial X-rays. In an additional patient, tube aspirate from the terminal ileum contained no radioactivity. The results indicate that cholic acid is converted to deoxycholic acid in the human colon, and both of these bile acids are absorbed from the human large bowel in significant amounts. These data establish the previously unproved concept that significant absorption of bile acids takes place from the large bowel of man.

INTRODUCTION

According to available evidence bile acids are absorbed or reabsorbed from the lower part of the small intestine in man (2). The human liver synthesizes cholic acid and chenodeoxycholic acid (3, 4), whereas the human gall bladder contains in addition to these two compounds a variety of bacterial transformation products, mainly deoxycholic acid and minute amounts of lithocholic and

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TABLE I

Pertinent Laboratory and Other Experimental Data in Eight Patients Studied

Patient	Age	Sex	Laboratory data at time of experiment: Total serum bilirubin	Alk. phosphat. Kau.*	SGOT	SGPT	Thymol turb.	Serum albumin	Serum globulin	Tracer‡	Remarks and specific procedures
			mg/100 ml					*g/100 ml*			
C. C.	60	F	0.3	9.1	—	—	1.2	5.4	1.3	Cecum	—
M. P.	50	M	0.6	20.0	38	40	—	—	—	Hepatic flexure of colon	Radiopaque material§
D. C.	34	F	0.1	6.2	25	10	2.2	3.9	3.2	Cecum	Gantrisin 4 g/day preoperatively
A. S.	31	M	0.5	7.0	—	—	1.1	—	—	Hepatic flexure of colon	Radiopaque material§
B. W.	55	F	0.7	10.0	14	12	0.1	4.0	2.6	Hepatic flexure of colon	Radiopaque material,§ 10 mg of cholic acid added‖
B. S.	72	F	14.3	36.0	440	80	1.6	3.7	2.8	Transverse colon	Chloramphenicol, 2 g/day, 20 mg of cholic acid added‖
H. A.	36	M	0.4	5.0	—	—	1.1	—	—	Hepatic flexure of colon	Contents of terminal ileum tube aspirated
L. A.	79	M	5.8	34.6	165	—	—	4.2	3.5	Enema through rectum	Procedure done 5 days postoperatively

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
* King–Armstrong Units.
‡ Site of administration of cholic acid-^{14}C solution.
§ 25 ml of diatrozoate sodium mixed with tracer.
‖ Nonradioactive cholic acid added to the solution of tracer.

other cholanic acids (4–6). Transformation of cholic into deoxycholic acid, and conversion of chenodeoxycholic into lithocholic acid, has been demonstrated "in vitro" by the incubation of the primary bile acids with fecal material or with bacterial cultures (7–10). Attempts to isolate and identify the various bacteria responsible for these changes have been made (8–10).

The presence of bacterial transformation products of primary bile acids in the human gall bladder implies that either significant absorption or reabsorption of bile acids occurs from the bacterium-rich large bowel in man, or that significant numbers of bile acid–transforming bacteria are present in the human small intestine. In the present study, the absorption of bile acids from the large bowel in man was investigated.

METHODS

Eight patients were studied. The age, sex, and pertinent laboratory data are included in Table I. Each participant had cholecystitis and cholelithiasis with a previous history of common duct obstruction, so that cholecystectomy and the exploration of the common hepatic duct became necessary and was surgically indicated. At the time of surgery six of the subjects had normal serum bilirubin concentrations, and two patients had clinical jaundice with an elevated serum bilirubin level (Table I). The patients were afebrile and ambulatory just before surgery. The experimental procedure was explained to them in detail, and informed consent was obtained. Two additional patients were approached but refused to participate in the study. One patient (D.C., 34 F) was given 4 g of sulfisoxazole by mouth daily for 6 days before surgery. Another (B.S., 72 F) received 1 g of chloramphenicol by mouth daily for 2 days before surgery, and the antibiotic was continued intravenously for the duration of the experiment after the removal of the gall bladder. The remaining six patients received no antibiotics.

Cholic acid-carboxyl-^{14}C [1] (56 mc/mmole) was purified by thin-layer chromatography (TLC) (11). Appropriate tracer amounts were dissolved in 20 ml of water and were sterilized in sealed vials. After sterilization the tracer was rechromatographed (TLC) and was found to be 98.5% pure on radioassay and yielded a single peak on gas-liquid chromatography.

Cholecystectomy was performed in each patient, and a T tube was placed in the common hepatic duct by the

[1] New England Nuclear Corp., Boston, Mass.

surgeon. In two patients appendectomy was performed, and 9.6–22 μc of cholic acid-^{14}C was injected into the lumen of the cecum through the appendiceal stump, under the visual control of the surgeon (Table I). In four additional patients (Table I) the tracer was injected into the lumen of the hepatic flexure of the colon through the wall of the intestine with a fine needle. A purse string suture was placed over the needle hole in this area to close it securely. In another patient (Table I) the labeled cholic acid was injected into the lumen of the transverse colon about 10 cm distal to the hepatic flexure. In three patients (Table I) 25 ml of radiopaque material (diatrizoate sodium) was mixed with the tracer and was injected into the lumen of the hepatic flexure of the colon. Serial X-rays (flat plate of the abdomen) were taken during the first 48 hr after surgery (every 6 hr during the first 18 hr, then every 10 hr). In two patients (Table I) 10 and 20 mg of nonradioactive cholic acid, respectively, was added to the tracer solution and was injected into the lumen of the large bowel together with the labeled material.

T tube bile was collected daily for 5 days in six patients. The bile was drained into sterile bottles containing 100 ml of ethanol, in 24-hr portions. In one of these subjects (B.W., 55 F), the bile output during the first 24 hr was collected in four separate 6-hr portions; the bile output from the 2nd–5th day was collected as 24-hr specimens. In another patient (H.A., 36 M) a tube was passed into the terminal ileum, facing the ileocecal valve. Ileal contents were aspirated, and T tube bile was collected for 10 hr after surgery in this patient. The ileal aspirate was processed like the T tube bile, to test for the possible presence of radioactivity as described below.

In the eighth patient (L.A., 79 M) the experiment was carried out 5 days after surgery. At this time, the patient was afebrile, out of bed, and was fed by mouth. Cholic acid-^{14}C was mixed with 250 ml of an aqueous 20% diatrozoate sodium solution, and was administered into the large bowel as an enema through a rectal tube with an inflated balloon. The progress of the fluid was monitored by X-ray fluoroscopy, and periodic flat plates of the abdomen were taken. The mixture filled the large bowel uniformly from the rectum to the hepatic flexure of the colon, and faintly outlined the cecum. There was no evidence of regurgitation of the fluid into the small bowel. The enema fluid was retained for 1½ hr, was then allowed to flow out through the rectal tube, and was collected in a plastic bag. The enema fluid was processed for quantitative radioactivity as outlined below, and the amount of radioactivity retained in the large bowel was determined. Bile was collected from a T-tube, inserted during surgery 5 days earlier. The bile collection began at the time of the start of the enema, and was carried out for 48 hr in two separate 24-hr containers as described above.

Extraction of bile acids. The volume of the bile specimens was measured. All samples were brought to constant volume with hot ethanol and were stored at 4°C until extracted. Two suitable aliquots (10–50 ml) of equal volume were taken, and a known amount of cholic acid-^{14}C was added to the second sample of each pair of specimens to monitor recovery. The per cent recovery of the added cholic acid-^{14}C, extracted parallel with each of the bile acid samples, varied from 72 to 102% in the eight patients, with an average of 89.5%. The data shown in the results section have been corrected for recovery. The specimens were filtered, and 10–30 ml of a solution of 33% KOH was added to bring the pH to 11. The mixtures were put in a steam bath at 75–80°C for 1 hr under a gentle nitrogen stream. This procedure resulted in the evaporation of most of the ethanol and the hydrolysis of any cholesterol esters which might have been present. Neutral steroids were removed from the aqueous solution with equal volumes of hexane by three extractions. The hexane was backwashed with water and discarded, and the water was added back to the original solution. The remaining aqueous solution was autoclaved in a pressure cooker at 15 lb of pressure for 3 hrs. The solution was cooled in an ice bath, and methanol was added in a ratio of water : methanol of 5 : 2. The cooled solution was acidified (Congo red) with 50% sulfuric acid. Fatty acids were removed by two extractions with an equal volume of hexane; the hexane was backwashed with water and was discarded. Bile acids were extracted from the water : methanol layer by shaking five times with an equal volume of peroxide-free ethyl ether. The ether was washed with water and was evaporated at 30–40°C under a gentle nitrogen stream. The residue containing the bile acids was dissolved in 100 ml of methanol and stored at 4°C. Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer, in a toluene solution containing 4 mg of 2,5-diphenyloxazole and 0.3 mg of 1,4-*bis*-2-(5-phenyloxazolyl)-benzene per ml. For each unknown amount, a parallel sample was run with known amounts of radioactivity in the vial to determine quenching (internal standard method).

The amount of radioactivity recovered by the above method from the different fractions was determined as follows: a known amount of cholic acid-^{14}C was added to a nonradioactive bile specimen, which was processed in quadruplicate. The neutral steroid fractions (hexane extraction, alkaline medium) contained an average of 1.06% of the total radioactivity present. The corresponding average figure for the fatty acid fractions (hexane extraction, acid medium) was 1.75%, and the bile acid fractions (ethyl ether extraction, acid medium) was 88.1%, respectively. The remainder (water : methanol layer) contained but trace amounts of radioactivity.

Thin-layer chromatography (TLC). TLC was carried out on 20 × 20 cm silica gel G plates. Each bile acid extract was chromatographed on a separate plate with a solvent system of isooctane : isopropyl ether : acetic acid, 50 : 25 : 25 (11). Known bile acids (cholic, deoxycholic, and chenodeoxycholic) were run on each plate, together with the unknown samples. No attempt was made to account for the small percentage (less than 5%) of radioactivity which may have been present in the bile in ketonic bile acids produced by bacterial action. The plates were sprayed with a 10% solution of phosphomolybdic acid in ethanol. Each row of spots, corresponding to a

TABLE II

Excretion of Radioactivity in T Tube Bile (Bile Acid Fraction of Extracts) in Six Patients for 5 Days, after the Injection of Cholic Acid-^{14}C into the Lumen of the Large Bowel

Day	C.C.	M.P.	D.C.	A.S.	B.W.	B.S.	Average‡
				% recovered*			
1	29.4	78.9	44.2	55.9	57.4	39.3	50.9
2	5.5	1.8	3.5	4.2	3.2	5.6	4.0
3	2.5	2.6	1.4	1.0	0.3	1.8	1.6
4	2.9	1.1	4.3	0.5	0.1	1.0	1.7
5	3.3	0.2	1.1	0.3	0.1	0.4	0.9
Total§	43.6	84.6	54.5	61.9	61.1	48.1	58.9

* Per cent of total administered radioactivity, recovered from T tube bile.
‡ Averages of the six patients.
§ Total 5-day excretion.

specific bile acid, was scraped quantitatively from the plates into a liquid scintillation counter vial. 4 ml of methanol was added to the gel, and the mixture was heated to 50°C for 30 min. After cooling, toluene scintillation solution (see above) was added to the tubes, and radioactivity was determined. Quenching was monitored automatically with an external standard source of radioactivity. The total radioactivity disintegrations per minute recovered from each plate was calculated, and the results were expressed in per cent of radioactivity distributed among the various bile acids.

Gas-liquid chromatography (GLC). Bile acids were esterified with methanol by standing overnight at room temperature in 2% sulfuric acid in methanol. After methylation, the solution was diluted with an equal volume of water. The methyl esters of the bile acids were extracted three times with equal volumes of ethyl ether: benzene (1:1). The extracts were washed with aqueous saturated sodium bicarbonate solution, and then with water, until neutral. The organic solvent was evaporated at 60°C under a gentle air flow. The methyl esters of the bile acids were dissolved in ethanol, the solution was brought to constant volume, and an aliquot was taken for radioactivity count. The radioactivity of this sample was compared to the activity of the original methanol solution of the bile acids, to correct for recovery (85–95%). A measured aliquot of the solution containing the bile acid methyl esters was used for GLC on a 6 ft × 4 mm column packed with 3% QF-1 (methylfluoroalkyl silicone) on 100/120 mesh gaschrom Q.[2] The instrument used was a Barber-Coleman, model 5000, with flame ionization detectors. Known quantitative standards of methyl cholate, deoxycholate, and chenodeoxycholate were chromatographed under the same conditions, and the amount of the individual bile acids in each unknown was calculated by comparison with the standards by a disc integrator. Corrections for recovery were done as indicated above.

[2] Applied Science Laboratories Inc., State College, Pa.

RESULTS

The procedure was well tolerated by each patient, and no ill effects were encountered at any time because of the experiment. On the 2nd day after surgery the patients were out of bed. On the 3rd postoperative day they were given clear fluids to drink, and on the 4th or 5th day they were fed solid food. On the 3rd postoperative day the patients were given an enema. Spontaneous bowel movements, if any, began on the 4th or 5th postoperative days. The temperature of the patients did not exceed 100.5°F at any time during the postoperative period.

Absorption of bile acids from the large bowel. Significant amounts of radioactive bile acids appeared in T tube bile in each patient. Table II shows the results of the excretion of radioactivity through the T tube in six patients, who were studied for the full 5 day duration of the experiment. The total amount of labeled material excreted during the 5 days varied from 43.6 to 84.6% of the administered dose. The average excretion for the group was 58.9% of the radioactive dose given. The major part, 29.9–78.9%, was excreted through the T tube during the first 24 hr. The average excretion for the 1st day was 50.9% of the administered dose of label in the six patients. Smaller amounts were excreted from the 2nd–5th

TABLE III

Excretion of Radioactivity in T Tube Bile, and Results of the Analysis of Bile Acids in Patient B.W., 55 Yr Female, during the First 24 Hr of the Experiment

Procedure	Unit	Hours after injection of tracer 0–6	6–12	12–18	18–24
Total radioactivity recovered	*dpm*, % of total given	15.1	24.1	10.4	7.8
TLC; distribution of radioactivity; % of total *dpm* (per plate)	Origin	1.6	2.5	0.7	1.1
	Cholic acid	34.3	15.8	5.1	4.3
	Chenodeoxycholic	1.7	1.7	2.0	2.1
	Deoxycholic	62.0	79.8	92.0	92.1
	Solvent front	0.3	0.2	0.3	0.4
GLC; mass of bile acid methyl esters, *mg* (total per sample)	Cholic	83.9	85.8	44.9	35.9
	Chenodeoxycholic	56.7	40.6	24.2	11.4
	Deoxycholic	46.4	35.0	22.7	10.8

TLC, thin layer chromatography; GLC, gas-liquid chromatography.

TABLE IV

Average Per Cent Distribution of Radioactivity in T Tube Bile (Bile Acid Extracts) by Thin-Layer Chromatographic Analysis in Six Patients during the 1st and 2nd Days of the Experiment

	Day 1		Day 2	
	Average	SE	Average	SE
	%		%	
Origin	1.8	0.3	3.7	0.9
Cholic acid	34.4	9.1	11.5	2.3
Chenodeoxycholic acid	5.0	1.8	7.8	1.3
Deoxycholic acid	58.0	33.1	76.7	3.5
Solvent front	0.1	0.04	0.4	0.28

From the 3rd–6th days of the study the number of counts in most specimens (see text) was insufficient under the experimental conditions given (less than 3000 counts/100 min per TLC plate.)

days. The peak excretion period was between the 6th and 12th hr of the 1st day after the administration of the labeled material in one patient (Table III).

In four patients (C.C. 60 F, D.C. 34 F, A.S. 31 M, and B.S. 72 F) serial blood samples were drawn during the experiment every 4 hr during the 1st day, and then daily thereafter. 1 ml of serum of each sample was checked for the presence of radioactivity. There was no demonstrable radioactivity present in the serum of the patients.

Analysis of bile acids. The results of TLC analysis of the bile acid extracts of T tube bile are shown in Table IV in the six patients studied for 5 days. In the bile acid extracts of the first 24 hr of the experiments an average of 34.4% of the radioactivity migrated to the spot corresponding to cholic acid, and an average of 58% of the label was recovered from the spot corresponding to deoxycholic acid (Table IV). The corresponding figures for the 2nd day averaged 11.5 and 76.7% respectively. During the 3 following days the amount of radioactivity recovered from the TLC plates was too low to be measured, except in one patient. In this subject only 1.1% of the radioactivity migrated to the spot corresponding to cholic acid, and 88.7% of the label was recovered from the spot corresponding to deoxycholic acid on the 5th day of the study. There were only small amounts of radioactive material recovered from the origin, solvent front, and the spots corresponding to chenodeoxycholic acid (Table IV). Since it may be presumed that labeled cholic acid was not transformed to chenodeoxycholic acid, the 5 and 7.8% of radioactivity recovered from the spots corresponding to chenodeoxycholic acid may represent overlaps due to the imperfection of the method or unidentified ketonic acids referred to in Methods. While it has been suggested that pure cultures of certain fecal anaerobes can dehydroxylate cholic acid in the 12-position to form chenodeoxycholic acid (12), there presentely exists no evidence that chenodeoxycholic or lithocholic acids can be formed from cholic acid in man (13). Table III includes the distribution of radioactivity in the bile extracts of the fractions of the first 24 hr of the experiment in one patient.

The average daily volume of bile excreted through the T tube was 320, 281, 263, 372, and 470 ml/24 hr, respectively, from the 1st–5th postoperative days in six patients. The results of the GLC analysis of T tube bile are shown in Table V. The daily excretion of total bile acids decreased sharply during the 2nd and 3rd days after surgery. During the 4th and 5th postoperative days, the amount of total bile acids excreted increased several fold (Table V). Although there was an increase in the volume of bile flow as well, the concentration of bile acids in the T tube bile increased two to threefold from the 1st to the 5th day after surgery. The amount of cholic acid excreted through the T tube increased from an average of 382 mg on the 1st day, to an average of 2391 mg on the 5th day. Chenodeoxycholic acid excretion showed less of a change (Table V). The excretion of deoxycholic acid decreased from an average of 152 mg on the 1st postoperative day to 22 mg on the 5th day. Table III includes the excretion of bile acids during the fractions of the first 24 hr in one subject.

Site of absorption. In three patients radiopaque material was injected into the lumen of the hepatic flexure of the colon together with the tracer (Table I). Serial X-rays of the abdomen indicated that the radiopaque material filled the hepatic flexure, the transverse colon, and faintly outlined the cecum during the 48 hr of monitoring. There was no evidence of regurgitation of the radiopaque material through the ileocecal valve into the small bowel on any of the X-rays.

In one patient, the contents of the terminal ileum

TABLE V

Gas-Liquid Chromatographic Analysis of the Daily Excretion of Bile Acids (mg/24 hr) in T Tube Bile (Bile Acid Extracts) in Six Patients during 5 Days of the Experiment

Patient	Day 1	2	3	4	5
			mg/24 hr		
Methyl cholate					
C. C.	227	68	76	152	229
M. P.	26	578	449	1593	3213
D. C.	165	22	332	2411	1528
A. S.	842	389	315	2983	7327
B. W.	251	105	72	776	1234
B. S.	783	199	398	1043	817
Average	382	227	274	1493	2391
Methyl chenodeoxycholate					
C. C.	178	50	31	44	59
M. P.	41	78	138	372	568
D. C.	94	18	68	582	417
A. S.	556	181	67	384	1027
B. W.	133	59	43	128	152
B. S.	236	111	73	189	134
Average	206	83	70	283	393
Methyl deoxycholate					
C. C.	240	40	13	26	55
M. P.	13	9	17	25	0
D. C.	147	11	107	79	31
A. S.	310	18	0	20	41
B. W.	121	18	8	0	0
B. S.	79	28	6	12	6
Average	152	21	25	27	22
Daily total bile acid methyl esters					
C. C.	645	158	120	222	343
M. P.	81	665	604	1990	3781
D. C.	406	51	507	3072	1976
A. S.	1708	588	382	3387	8395
B. W.	505	182	123	904	1386
B. S.	1098	338	477	1244	957
Average	741	330	369	1803	2806
Average *mg/ml* bile*	2.3	1.2	1.4	4.8	5.9

* Average amount of bile acids (mg) per 1 ml of T tube bile excreted.

T tube aspirated for 10 hr after the injection of cholic acid-^{14}C into the lumen of the hepatic flexure of the colon (Table I). During this period 31% of the administered dose of radioactivity was excreted through T tube bile. There was no radioactivity present in the tube aspirate from the terminal ileum.

The effect of anesthesia. In one patient labeled cholic acid was administered in the form of an enema 5 days postoperatively. The enema fluid was retained in the bowel for 1½ hr. The outflowing enema fluid, collected in a plastic bag (see Methods), contained 40.2% of the total radioactivity added. During the first 24 hr after the administration of labeled cholic acid, 29.7% of the radioactivity retained in the large bowel was recovered from T tube bile. During the 2nd day 1.1% of the label appeared in the bile. Thus a total of 30.8% of the retained dose was absorbed during the initial 2 days of the experiment. The

amount of radioactivity recovered from T tube bile during the 2 days was 18.5% of the total amount of label initially added to the enema fluid.

Other results. The addition of 10 mg and 20 mg, respectively, of nonradioactive cholic acid to the tracer in two patients (Table I) had no visible influence on the rate of absorption or on other results of the study.

The preoperative administration of sulfisoxazole or the pre- and postoperative administration of chloramphenicol had no detectable influence on the results of this investigation.

DISCUSSION

The data of the present study support the previously unproved concept that significant amounts of bile acids are absorbed from the large bowel in man. About one-half of the labeled bile acids injected into the lumen of the large bowel was absorbed and recovered from T tube bile. The absorption seemed to be relatively rapid, most of the material being absorbed during the first 24 hr. Cholic acid is relatively rapidly converted to deoxycholic acid in the human large bowel (Table III). Both of these bile acids are absorbed or reabsorbed from the human colon in significant quantity. The colonic site or sites of absorption cannot be ascertained with certainty from the present study. The cecum, ascending colon, hepatic flexure, and transverse colon may be sites of reabsorption, in whole or in part. There is no information on the remaining parts of the large bowel from the present data.

After the insertion of a T tube into the common hepatic bile duct the total mass of excreted bile acids and the concentration of bile acids in the bile decreased during the 2nd and 3rd postoperative days. By the end of the 5th day, however, the total mass of excreted bile acids increased about fourfold, and the concentration of biliary bile acids increased about threefold (Table V). The reasons for these phenomena are not apparent. Whether local edema of the tissues, the operative procedure itself, the absence of oral feeding during the first 48 hr, or the absence or decrease of feedback inhibition of hepatic bile acid biosynthesis during the latter part of the experiment are in part responsible for this phenomenon is not quite clear from these data. However, it is known that in patients with choledochostomy drainage (3) and in the bile fistula rat (14, 15) interruption of the enterohepatic circulation of the bile produces a marked increase in bile acid excretion. These data suggest that a feedback mechanism of bile acid production is operative in man as well as in the rat. It has already been shown that in bile fistula rats not only cholesterol biosynthesis from acetate (15, 16), but also the 7α-hydroxylation of cholesterol, the initial step in bile acid formation, is markedly increased (17, 18).

Data in Table V indicate a marked increase of the amount of excreted cholate in T tube bile from the 1st–5th day of the experiment. The corresponding amounts of deoxycholate showed a sharp decrease. This finding suggests that most of the bile acids were excreted to the outside through the T tube, and thus only very small quantities of cholic acid were transported to the sites of dehydroxylation. This conclusion, however, must be qualified, since the rates of reabsorption of cholic and deoxycholic acids from the human large bowel are not known, and the figures obtained may be influenced by this factor. Ekdahl and Sjovall (3) analyzed T tube bile acids in patients with choledochostomy drainage and reported that deoxycholic acid was regularly found during the 1st postoperative day but then decreased or disappeared. The results of the present study are in agreement with this finding.

The distribution of radioactivity between different bile acids is included in Table IV. It must be pointed out that the actual distribution of the label in T tube bile may not represent the degree of conversion of cholic to deoxycholic acid within the lumen of the large bowel. There is no information available on the comparative rates of absorption of cholic and deoxycholic acids from the human large bowel. Since differences in this respect may influence the distribution of radioactivity in the excreted bile, the data in Table IV do not necessarily reflect the rate of conversion, but show only the relative percentage of respective labeled materials in the excreted bile.

It should be pointed out that the administration of sulfisoxazole and chloramphenicol (Table I) failed to alter the per cent recovery of radioactivity in deoxycholic acid. The dehydroxylase activity presumably resides in anaerobic organisms (9, 10, 12), and these organisms are resistant to most of the commonly used antibiotics.

It was clearly shown that in the present studies the tracer was absorbed from the large bowel, and that there was no regurgitation of the radioactive material through the ileocecal valve. The experiment carried out 5 days after surgery, showing a significant absorption of the tracer from enema fluid, ruled out the possibility that the process of anesthesia, the initial postoperative absence of oral feeding, or the operative process per se were responsible for the absorption of the labeled bile acids.

In the rat, it has been shown that significant amounts of bile acids are absorbed from the cecum. Lindstedt and Samuelsson estimated that in these animals 51% of the total amount of cholic acid is converted into deoxycholic acid per day (19). They further estimated that of the total amount of cholic acid which is transported to the large intestine per day, 70% is absorbed and transported back to the liver (19). Norman and Sjovall injected labeled bile acids into the lumen of the cecum of bile duct–cannulated rats (20). The bile of the animals contained 42–79% of the labeled material. The order of magnitude of these figures is in agreement with the data in man observed in the present study. The absorption of bile salts from the large bowel of the rat was confirmed by Holt (21) and by Sullivan (22).

During the enterohepatic circulation of bile, most of the bile acids excreted by the liver are reabsorbed during transit in the small bowel, are carried back to the liver by the portal vein, and are eventually reexcreted into the gall bladder and used again. The pool of bile acids is circulated 6–10 times daily through the enterohepatic cycle. At each cycle a small fraction of transiting bile acids will escape reabsorption in the small bowel, will find its way into the large intestine, and will appear in the feces. According to published data, daily fecal excretion of bile acids varies from 87 mg (cholic acid metabolites only) to 2114 mg, depending on the methods of measurement used, and on the regimen given to the patient under study (23). If the average daily excretion of bile acids is assumed to be of 500 mg in an average adult, of this amount about 200 mg is deoxycholic acid and 300 mg is nonabsorbable lithocholic acid (21). Then, in the context of the present study, an approximately equal amount of deoxycholic acid should be reabsorbed from the large intestine during daily transit. This implies that about 700 mg of bile acids escape absorption from the small bowel daily and reach the human large intestine. Of this, 200 mg are reabsorbed during transit in the large bowel. Considering that the total pool size of bile acids is about 1.2–4 g in man (25, 26), these data suggest that about 5–20% of the pool is exposed daily to the effects of bacteria in the large bowel before reabsorption takes place. The presence of significant amounts of bacterial transformation products of the primary bile acids in the human gall bladder can be explained on the basis of these data.

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INFLUENCE OF BILE SALTS ON THE MOVEMENTS OF THE DIGESTIVE TRACT (I)

Influence of Bile Salts on the Movements of the Stomach

By

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Introduction

While there have been many reports regarding the effects of bile or bile salts on the movements of the digestive tract, most of them are concerned with the effects on the movements of the small and large intestines and there has been a paucity of information concerning those on the movements of the stomach.

Hayashi[1] studied roentgenologically the effects of sodium glycocholate and taurocholate upon the motor function of the alimentary tract and found that an intravenous injection of those bile salts caused an increase in the tonus and peristalsis of the stomach, while an oral administration caused, on the contrary, a slight decrease in the movements of the stomach. Still and Carlson[2] reported that an intravenous injection of bile at high dosage caused a transitory inhibition of gastric movements. Hosono and Maruno[3] stated that successive oral administrations of sodium glycocholate and taurocholate stimulated the gastric movements of the rabbit. Takasugi and Yomogita[4] reported that an oral administration of taurocholate or an intravenous injection of dehydrocholate (Decholin) slightly accelerated the emptying of the human stomach. Terauchi[5] also observed that an intravenous injection of bile or bile salts stimulated the gastric movements and Matsuura[6] stated that an intravenous or oral administration of glycocholate caused an increase at low dosage and a decrease at high dosage in the gastric movements of the rabbit, while an intraperitoneal injection always caused a decrease, even at very low dosage.

This paper deals with the results of a series of experiments planned to investigate the effects of bile salts on the movements of the stomach.

Experimental Part

Materials and Methods

The bile salts used were sodium glycocholate, taurocholate and dehydrocholate. The experiments were performed on male or female rabbits weighing about 2 kilograms, which had been fasted for 24 or 48 hours. The contractions of the empty stomach were studied by the balloon method. The experiment was initiated after the lapse of 30 to 60 minutes following the insertion of the balloon which is necessary to obtain a constant gastric movement.

Results

I. *Effect of the intravenous injection of bile salts.*

Aqueous solutions of bile salts were injected into the ear vein of the rabbit. The results are presented in Table 1 and illustrated in Figures 1 to 5. The intravenous injection of glycocholate or dehydrochlate in

Table 1. Effect of the intravenous injection of bile salts

Rabbit No.	Body weight of rabbit (kg)	Sex		Dose of bile salts injected (mg/kg)	Findings of gastric movements
1	2.54	♂	Glycocholate	10	A slight increase in amplitude of gastric movements.
2	1.85	♀		10	No remarkable change.
3	2.05	♀		20	An increase in amplitude of gastric movements.
4	2.25	♂		20	A slight increase in amplitude, illustrated in Fig. 1.
5	1.90	♀		40	An increase in amplitude.
6	2.14	♀		40	No remarkable change.
7	2.15	♀		60	A slight decrease in amplitude.
8	1.74	♂		60	A slight decrease in amplitude.
9	1.75	♀	Taurocholate	10	No remarkable change.
10	2.45	♂		10	No remarkable change.
11	2.16	♂		20	An increase in amplitude.
12	2.25	♀		20	An increase in amplitude.
13	1.75	♀		40	No remarkable change.
14	2.20	♂		40	No remarkable change.
15	2.07	♂		60	A decrease in amplitude, illustrated in Fig. 2.
16	1.85	♂		60	A marked decrease in amplitude.

Rabbit No.	Body weight of rabbit (kg)	Sex	Dose of bile salts injected (mg/kg)		Findings of gastric movements
17	2.40	♀	Dehydrocholate	10	No remarkable change.
18	1.93	♂		10	A slight increase in amplitude.
19	2.50	♀		20	A marked increase in amplitude.
20	1.90	♂		20	A transitory increase in tonus and an increase in amplitude, illustrated in Fig. 3.
21	2.20	♀		40	A marked increase in amplitude.
22	2.55	♀		40	A slight increase in amplitude.
23	2.10	♀		60	A slight decrease in amplitude.
24	1.60	♀		60	A marked decrease in amplitude, illustrated in Fig. 4.

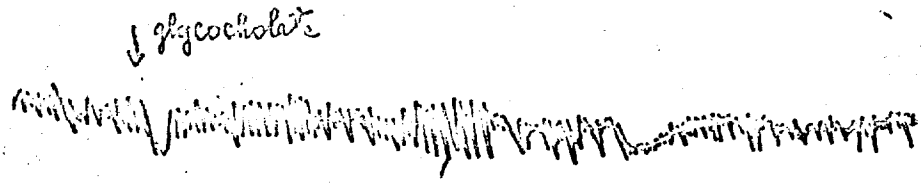

Fig. 1. 2 minutes after the intravenous injection of 2.3 c.c. of a 2 % glycocholate solution, a slight increase in amplitude of gastric movements was found.

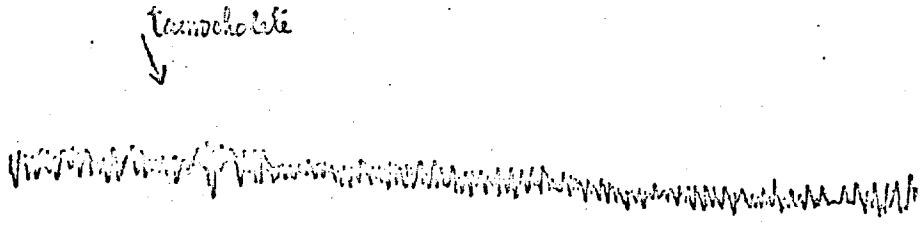

Fig. 2. An intravenous injection of 6.1 c.c. of a 2 % taurocholate solution caused a decrease in amplitude about 3 minutes after injection.

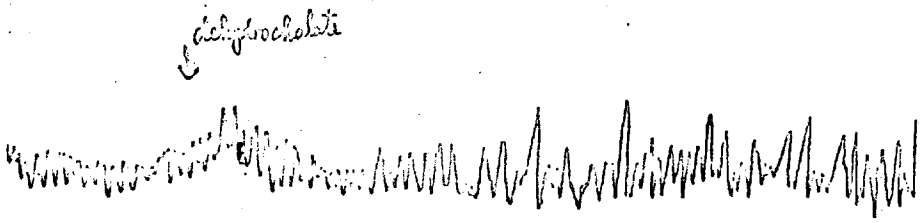

Fig. 3. 1.9 c.c. of a 2 % dehydrocholate solution was injected. Shortly after injection a transitory increase in tonus and an increase in amplitude were found.

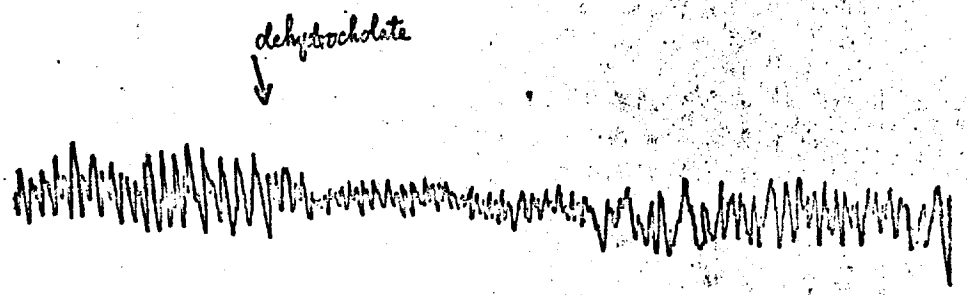

Fig. 4. 5.4 c.c. of a 2 % dehydrocholate solution was injected. 1 minute after injection the amplitude of gastric movements was markedly decreased and about 10 minutes thereafter it returned almost to its previous level.

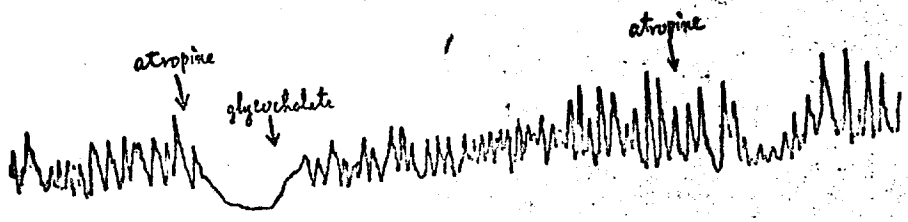

Fig. 5. Immediately after the intravenous injection of 0.5 c.c. of a 0.1 % atropine solution a pronounced decrease in amplitude and tonus was found. The contractions of the stomach, however, appeared again after the intravenous injection of 2.1 c.c. of a 2 % glycocholate solution.

amounts equivalent to 10 mg. per kilogram of body weight caused no remarkable change or a slight increase in amplitude of gastric movements a few minutes after the injection and that of 20 to 40 mg/kg caused a moderate increase in it, while that of 60 mg/kg, on the contrary, caused a decrease. The intravenous injection of 10 mg/kg taurocholate caused no remarkable change and that of 20 mg/kg an increase in amplitude of gastric movements, while that of 60 mg/kg caused a decrease. The tonus of the stomach was generally little or not influenced by the intravenous injection of bile salts, though a slight increase in the tonus of the stomach was observed after the injection of 20 mg/kg of dehydrocholate. Figure 5 shows that the contractions of the stomach, which had been pronouncedly inhibited by the intravenous injection of 0.5 c.c. of a 0.1 per cent atropine solution, became active again by the intravenous injection of 20 mg/kg of dehydrocholate and these activated contractions were affected no longer by the second injection of atropine.

II. *Effect of the oral administration of bile salts.*

Aqueous solutions of bile salts which were previously warmed at body temperature were introduced into the stomach through a Nelaton's catheter. The results are presented in Table 2.

Table 2. Effect of the oral administration of bile salts

Rabbit No.	Body weight of rabbit (kg)	Sex	Dose of bile salts administered (mg/kg)		Findings of gastric movements
25	2.70	♂	Glycocholate	20	A slight increase in amplitude.
26	1.65	♀		20	An increase in amplitude.
27	1.90	♀		40	A slight increase in amplitude.
28	1.70	♂		40	A slight decrease in amplitude.
29	1.74	♂		40	A decrease in amplitude.
30	2.52	♂		60	A decrease then a slight increase in amplitude.
31	1.64	♂		60	A decrease in amplitude.
32	1.76	♀		60	A decrease in amplitude.
33	2.15	♀		60	A decrease in amplitude.
34	1.80	♀	Taurocholate	20	A slight increase in amplitude; illustrated in Fig. 10.
35	2.07	♀		20	No remarkable change.
36	1.90	♀		40	No remarkable change.
37	2.35	♂		40	No remarkable change.
38	1.75	♂		60	A decrease in amplitude.
39	2.12	♂		60	No remarkable change.
40	2.30	♂		60	A decrease in amplitude.
41	2.10	♂	Dehydrocholate	20	A slight increase in amplitude.
42	2.60	♂		20	A slight increase in amplitude.
43	2.25	♂		20	A slight increase in amplitude.
44	2.45	♀		40	A slight increase in amplitude.
45	2.10	♂		40	No remarkable change.
46	2.20	♂		60	No remarkable change.
47	1.80	♀		60	A decrease in amplitude.
48	1.76	♂		60	No remarkable change.

The oral administration of 20 mg/kg of glycocholate or dehydrocholate caused an increase in amplitude of the gastric movements a few minutes after the injection and that of 40 mg/kg caused sometime a slight increase and sometime a slight decrease, while that of 60 mg/kg caused a decrease. The oral administration of 20 to 40 mg/kg of taurocholate caused no remarkable change or a slight increase in amplitude of the gastric movements, while that of 60 mg/kg caused a decrease. The tonus of the stomach was not influenced by the oral administration of each bile salt.

III. *Effect of the intraperitoneal injection of bile salts.*

Aqueous solutions of bile salts, which were previously warmed at body temperature were intraperitoneally injected at the right hypocondriac region. The results were shown in Table 3 and Figures 6 to 8.

Table 3. Effect of the intraperitoneal injection of bile salts

Rabbit No.	Body weight of rabbit (kg)	Sex	Dose of bile salts injected (mg/kg)		Findings of gastric movements
49	1.85	♀	Glycocholate	1	No remarkable change.
50	1.66	♀		1	No remarkable change.
51	2.10	♂		5	A slight decrease in amplitude.
52	2.34	♂		5	A slight decrease in amplitude.
53	1.75	♂		5	No remarkable change.
54	1.74	♀		10	A decrease in amplitude.
55	1.96	♂		10	A marked decrease in amplitude.
56	2.50	♂		10	A slight decrease in amplitude.
57	1.85	♂		20	A standstill of movements.
58	2.07	♂		20	A standstill of movements.
59	2.40	♀		20	A standatill of movements, illustrated in Fig. 6.
60	2.25	♂	Taurocholate	1	No remarkable change.
61	2.10	♂		1	No remarkable change.
62	2.15	♂		5	No remarkable change.
63	1.85	♀		5	A slight decrease in amplitude.
64	1.80	♀		5	A slight decrease in amplitude.
65	2.21	♀		10	A marked decrease in amplitude.
66	2.54	♂		10	A decrease in amplitude, illustrated in Fig. 7.
67	1.70	♀		10	A slight decrease in amplitude.
68	2.35	♀		20	A decrease in amplitude.
69	2.25	♂		20	A marked decrease in amplitude.
70	2.27	♂		40	A decrease in amplitude.
71	1.60	♂		40	A decrease in amplitude.
72	2.10	♂	Dehydrocholate	2	A slight increase in amplitude.
73	2.05	♀		2	No remarkable change.
74	2.70	♂		5	No remarkable change.
75	2.52	♂		5	No remarkable change.
76	2.05	♀		10	A slight decrease in amplitude.
77	1.76	♂		10	No remarkable change.
78	1.87	♂		20	A slight decrease in amplitude.
79	2.14	♂		20	No remarkable change.
80	2.42	♂		40	A decrease in amplitude.
81	1.72	♀		40	A marked decrease in amplitude.

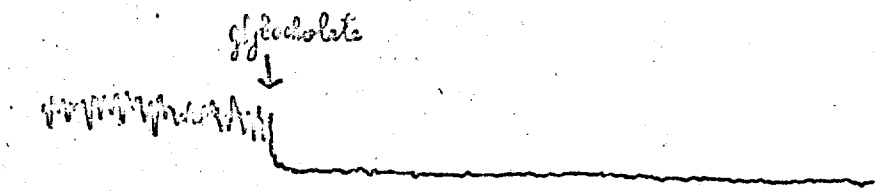

Fig. 6. 2.4 c.c. of a 2 % glycocholate solution was intraperitoneally injected. Immediately after injection a standstill of gastric movements occurred.

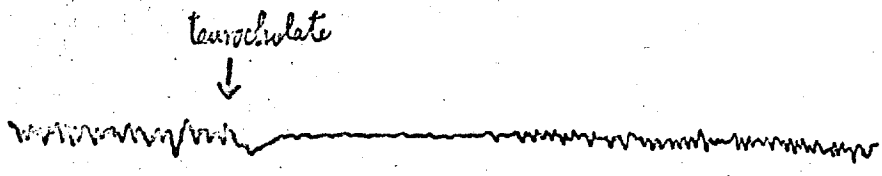

Fig. 7. 1.3 c.c. of a 2 % taurocholate solution was intraperitoneally injected. Immediately after injection a marked decrease in amplitude was found.

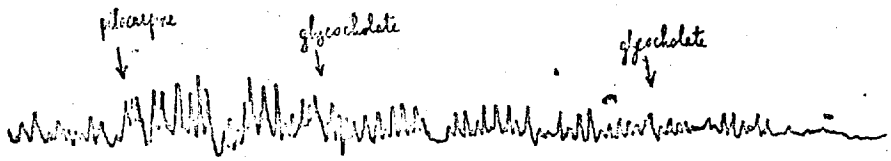

Fig. 8. The contractions of the stomach, which had been markedly stimulated by the intravenous injection of 0.6 c.c. of a 0.1 % pilocarpine solution, were gradually inhibited by the intraperitoneal injection of 2.8 c.c. of a 2 % glycocholate. The second intraperitoneal injection of the same dose of glycocholate caused a standstill of gastric movements.

The intraperitoneal injection of 1 mg/kg of glycocholate caused no remarkable change, that of 5 mg/kg a slight decrease, that of 10 mg/kg a marked decrease in amplitude of gastric movements and finally that of 20 mg/kg caused a complete standstill of the gastric movements. The intraperitoneal injection of 1 mg/kg of taurocholate caused no remarkable change, that of 5 mg/kg a slight decrease, that of 10 mg/kg a moderate decrease and that of 20 to 40 mg/kg a marked decrease in amplitude of gastric movements. The intraperitoneal injection of 2 to 5 mg/kg of dehydrocholate caused no remarkable change, that of 10 to 20 mg/kg a slight decrease and that of 40 mg/kg a marked decrease in gastric movements. As shown in Figure 8, the contractions of the stomach, which had been

markedly stimulated by the intravenous injection of 0.6 c.c. of a 0.1 per cent pilocarpine solution, were gradually inhibited by the intraperitoneal injection of 20 mg/kg of glycocholate and finally led to a standstill by the second intraperitoneal injection of 20 mg/kg of glycocholate.

Summary

Performing some experiments concerning the effect of bile salts on the gastric movements, the following results were obtained.

1. The intravenous injection or oral administration of a small dose of sodium glycocholate, taurocholate and dehydrocholate caused an increase and that of a large dose a decrease in the movements of the rabbit's stomach, while the intraperitoneal injection of a small dose always caused a decrease and that of a large dose caused a complete standstill of the gastric movements. In other words, application of bile salts to the mucosa of the rabbit's stomach in situ caused stimulation, except at high dosage of bile salts, while application to the serosa inhibited activity.

2. Bile salts, except at high dosage, have more of a stimulating effect on the rhythmic contractions than on the tonus of the stomach.

3. In the stimulating action of the bile salts when applied to the mucosa, dehydrocholate is the strongest and taurocholate the weakest, whereas in the inhibiting action when applied to the serosa, glycocholate is the strongest and taurocholate the weakest.

4. From the facts that the contractions of the stomach pronouncedly inhibited by atropine were stimulated by the intravenous injection of dehydrocolate and that the contractions of the stomach markedly stimulated by pilocarpine were inhibited by the intraperitoneal injection of glycocholate, it may be concluded that bile salts act directly on the musculature of the stomach and not through the autonomic nervous system.

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THE MINIMUM MORTAL DOSE OF SODIUM TAUROCHLORATE ADMINISTERED ENDOPLEURICALLY

(Experimental research)

Dr. Paolo Schiaparelli

Sodium taurocholate has an important use in the cure of purulent pleurisy in infancy, following the original method proposed by C. Cocchi. The sodium taurocholate is injected in appropriate dilution into the thoracic cavity, where it releases a bactericidal effect on the pneumococcus and a lytic one on the exudate, as was proved in experiments in vitro.

This biliary salt, as we know, is the combination of aminoethanesulfonic acid, or taurine, and cholalic acid; like all the biliary salts, when injected into the circulation it determines hemocytolysis and works an exciting influence on the parasympathetic system. (Rondoni)

The toxic action of this salt in circulation appears to be diminished by the proteins of the blood serum.

The minimum mortal dose (m.m.d.) of sodium taurocholate administered intravenously has been established by Roger in the rabbit at 0.40 g for every kg of weight.

Cocchi, in experimental diplococcic septicemia, was able to inject intravenously 0.50 g of taurocholate in a 5% solution into rabbits of around 2.500 kg body weight, without provoking pains in the animal, and in the cure of diplococcic empyema he was able to inject into the thoracic cavity of children as much as 0.70 g sodium taurocholate for every kg of weight, in both 10% and 20% solutions.

Castellanos too, using sodium taurocholate in the cure of empyemas, arrived at corresponding doses.

Recently Galeotti-Flori established that the m.m.d. of sodium taurochlorate in a physiological solution of 10% administered to the rabbit intravenously is 0.50 g per kg, and that this dose is rendered insufficient if there is added to the solution of sodium taurocholate 20% of homologous blood serum.

I could not find any evidence that there had been performed other experiments on the toxicity of this biliary salt, nor that the toxicity and effect of this salt when introduced into the thoracic cavity had been experimentally studied.

I therefore considered it interesting to establish the minimum lethal dose of sodium taurcholate introduced endopleuric-ally.

The research had necessarily to be carried out using animals. And since we are not able to reproduce experimentally a fibro-purulent pleurisy which would be, in anatomo-pathological and clinical characteristics and in development, comparable to spontaneous human fibbrino-purulent pleurisy, the minimum mortal dose could only be determined by experimenting on the effect of the substance injected into a normal pleura.

Given the dissolving action exercised by the sodium taurocholate on the fibrin, it was to be anticipated that rather considerable lesions would be produced in healthy tissues as well. Besides this, it had to be taken into consideration that there was a possibility of a general action, either as repercussion and consequence of the possible local lesion, or because of the effect worked on the tissues of the organism by the sodium taurocholate resorbed by the thoracic cavity.

Method. -- I carried out the endopleural injections with common Pravaz syringhes; first I introduced the needle in correspondence with the fourth intercostal space; then to the needle I grafted a small water manometer, the oscillations of which indicated whether the point of the needle was exactly in the cavity; then I joined the syringhe to the needle and introduced the solution of sodium taurocholate. Before the treatment, the animals had not eaten for 5 or 6 hours. I used pure sodium taurocholate supplied by the firm of Carlo Erba; in a solution with liquid of Ringer, this has a pH of 5.8 (with the comparator of Hellige).

GROUP I

In a first group of experiments I wished to test the tolerance of the rabbit of an endopleural injection of sterile physiological solution, in order to ascertain whether the solution may not be of itself harmful to the animal in the injected volume.

For this the following was necessary:

to	rabbit	Nr.	1	I	injected	endopleurally	on	the	right	side	2	cc
"	"	"	2	"	"	"	"	"	"	"	5	"
"	"	"	3	"	"	"	"	"	"	"	10	"
"	"	"	4	"	"	"	"	"	"	"	15	"
"	"	"	5	"	"	"	"	"	"	"	30	"

(Physiological solution)

After the injection, the rabbits showed no disturbance; after about four hours after the treatment, explorative punctures yielded negative results.

To rabbit Nr. 5 (kg 1.160), I injected 45 cc physiological solution: this greater volume only provoked a slight temporary dyspnea; after a few minutes, the respiration was again normal. After 4 hours, an explorative puncture yielded a small amount of slightly hemorraghic liquid; after 24 hours, a successive puncture was negative. I then killed the animal: the necroscopic examination demonstrated complete absence of liquid in the serous cavity; pleura and lungs completely whole. The hemorraghic aspect of the liquid extracted with the first puncture thus must have been of a

traumatic nature, due to the punctures themselves and a lesion so slight that it could not be traced. Thus it was demonstrated how the endopleural injections of physiological solution, even in greater quantity in relation to the weight of the animal (cc 45), are not harmful to the rabbit: the resoption of the liquid occurs very rapidly.

GROUP II

I then considered it opportune to test the toxicity of sodium taurocholate introduced intravenously, in order to avoid doubts regarding the quality of sodium taurocholate used (I used taurocholate that I still had on hand), or the particular reactivity of the rabbits.

Rabbit No. 6. -- Kg 2.400.

I injected 0.24 g sodium taurocholate dissolved in 2 cc physiological solution (0.10 g per kg). Injection intravenous.

Rabbit No. 7. -- Kg 1.500.

Intravenously injected 0.30 g sodium taurocholate dissolved in 3 cc physiological solution (0.20 g per kg).

Rabbit No. 8. -- Kg 2.000.

Intravenously injected 0.60 g sodium taurocholate dissolved in 3 cc physiological solution (0.30 g per kg).

The rabbits support these injections very well, and present no symptoms of suffering.

I did not consider it necessary to continue the tests in order to arrive at the mortal intravenous dose (recently also established by Galeotti-Flori at 0.50 g per kg), having already exceeded by about 2 and a half times the dose which was shown to be mortal in endopleural administration.

GROUP III

In this group of experiments I wished to determine the m.m.d. of sodium taurocholate administered endopleurally. I used sodium taurocholate in a sterile physiological solution, maintaining a constant volume of 5 cc. The injections were also made in the right hemithorax.

Rabbit No. 9. -- Kg 1.6000

Endopleurally injected 0.80 g sodium taurocholate in 5 cc physiological solution (0.05 g per kg).

The animal symptoms of suffering immediately after the injection: it screams and runs wildly about the room. The disturbances disappear, however, in a short time.

Rabbit No. 10. -- Kg 1.800.

Injected 0.16 g sodium taurocholate in 5 cc physiological solution (0.10 g per kg).

The sufferings of the animal after the injection are more accentu-

ated; the animal undergoes some convulsive attack of the duration of a few minutes; after about a quarter of an hour, all disturbance seems to disappear; there remains only a slight dyspnea.

Rabbit No. 11. -- Kg 1.600.

Endopleurally injected 0.24 g sodium taurcholate in 5 cc physiological solution (0.15 g per kg).

Immediately after, the animal screams, moves violently, runs about the cage; it is in the clutches of a strong dyspnea, from the mouth there issues a bit of foaming hemorraghic liquid.

The rabbit dies after about 15 minutes, in the grip of convulsions and tremors. A necroscopic examination reveals: on the right side of the thoracic cavity about 5 cc dark yellowish liquid, hemorrhagic. The right parietal pleural layer shows some slight superficial hemorrhage; the visceral one, and the surface of the right lung are strongly congested. The pleura and lungs of the left are intact.

Rabbit No. 12. -- Kg 2.500.

Injected 0.25 g sodium taurocholate in 5 cc physiological solution (0.10 g per kg).
Same as in rabbit No. 10, and it survives.

Rabbit No. 13. -- Kg 1.320.

Injected 0.13 g sodiun taurocholate in 5 cc physiological solution (0.10 gr per kg).

The rabbit dies one half hour after the injection, with the same symptomatology and the same findings of rabbit No. 11.

Rabbit No. 14. -- Kg 1.700.

Injected 0.17 g sodium taurocholate in 5 cc physiological solution (0.10 g per kg).

Same as rabbit No. 10, and survives.

Rabbit No. 15. -- Kg 1.500.

Injected 0.19 g sodium taurocholate in 5 cc physiological solution (0.12 g per kg).

The rabbit dies after about 10 minutes. Manifestations and findings as in rabbit No. 11.

Rabbit No. 16. -- Kg 2.500.

Injected 0.37 g sodium taurocholate in 5 cc physiological solution (0.15 g per kg).

The rabbit dies after about 10 minutes.

Rabbit No. 17. -- Kg 1.500.

Injected 0.22 g sodium taurocholate in 5 cc physiological solution (0.15 g per kg).

The rabbit dies after about 10 minutes.

Rabbit No. 18. -- Kg 1.800.

Injected 0.18 g sodium taurocholate in 5 cc physiological solution

The rabbit, like No. 10, is well after a few minutes.

Rabbit No. 19. -- Kg 1.700.

Injected 0.17 g sodium taurocholate in 5 cc physiological solution (0.10 g per kg).

Same as No. 10.

It is revealed that the rabbits who underwent endopleural injections of a solution of sodium taurocholate corresponding to 0.05-0.10 g of this salt for every kg of body weight (with the exception of No. 13) supported the injection well, while the rabbits which received 0.12 and 0.15 g sodium taurocholate for every kg of body weight died after a few minutes following the injection.

Therefore I believe that the m.m.d. of sodium taurocholate administered endopleurally to the rabbit can be fixed at around 12-15 cg per kg of weight.

GROUP IV

I used solutions of sodium taurocholate less concentrated, in order to see whether the dilution has an influence on the minimum mortal dose; I brought the dilution to 4 or 8 times that used in the experiments of group III.

Rabbit No. 20. -- Kg 1.800.

Injected 0.18 g sodium taurocholate in 10 cc physiological solution (10 g per kg).

The rabbit supports this injection like No. 10.

Rabbit No. 21. -- Kg 2.000.

Injected 0.24 g sodium taurocholate in 10 cc physiological solution (0.12 g per kg).

The rabbit dies immediately after, like rabbit No. 11.

Rabbit No. 22. -- Kg 1.880.

Injected 0.29 g sodium taurocholate in 10 cc physiological solution (0.15 g per kg).

The rabbit dies immediately after, like No. 11.

Rabbit No. 23. -- Kg 1.300.

Injected 0.19 g sodium taurocholate in 20 cc physiological solution (0.15 g per kg).

The rabbit dies after a few minutes, like No. 11.

Rabbit No. 23(a). -- Kg 1.400.

Injected 0.21 g sodium taurocholate in 45 cc physiological solution (0.14 grams per kg).

The animal dies after about 10 minutes, like No. 11.

GROUP V

I referred to the recent research of Galeotti-Flori, who found that the addition of blood serum to the solution of sodium taurocholate notably decreases the hemolytic action of the sodium taurocholate itself; I wished to test whether the proteins of the blood would also diminish the action of the sodium taurocholate when it is administered endopleurally.

To this end, I practiced endopleural injections of sodium taurocholate in physiological solution, in combination with a certain quantity of whole blood citrated of the rabbit, or of homologous blood serum. The injections were carried out after the sterilized solution of sodium taurocholate had been in contact with the blood or with the serum for 2 hours in a thermostat at 37°C.

Rabbit No. 24. -- Kg 1.900.

Injected 0.20 g sodium taurocholate in 5 cc physiological solution and 2.5 cc citrated blood of the rabbit (0.10 g per kg).

The rabbit supports the injection well.

Rabbit No. 25. -- Kg 1.750.

Injected 0.26 g sodium taurocholate in 5 cc physiological solution and 2.5 cc citrated blood of the rabbit (0.15 g per kg).

The rabbit dies after a few minutes, like No. 11.

Rabbit No. 26. -- Kg 1.600.

Injected 0.16 g sodium taurocholate in 5 cc physiological solution and 2 cc blood serum of the rabbit (0.10 g per kg).

The rabbit bofe the injection well.

Rabbit No. 27. -- Kg 1.200.

Injected 0.19 g sodium taurocholate in 5 cc physiological solution and 2 cc blood serum of the rabbit (0.15 g per kg).

The rabbit dies after 10 minutes, like No. 11.
From these tests it is shown that neither whole blood nor blood serum of the rabbit diminish the toxicity of sodium taurocholate administered endopleurally.

GROUP VI

Endopleural injections on the left side of solutions of sodium taurocholate in 2 rabbits (No. 9 and No. 10) already treated on the right side 15 days before, with 0.05 g and 0.10 g respectively of sodium taurocholate per kg of weight.

Rabbit No. 9. -- Kg 1.500.

Injected 0.075 g sodium taurocholate in 5 cc physiological solution (0.05 g per kg).

Light symptoms of suffering (the animals screams and runs wildly about the cage) immediately after the injection; however, the disturbances disappear after about 15 minutes.

Rabbit No, 10. -- Kg 1.500.

Injected 0.15 g sodium taurocholate in 5 cc physiological solution (0.10 g per kg).

The rabbit dies shortly afterward.
In the necroscopic examination we noted: in the thoracic cavity of the left about 5 cc dark yellowish, hemorraghic liquid; the surface of the left lung is strongly congested. In the thoracic cavity of the right there is found a notable quantity of dark hemorrhagic liquid (about 20 cc). The right lung is reduced to a small hard carneous mass, sharp on the edge, strongly leaning on the mediastinum, with some fibrous cusps between the parietal and visceral pleura.

All the rabbits which survived the endopleural injection of sodium taurocholate were later killed at various intervals of time. Necroscopic examinations revealed the following.

Rabbit No. 18, treated endopleurally with 0.10 g sodium taurocholate per kg, shows, after two days, a pleural effusion on the right, controlled with evacuative punctures, with which there were extracted about 10 cc of reddish, slightly muddy liquid. After 4 days following the treatment, the animal was killed, and under the necroscope there were found in the right thoracic cavity about 35 cc sanguinolent liquid, which, centrifuged, appears to have a lemony color; the pleura is apparently intact, except for some fibrous accretions.
The lung of the same side shows a surface of a dark color, and manifest signs of an initial process of atelectasis.

Rabbit No. 19 (0.10 g sodium taurocholate per kg) shows, after 2 days, an abundant pleural effusion.

Sacrificed after 8 days, there is revealed under the necroscope 35 cc of liquid in the right thoracic cavity; the lung is intensely atelectasic and retracted, hard, carnified with notable pleural accretions.

Rabbit no. 20 (0.10 g sodium taurocholate per kg) dies from recurrent illness after 12 days. The necroscopic examination reveals the same findings as in rabbit No. 19.

Rabbit No. 24, treated endopleurally with 0.10 g sodium taurocholate per kg in combination with 2.5 cc homologous whole blood, was sacrifieed after 15 days. Necroscopic findings like those of rabbit no. 19.

Rabbit no. 12 (0.10 g sodium taurocholate per kg) was sacrificed after 20 days following the treatment.

Rabbit No. 14 (0.10 g sodium taurocholate per kg) was sacrificed after 25 days.

The necroscopic examinations of these last two rabbits revealed the atelectasic state of the right lung to be much more accentuated; the lung is harder, much reduced in volume, with a dark, hemorrhagic surface with more intense pleural accretions.

Rabbit No. 26, treated endopleurally with 0.10 g sodium taurocholate per kg in combination with 2 cc homologus blood serum,

was sacrifieed 60 days after the treatment.

In the necroscopia were noted: on the right, 30 cc pleural, hemorrhagic effusion; the right lung is reduced to a small mass of hard flesh, leaning on the mediastinum, with numerous fibrous cusps between the parietal and visceral pleura.

From these protocols it is revealed that I conducted the experiments on 29 rabbits, and of these 29, 20 were treated with endopleural solutions of sodium taurocholate

On all the rabbits, those which died immediately after the endopleural injections, as those sacrificed some time after the treatment, I performed a histological examination of the pleuras and the lungs. In some rabbits, the examination was completed with the histological observation of the liver, the kidneys, the adrenals, the heart and the spleen.

The histological findings were different, according to the time elapsed between the injection of sodium taurocholate and the death of the animal. But in the animals which were found in the same conditions, the results were perfectly constant. I therefore limit myself, for the sake of brevity, to reporting the findings which can demonstrate the evolution of the verified lesions.

Rabbit No. 21 (0.15 g sodium taurocholate per kg, dead after 10 minutes following treatment).

Right lung. -- In the central part of the lung there exist lobules in acute vescicular emphysema, and here and there in some circumscribed zone, the alveoles appear to be full of a homogeneous plasmatic substance, similar to that which is found in common edemas. The perilobular capillary ansas are full of blood, especially in the tracts where the emphysema is less outstanding. Proceeding toward the periphery, the aspect of the lung is modified in that the emphysema is less manifest, while the alveoles containing the substance already seen here and there in the central part become more numerous, assuming here, however, an hemorrhagic aspect, due to the presence of numerous red corpuscles. In the interstitial tissue as well we find abundant erythrocytes.

Proceeding toward the exterior of the organ, the histological alterations are more serious, in that all the alveoles are occupied by the plasmatic exudation which prevails in the hemmorhagic phenomena, and concomitant with that, there are regressive phenomena in the cellular elements, so that the cells appear pyknotic in some ways, and in others badly defined. On the other hand, on the immediate periphery, the edema begins to cease, and there are no hemorrhagic manifestations, though the same regressive phenomena in the cellular elements still remain. No appreciable pleural reaction.

Rabbit No. 18 (0.10 g sodium taurocholate per kg, dead 4 days after the treatment).

Right lung. -- In the central part it is noted that the alveoles tend in some places to relax; this tendency has an initial

process of retraction and is more evident toward the periphery of the section, where the alveolar design has nearly disappeared. Some fibroblastic interalveolar elements are noted. The pleura is slightly thickened. Nothing of particular note in the other organs.

Rabbit No. 20 (0.10 g per kg, sacrificed 12 days after the treatment).

Right lung. -- In the central part it is noted how the alveoles are slightly distended and tend in various places to relax. Proceeding toward the exterior, the alveolea are all closer together in consequence of an initial process of retraction , and many of them present in their centers large cells of alveolar origin, desquamated, many of them vacuolized.

Even more toward the periphery it is noted that the pulmonary design is nearly completely vanished, the tissue of the organ having been transformed into a nearly homogeneous tissue (plasma with fibrils), where some fibroblastic elements are observed, as well as various nuclear debris. The interalveolar septa appear thickened by a slight connectival hyperplasia.

On the surface there is found a thin layer of fibrillary structure (which probably represents the thickened pleura).

All the fleshy pulmonary residue is slightly sanguified.

Rabbit No. 12 (0.10 g per kg, sacrificed 15 days after the treatment).

Right lung. -- The central part reveals the same processes of atelectasis found in rabbit No. 20. In some places around the vessels, slight inflammatory reactions.

In the peripheral part of the lung the endothelial alveolar elements are no longer recognizable, and the alveolar septa are thickened by the neoformation of connective tissue.

From the underside of the thickened pleura, in certain places branches of the connective penetrate into the parenchyma. The pleural thickening is already proceeded to a notable degree, though the characteristics are those of a beginning (not very thick, nor crowded, rich in nuclei.)

Liver. -- Degenrative phenomena are not very evident. In some cells of the reticula there is pigment of an ochrous color.

Adrenal glands. -- Except for a certain degree of sanguinary repletion of the medulla, there is no particular alteration.

Rabbit No. 14 (0.10 g per kg, sacrificed 25 days after the treatment).

Right lung. -- The tissue appears to be compact, pparticularly in the central part, where the alveolar design is no long visible, revealing thus an aspect of thickening and carnification. The vessels and the bronchi also tend to relax, causing the disappearance of their light. On the other hand, in the periphery, immediately below the pleura, the alveolar design, though modified, is recognizable, though the septa appear to be notably thickened by hyperplasia of the connectival elements; on the other hand, however, here as well there are more evident epithelia of integument

On the whole, the alveolar structure is determined by branches of connective tissue which intertwine. On the surface, the pleural crust has thickened notably, formed from lamella of a connectival nature, rich in young elements with some vasa. In certain points, between the lamella, there are tracts which have a homogeneous aspect.

Liver. -- Examining the liver under slight magnification, it is noted that the trabecula of the hepatic cells are at a large distance from one another, as though there existed a certain degree of edema; here and there are found circumscribed zones in which the tissue appears very altered. The normal structure is nearly vanished. In these zones, as is noted under greater magnification, the cellular alteration has reached a degree so elevated that is is only possible to observe the residue of disgregated nuclei, in a substance which probably represents the residue of the propoplasmatic dissolution.

Where the alteration has not reached such an advanced stage, it is noted that the cell have lost the clearness of their contours, the protoplasma appears vacuolized, and often even the nucleus, in the grip of outstanding regressive phenomena, tends to disappear.

Kidneys. -- No particular phenomenon in the glomerules. Cells of the ascending crus of Henle are in the grips of a slight, but manifest roiled swelling.

Rabbit No. 26 (0.10 g per kg, sacrificed 60 days after the treatment).

Right lung. -- The pulmonary tissue appears to be totally compacted, with the aspect of intense carnification. The alveolar design has completely disappeared, because the walls of the alveoles relax. In the more peripheral part there is seen intense connectival intrafiltration in branches which intertwine into all the areas. The organ is all very slightly sanguified. The pleura is even more thickened than in the case of rabbit 14, and the connectival lamella of homogenous aspect are more abundant. The histological report of the other organs is similar to that of rabbit No. 14.

DISCUSSIONS AND CONCLUSIONS

Summarizing the data which I obtained in my experiments, we see that the rabbits treated endopleurally with a dose of sodium taurochlorate corresponding to 0.05 g, 0.08 g, 0.10 g for every kg of the animal's weight, in solution with 5 cc and 10 cc of a physiological solution (with the sole exception of rabbit No. 13) survived this injection; while the rabbits treated with a dose of sodium taurocholate corresponding to 0.12 and 0.15 g per kg (in solution with 5, 10, 20, 30, 45 cc physiological solution) died after 10 or 15 minutes.

The minimum mortal dose of sodium taurocholate administered endopleurally in the rabbit can thus be established as a dose of 12 cg per every kg of weight, regardless of the concentration of the solution of sodium taurocholate; this dose is notably less than that minimum mortal dose of sodium taurocholate administered intravenously (established at 50 cg per kg).

It is difficult to be able to say why this biliary salt is more toxic when administered endopleurally than it is when administered intravenously.

The necroscopic findings and the numerous controls with Ringer liquid authorize me to exclude the possibility that the traumatic factor might be the cause of death for the animal.

We can think of the fact that sodium taurocholate intoduced into the blood system is diluted in the blood in such high proportion that its toxic activity is much diminished. But from the experiments of group IV we see that the m.m.d. of endopleural administration does not modify upon dilution: instead I calculated the dilution which the mortal dose of sodium taurocholate injected intravenously undergoes in distributing itself in the blood, and I tested the toxicity of sodium taurocholate introduced endopleurally in a dilution slightly greater than that which the intravenous mortal dose undergoes in diluting with the blood. Thus we can exclude the possibility that the more intense action is the effect of the concentration.

However, the sodium taurocholate provokes serious alterations in the pleuropulmonary tissues. The alterations assume a different aspect according to whether the animal died immediately after the injection, or was sacrificed later.

In the animals dead immediately after the injection I noted: acute vescicular emphysema in the central section of the parenchyma with repletion of the perialveolar vascular ansas; toward the periphery of the lung, the lesions appear more gerious; the interstitial hemorrhages and the acute edema of the alveoles, which are full of blood, become more evident; even further in the periphery, that is in the surface layer of the lung, we see rather intensive regressive cellular phenomena. Nothing of particular note in the pleural layers.

In the rabbits which survived the endopleural injection, I notedsome very interesting pulmonary histological lesions, which demonstrated how even a very small dose of this biliary salt can wound the healthy pulmonary tissue.

It is important to note that the sodium taurocholate modifie the power of resorption of the pleura of the injected liquid in an outstanding manner. While the physiological solution is resorbe very rapidly (see experiments of group I), a solution of sodium taurocholate (less than the mortal dose), though diluted, determin within a few hours, an alteration of the tissues in which the resorption cannot be verified as usual. In fact, within the pleura are found notable quantities of liquid, which present characteristics mixing those of a transudate and exudate, and seems to contain the injected sodium taurochlorate, even after some time. The sodium taurocholate may thus act at length and with intensity, exercising its influence on the tissues.

By sacrificing the rabbits treated with doses of sodium taurocholate inferior to the minimum dose, at various intervals after the day of the endopleural injection, and examining the lungs and other organs of the animal histologically, I was able to note the gradual increase of the pulmonary lesions.

In fact, as soon as a few days after the treatment (4 or 5), the pulmonary tissue is seen to be notably atelectasic, especially in the central section of the organ, where the alveoles relax into one another, so that the tissue assumes a compacted aspect. Peripherally, below the strongly thickened, sclerotic pleura, there are evident phenomena of necrosis in the cells of the pulmonary tissue.

Then examining the lung of the rabbit, in whose thoracic cavity the sodium taurocholate solution was "in sito" for 15 days, I noted that the pleural corium, formed from lamella and young connectival elements, was much more thickened, and the pulmonary parenchyma had completely lost every alveolar design, assuming an aspect of carnification. If then the rabbits sacrificed after 30, 45, and 60 days following the endopleural treatment are examined, both the pleural corium and the atelexia and carnification of the lung appear in progressive increase to such a point that the pulmonary tissue is reduced to a small block of hard, fibrous flesh, leaning on the mediastinum.

The pleura is full of liquid; between the parietal pleura layer and the pleuropulmonary residue there are fibrous cusps.

The other organs present faint alterations, except for the liver, where I noted some degenerative phenomena in the parencymatc cells and some accumulation of ochre-colored pigment in the tissue of the sustentaculum.

It is not possible to say whether the lesions verified in the lungs of the rabbits dying from the injection of a mortal dose of sodium taurocholate are sufficient to explain the cause of the animal's death. The importance of these lesions is difficul to define, and, moreover, there is no end to the comparison even for the argument of analogy with other lesions, since those verifie by me are not comparable to the common pulmonary lesions and are not classifiable.

Then it must be considered that the rabbits inoculated with the sodium taurocholate endopleurally die in convulsions, which would demonstrate the participation of the central nervous system. We can think of phenomena of "shock", or reflex phenomena in the nervous centers, or an action of the sodium taurochlorate on the nervous mediastinic ganglia.

It must be remembered that even the rabbits injected intravenously with a sufficient dose die in convulsions. But if in this case it can be admitted that the sodium taurocholate succeed in acting on the central nervous system in sufficient concentration, this appears less probable in the case of endopleural injecti because of the minimum doses and the slight resorption. The point of departure of the complex of phenomena which leads to the death of the animal seems to be the pleura. That from the contact of the sodium taurocholate and the lung there may generate a toxic substance can be excluded, since I tried to inject a colliquated lung extract of socium taurocholate and was unable to find any toxicity of the preparation which exceeded that of the sodium taurocholate alone. The mechanism of death thus appears to be very obscure.

However, there remains the fact that the sodium taurocholate introduced endopleurally into healthy pleura is notably more toxic

blood serum to a solution of sodium taurocholate seems to diminish the toxic power of this salt when administered intravenously (Galeotti-Flori), this addition determines no appreciable variation in the toxicity of the salt administered endopleurally (see Group V).

Finally, another element must be taken into consideration: that is the reaction of the substance. I used the sodium taurocholate of the Firm of C. Erba, which, in solution, has a pH of 5.8. Since in therapy one will use sodium taurochlorate in solution without modifying its reaction, I conducted all the experiments with the substance at an incorrect pH.

Whether and what importance this may have on the toxicity of sodium taurocholate and on its therapeutic activity, will be shown by experiments which I now have in progress.

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The sodium taurocholate exercises a characteristic lytic action on the pneumococcus and the fibrino-purulent exudate. Because of this activity it has been used in the cure of fibrino-purulent infantile pleurisy, and especially in smaller children for whom an operation is dangerous and contraindicated. Since the therapeutic activity of sodium taurocholate is narrowly bound to a lytic property so outstanding that it even loosens the fibrin, it is not surprising if even the normal tissues do not escape the aggressive action of this substance.

In my experimental research, because of the impossibility of reproducing a fibrino-purulent pleurisy comparable to the human, I necessarily had to work on normal pleuras.

The normal tissues thus came into direct and immediate contact with the sodium taurocholate neither being able to escape the action of the substance retained in the closed serous cavity which was altered in its capacity of absorption.

In the natural disease, the conditions are very different; the thoracic cavity contains exudate which dilutes and in part retains the sodium taurocholate but the sodium taurocholate can not come into contact with the tissues, especially because they are protected by the fibrinous layers.

The colliquation which the exudate undergoes because of the medicament introduced in a discontinuous manner is gradual, and does not reach the point of leaving the tissues undefended.

The sodium taurocholate if during the injection it comes into contact with living sensitive tissue, is immediately noticed by the child, who reveals, through his behavior, a vivid pain; he is racked by an irritating cough, and emits, with the cough, a small quantity of mucous, foaming excrete, slightly marked with blood. The response to the stimulus is, as I said, immediate; therefore the injection can be interrupted immediately.

The pain, the cough, the emission of excreate, last a few minutes; the consequences of the injection are rapidly resolved without leaving aftermaths. This I was able to observe in a case of circumscribed purulent pleural collection cured by sodium taurocholate in which, because of a sudden movement of the patient during the injection, the point of the needle moved from the cavity, already emptied of exudate, toward the tissues.

But in intense congestions, the possibility of conducting the sodium taurocholate into contact with the sensitive tissues cannot occur; it is even difficult to carry out a proper injection. It is however opportune to carry out the introduction of the sodium taurocholate very slowly, and with a weakening of pressure; to allow instead, if possible, the solution to be spontaneously inspired from the syringhe by the negative pressure which is verified in the inspiratory phases of the pleura, after the subtraction of a sufficient quantity of exudate, because thus there is the best guarantee that the point of the needle is inside the cavity.

With these cautions in the method of injection of sodium taurchlorate we not only avoid danger, but also any kind of inconveniences.

Summary:

The minimum mortal dose of sodium taurocholate injected endopleurally into the normal rabbit, with healthy pleuras, is 12 cg for every kg of weight.

This does not vary with the variation of the concentration of the dilution of the injected salt. This dose is considerably inferior to the minimum mortal dose of the salt injected intravenously (50 cg per kg).

The anatomical and histo-pathological examination of the lungs, both of the animals dead immediately after the treatment and those sacrificed after some time, demonstrated the existence of interesting alterations. In the rabbits which died immediately there were pulmonary lesions so slight that they could not of themselves be explanations for the death of the animal.

The findings verified in the rabbits treated with doses of sodium taurocholate inferior to the minimum lethal dose placed in evidence:

that the sodium taurocholate (at a pH of 5.8) modifies the power of absorption of the pleura, so that the taurocholate persists at length in the serous pleura;

that the sodium taurocholate determines, in the lung, outstanding progressive alterations which lead, 30-60 days after the treatment, to a carnification and total retraction of the lung.

The blood serum does not diminish the toxicity of the sodium taurocholate administered endopleurally.

The experiments were conducted with animals with normal pleuras. The characteristic activity of the sodium taurocholate is bound to the property of not only determining the lysis of the pneumococcus, but also of loosening the fibrin; it is therefore natural that in experimenting on healthy pleuras the aggressive activity of the sodium chloride is not arrested in the face of normal tissues. Nonetheless, the aggressive action, though being inseparable from the therapeutic, is arrested in the child in conditions of natural disease, by the exudates which protect the pleura and the lungs. Therefore the use of sodium taurocholate presents no danger, and an appropriate technique avoids any inconveniences.

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Studies in Carcinogenesis. XV. Compounds Related to 20-Methylcholanthrene [1 2]

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Of the compounds examined for carcinogenic potency in this laboratory, a considerable number were structurally related to 20-methylcholanthrene (fig. 1, formula II). The results obtained with some of these compounds have been given in previous communications (2–6). This paper records the data [3] obtained following the injection of 22 additional compounds [4] into mice of inbred strains in experiments conducted in the period 1935–40.

As in previous experiments, the compounds were usually injected in the crystalline form moistened with glycerol. Unless otherwise specified, one injection of the crystalline material was given subcutaneously in the left flank of strain A mice, which were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The animals were usually about 3 to 4 months old when first injected. The properties of the specimens employed have been described in the papers from the cooperating laboratories of organic chemistry. Solutions in lard filtered at 38° C. were used in those instances in which not enough material was available for administration of the required amount in crystalline form, or when low doses were desired. The animals were fed a diet of Purina dog chow and tap water as in previous experiments in these studies.

In the tables the plus signs give the total number of mice in each experiment in which tumors had been induced at the injection site in the periods shown; the minus signs give the number of negative survivors at those times. In the tables and text the term "negative" designates the mice not bearing induced tumors at the site of injection. The term "tumor," as used in this paper in referring to lesions at the injection site, designates a

[1] *This work was carried out in the U. S. Public Health Service, Office of Cancer Investigations, then at the Harvard Medical School and at the Gibbs Memorial Laboratory, Harvard University.*

[2] *Presented (1) in part at the Third International Cancer Congress, Atlantic City, September 11, 1939. A summary was included in another paper (2).*

[3] *The results obtained in these biological experiments are in some instances announced in the papers of Fieser and coworkers in the Journal of the American Chemical Society, which described the syntheses of these compounds, and in the review by Fieser (7).*

[4] *These compounds, unless otherwise stated, were synthesized in the laboratory of Prof. L. F. Fieser, of Harvard University, as part of a cooperative project initiated by the U. S. Public Health Service in 1934.*

progressively enlarging mass having gross characteristics similar to other induced tumors in our experience which had been proven by transplantation and by histological examination to be sarcomas. In referring to lesions of the lung, the word "tumor" designates nodules having gross characteristics indistinguishable from histologically proven primary pulmonary tumors.

ALKYL DERIVATIVES

The structural formulas of cholanthrene (fig. 1, formula I), which may be regarded as the parent hydrocarbon, of 20-methylcholanthrene (fig. 1, formula II) and of five other alkyl derivatives are shown in figure 1. All of these compounds were found to be potent carcinogens. The most rapidly acting were cholanthrene and methylcholanthrene.[5] For ready comparison, the previously published data for the 20-ethyl[6] (fig. 1, formula III) and the 16, 20-dimethyl (fig. 1, formula IV) derivatives are included in table 1.

15,20-Dimethylcholanthrene (fig. 1, formula V).—The small available amount of 15,20-dimethylcholanthrene (*9*), synthesized by Dr. W. F. Bruce, of Cornell University, and Dr. L. F. Fieser, was dissolved in filtered lard to give a solution containing 5 mg. of the hydrocarbon per cubic centimeter. The injection (Dec. 11, 1937) of 1 mg. was repeated after 4 months.

Ulceration at the injection site occurred in half of the mice during the second

[5] *Since the only monomethyl derivative of cholanthrene dealt with in this paper is 20-methylcholanthrene, it is henceforth referred to simply as methylcholanthrene.*

[6] *In the report (5) of the carcinogenic activity of 20-ethylcholanthrene, the reference for the synthesis of this compound was given as "Bruce, W. F.: Unpublished work, 1936." The synthesis of this compound has since been published by Bruce and Kahn (8), of the Department of Chemistry, Cornell University.*

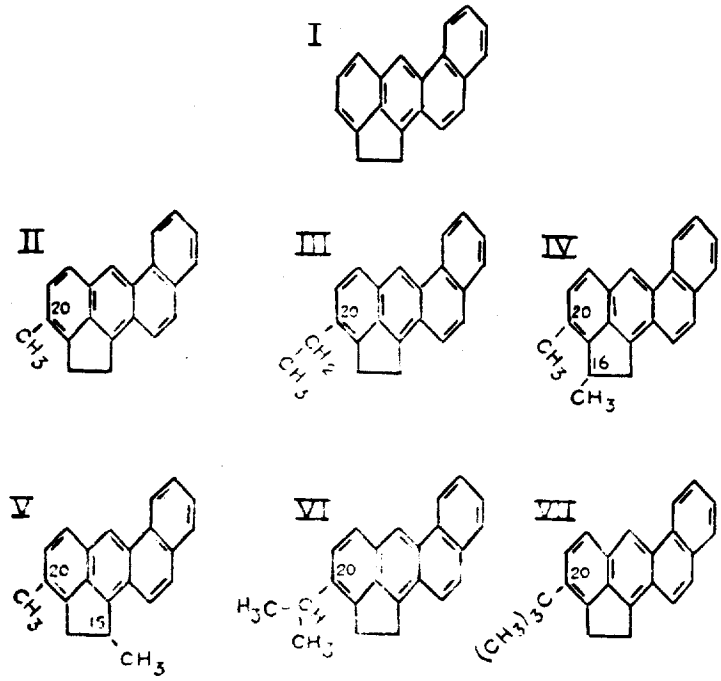


FIGURE 1.

TABLE 1.—*Alkyl derivatives of 20-methylcholanthrene*

[Production of subcutaneous tumors in strain A mice]

Substituent	Formula No.	Dose (crystals)	Mice		Total number of mice with tumors (+) and negative surviving mice (−), after various periods (in months)			
			Number	Sex	4 months	6 months	9 months	12 months
		Milligrams						
20-Ethyl	III	10	16	M+F	3+ / 12−	9+ / 5−	10+ / 1−	11+ / 0−
16, 20-Dimethyl	IV	20	14	M	1+ / 11−	4+ / 8−	8+ / 4−	10+ / 1−
15, 20-Dimethyl	V	[1] 2	16	M+F	3+ / 7−	7+ / 2−	8+ / 0−	---------- / ----------
20-*iso*-Propyl	VI	7	20	M	0+ / 18−	2+ / 16−	8+ / 5−	11+ / 0−
20-*t*-Butyl	VII	15	10	M	0+ / 9−	0+ / 8−	0+ / 6−	2+ / 3−

[1] In lard filtered at 38° C.

month. Severe ulceration was present in five of the six mice that died or that had to be killed because of poor condition during the first 4 months.

The first three tumors appeared during the fourth month. Tumors developed in four other mice during the fifth month. In the last survivor, a tumor developed in the eighth month (table 1). On the basis of previous experience with other compounds, it is possible that smaller doses might have given a lower early mortality and a higher yield of tumors.

*20-*iso*-Propylcholanthrene (fig. 1, formula VI).*—The 20-*iso*-propyl derivative, synthesized by Bruce and Todd (*10*), of Cornell University, was injected (Oct. 24, 1936) in a dose of 2 mg.; 6 months later another injection, of 5 mg., was given. The first tumor appeared during the fifth month. The experiment was terminated in the eleventh month at which time there was one negative survivor. The rate of tumor production and the survival data are given in table 1.

*20-*tertiary*-Butylcholanthrene (fig. 1, formula VII).*—The 20-*tertiary*-butyl derivative, synthesized by Fieser and Snow[7] (*11*), was injected (July 30, 1937) in a dose of 5 mg.; the same amount was injected after 5 and after 9 months.

The first tumor appeared in the tenth month, and another developed 1 month later. At the end of 11 months there were four negative survivors; none of these developed tumors at the injection site by the time the experiment was terminated in the fifteenth month (table 1).

OXYGEN-CONTAINING DERIVATIVES

15-Hydroxy-20-methylcholanthrene (fig. 2, formula VIII).—A dispersion of 15-hydroxy-20-methylcholanthrene was prepared by warming the compound in filtered lard. With a concentration of 2.5 mg. per cubic centimeter of lard at 38° C., some was dissolved and some remained suspended. The mice received (July 23, 1938) 0.4 cc. of this dispersion, which was equivalent to 1.0 mg. of the compound. Since no tumors had appeared in 3½ months, the mice received 5 mg. of the crystalline compound at this time.

The first 3 tumors appeared at the end of the fourth month; a tumor appeared in a fourth mouse at the end of 6 months.

[7] University of Chicago.

No further tumors developed until the tenth month, when tumors appeared in 5 more mice. By the end of a year tumors had been induced in 13 of the animals. The 2 negative survivors were killed after 13 months (table 2). Ulceration occurred in about one-third of the animals after 6 months. In 9 of the mice bearing tumors at the injection site, the lungs contained a strikingly large number of tumor nodules.

With the compound containing a hydroxyl group at the 15 position of methylcholanthrene, a marked reduction of the rate of tumor production at the injection site was obtained, as compared with methylcholanthrene.

15-Keto-20-methylcholanthrene (fig. 2, formula IX).—The first injection (Aug. 3, 1938) of 3 mg. of 15-keto-20-methylcholanthrene was followed by another, of 5 mg., 3 months later.

By the end of a year, tumors had been induced in only 3 mice; at this time there were 12 negative survivors (table 2). Another tumor developed after 14 months, and 3 more appeared in the sixteenth month when the experiment was terminated. A striking picture of multiple tumor nodules in the lungs was seen in only 1 mouse.

Since tumors were induced in only seven mice in 16 months, in spite of excellent survival, it is clear that the introduction of the keto group at the 15-position reduced the potency of methylcholanthrene even more markedly than introduction of the hydroxyl group. The London group (12) recently stated that epitheliomas were obtained with this compound, which they designate as 20-methylcholanthrene-15-one, thus confirming the potency it had exhibited with the subcutaneous technique.

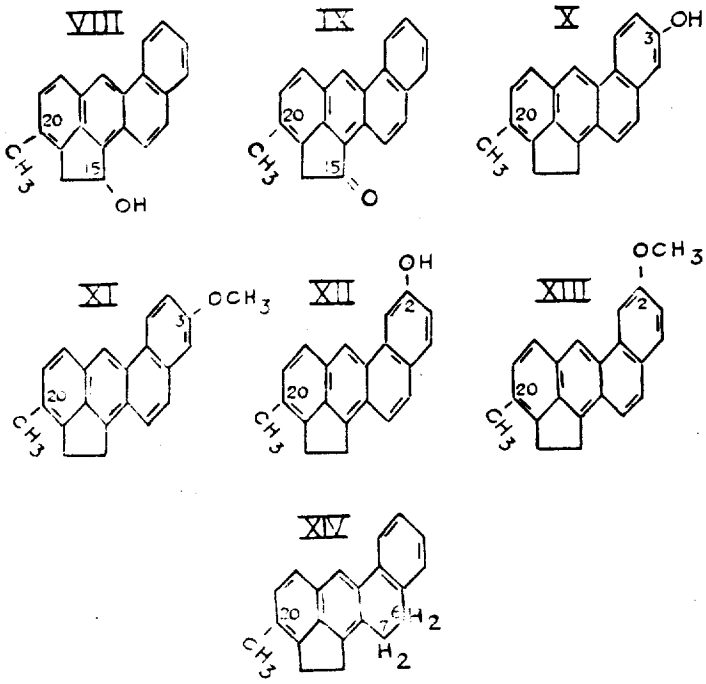


FIGURE 2

TABLE 2.—*Oxygen-containing derivatives of 20-methylcholanthrene*

[Production of subcutaneous tumors in mice [1]]

Substituent	Formula No.	Dose (crystals)	Total number of mice with tumors (+) and negative mice (−), after various period (in months)				
			4 months	6 months	9 months	12 months	15 months
		Milligrams					
17-Hydroxy	VIII	6	3+ 17−	4+ 16−	4+ 14−	13− 2−	
15-Keto	IX	8	0+ 19−	1+ 18−	2+ 15−	3− 12−	1− 4−
3-Hydroxy	X	10	0+ 20−	0+ 19−	0+ 17−	0− 12−	0− 8−
3-Methoxy	XI	15	0+ 10−	0+ 10−	0+ 10−	0− 7−	
2-Hydroxy	XII	1	0+ 20−	0+ 20−	0+ 19−	0− 13−	0− 6−
2-Methoxy	XIII	12	0+ 19−	0+ 18−	0+ 17−	0+ 11−	0− 7−
1,2-Dihydro	XIV	7	0+ 20−	0+ 19−	0+ 19−	0+ 19−	0− 11−

[1] These compounds were administered to 10 male and 10 female mice of the A strain, with the exception of the experiment with compound XI in which 10 C3H male mice were employed.

3-Hydroxy-20-methylcholanthrene (fig. 2, formula X). The injection, on August 16, 1937, of 5 mg. of crystalline 3-hydroxy-20-methylcholanthrene was repeated 6 months later. No tumors were induced at the site of injection (table 2), although 12 of the mice survived for a year and 8 were alive at 15 months. The experiment was terminated in the seventeenth month at which time the compound was seen in the subcutaneous tissues of the 3 survivors 11 months after the last injection.

This compound had also given negative results in the hands of the London investigators (*12*) in a skin-painting experiment. In both laboratories this derivative was considered of particular interest because the bile acids and sterols have a characteristic hydroxyl group [3] at position [illegible] and it was conceivable that, if ring closure took place in these biologically [illegible] compounds with the formation of compounds of the type of methylcholanthrene, the 3-hydroxyl group might be [illegible] *formulas XXII and XXIII.*

retained. However, neither skin painting nor subcutaneous injection of this compound yielded tumors.

3-Methoxy-20-methylcholanthrene (fig. 2, formula XI). The 3-methoxyl derivative of methylcholanthrene was injected (July 29, 1937) in a dose of 5 mg., which was repeated after 5 and 9 months. Survival was good (table 2), but no tumors had been induced by the time the last mouse was killed in the thirteenth month. This is in agreement with the negative results obtained in a skin-painting experiment with the same compound by the group in London (*12*).

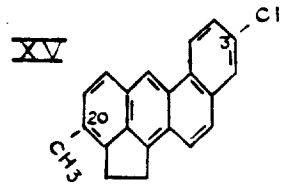
2-Hydroxy-20-methylcholanthrene (fig. 2, formula XII).—Only a small amount of the 2-hydroxyl derivative was available. Each mouse received one injection of 1 mg. Survival was good (table 2), but no induced tumors were noted. The last six survivors were killed at the end of the fifteenth month.

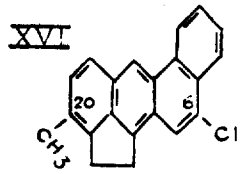
2-Methoxy-20-methylcholanthrene (fig. 2, formula XIII). [illegible]

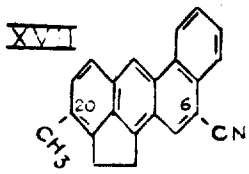
methoxyl derivative was injected (Mar. 24, 1938) as the first dose; 5 mg. was injected 7 and 12 months later. Eleven mice survived for a year, and seven were alive at 15 months (table 2). The last three survivors were killed at the end of 17 months. No induced tumors were obtained.

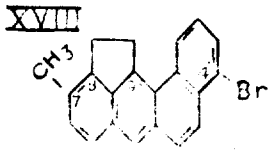
6,7-Dihydro-20-methylcholanthrene (*fig. 2 formula XIV*).—The first dose (Feb. 11, 1938) of the 6,7-dihydro derivative consisted of 2 mg.; a second injection, of 5 mg., was given 9 months later. The results are summarized in table 2.

Survival was quite good: 10 mice lived for a year, and 11 were alive at 15 months.









The last 9 survivors were killed in the seventeenth month. No induced tumors were obtained.

CHLORO, BROMO, AND CYANO COMPOUNDS

3-Chloro-20-methylcholanthrene (*fig. 3, formula XV*).—Since only a small amount of 3-chloro-20-methylcholanthrene was available, the experiment was restricted to 10 mice. Five milligrams was injected (July 20, 1937) as the first dose; this was repeated after 5 months. The results are summarized in table 3. No tumors were induced by the time that the last 2 survivors were killed in the fourteenth month (table 3).

6-Chloro-20-methylcholanthrene (*fig. 3, formula XVI*).—The first injection (Oct. 24, 1938) consisted of 2 mg.; another injection, of 5 mg., was given 6 months later. An epidemic infection brought this experiment to an early close (table 3). The last three sick survivors were killed in the tenth month. No induced tumors were noted in this period.

6-Cyano-20-methylcholanthrene (*fig. 3, formula XVII*).—The 6-cyano derivative of methylcholanthrene was injected (Oct. 24, 1938) in a dose of 2 mg. Survival was poor because of an epidemic infection. The nine sick survivors were killed in the ninth month. No induced tumors were noted in this period.

4'-Bromo-7-methyl-8,9-dimethylene-1,2-benzanthracene (*fig. 3, formula XVIII*).—It was previously reported (*1*) that 7-methyl-8,9-dimethylene-1,2-benzanthracene did not produce tumors in mice. Similarly negative results were obtained with the 4'-bromo derivative of this structural analogue of methylcholanthrene (table 3).

Since only a small amount of this compound was available, the experiment was restricted to 10 mice. The first injection

(July 20, 1937) consisted of 5 mg.; the rest of the supply was given 5 months later. The last 3 survivors were killed in the fifteenth month. No induced tumors were obtained.

ANTHRACENE DERIVATIVES

1, 2-Cyclopenteno-5, 10-aceanthrene (fig. 4, formula XIX).—To ascertain the importance of the angular 6-membered ring of cholanthrene (fig. 4, formula I) in tumor production, the 1, 2-cyclopenteno analogue was injected (Sept. 30, 1936) into mice. A solution containing 5 mg. of the hydrocarbon per cubic centimeter of filtered lard was employed. Four doses of 1 mg. each were given during the first 10 months.

The first tumor appeared in the sixth month. By the end of a year, tumors had been induced in 3 mice, and 10 mice were alive and negative (table 4). At 15 months there were 6 negative survivors; of these 1 developed a tumor in the seventeenth month. This tumor was successfully transplanted in other strain A mice for 2 transplant generations, and was then discarded. The experiment was terminated in the eighteenth month.

Since sarcomas had been induced in 4

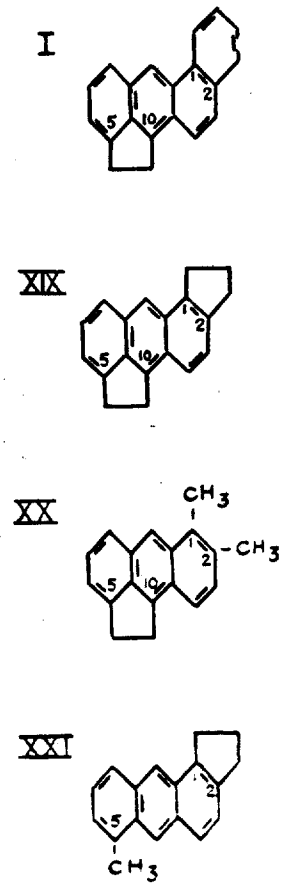


FIGURE 4.

TABLE 3.—*Chloro, bromo, and cyano compounds related to 20-methylcholanthrene*

[Results of subcutaneous injections in strain A mice]

Substituent	Formula No.	Dose per injection	Mice			6 months	9 months	12 months	15 months
			Number	Sex		After various periods			
		Milligrams							
6-Chloro	XV	10	10	M	+	0+	0+	0+	
					−	10−	6−	4−	
6-Chloro	XVI	7	20	M	+	0+	0+		
					−	16−	5−		
6-Cyano	XVII	2	20	M	+	0+			
					−	12−			
1-Bromo-7-methyl-8, 9-dimethylene-1, 2-benzanthracene	XVIII	7.5	10	M+F	+	0+	0+		
					−	8−	7−		

TABLE 4.—*Anthracene derivatives*

[Production of subcutaneous tumors in strain A mice]

Substituent	Formula No.	Dose (crystals)[1]	Mice		Total number of mice with tumors (+) and negative surviving mice (−), after various periods (in months)			
			Number	Sex	6 months	9 months	12 months	15 months
		Milligrams						
1,2-Cyclopentene-5,10-ace	XIX	4	20	M+F	1+ / 19−	2+ / 13−	3+ / 10−	3+ / 6−
1,2-Dimethyl-5,10-ace	XX	8	20	M	0+ / 19−	0+ / 18−	0+ / 14−	0+ / 10−
1,2-Cyclopentene-5-methyl	XXI	10	20	M	0+ / 19−	0+ / 14−	0+ / 14−	0+ / 12−

[1] Dissolved in lard filtered at 38° C.

of 20 mice with this compound, it is obvious that it has definite carcinogenic potency. Substitution of a 5-membered ring for a 6-membered ring in the 1,2-positions had, however, resulted in a marked diminution in potency.

1,2-Dimethyl-5,10-aceanthrene (fig. 4, formula XX).—On substituting 2 methyl groups for the 5-membered ring in the 1,2-positions of compound XIX, the potency was found to be completely obliterated. Three milligrams of 1,2-dimethyl-5,10-aceanthrene was injected (Dec. 1, 1935) as the first dose, and the remainder of the small amount available was given 3 months later.

Ten of the mice survived for 15 months. The experiment was terminated in the seventeenth month. No induced tumors were obtained.

1,2-Cyclopenteno-5-methylanthracene (fig. 4, formula XXI). When a 5-methyl group was substituted for the 5,10-ace group of compound XIX, the resulting compound was completely inactive. The first injection (Dec. 1, 1936) of 1,2-cyclopenteno-5-methylanthracene consisted of 3 mg. The rest of the available material was given 3 months later.

Survival was good (table 4). When the 10 survivors were killed in the seventeenth month, the compound was seen in the subcutaneous tissues of all the mice although the last injection had been given 15 months previously. No induced tumors were obtained, although 5-methyl-1,2-benzanthracene, its analogue with a 6-membered ring, produced (*5*) subcutaneous tumors in 16 of 20 mice in 9 months.

BILE ACIDS AND CHOLEIC ACIDS

Desoxycholic acid (fig. 5, formula XXII).—Crystals of desoxycholic acid, mixed with glycerol as usual, were injected (June 5, 1935) subcutaneously in 10 strain A mice in a dose of about 10 mg. Within 4 days all of the mice were dead. However, when a small amount of the dry crystals was given, the mice tolerated it. The amount injected was increased progressively so that the fifth injection, 6 months later, consisted of 7 mg. This latter dose was repeated 3 times during the next 5 months. No subcutaneous tumors were induced by the time this experiment was terminated after 13 months (table 5).

Two more groups of strain A mice were tested for response to an initial injection of about 10 mg. Six mice were injected with crystals moistened with glycerol; the next day five were dead. Six other mice received crystals moistened with water; within 3 days five were dead.

A solution containing 2.5 mg. of desoxy-

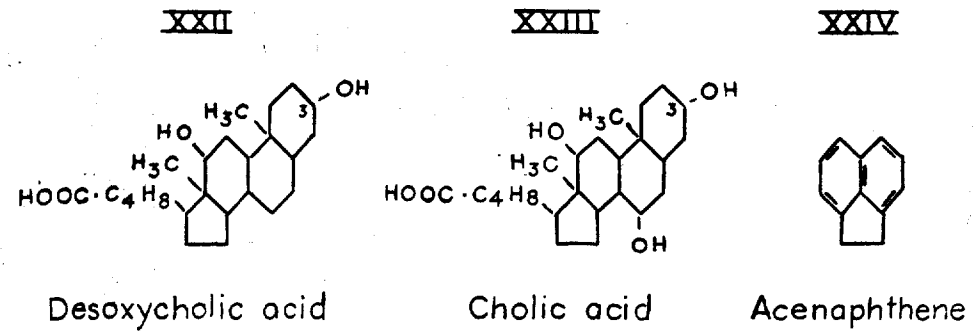


FIGURE 5.

cholic acid per cubic centimeter was prepared with dilute NaOH and was injected (June 5, 1935) in a dose of 1.25 mg. The mice received 7 injections over a period of 11 months. Survival was good: all 12 mice lived for 10 months, and 7 lived for 14 months. The experiment was terminated by killing the last 2 survivors after 18 months. No subcutaneous tumors were induced (table 5).

Loeffler (*13*) reported negative results as regards tumor production, on oral administration of desoxycholic acid and cholesterol to mice and rabbits for periods up to 130 days; the bile acid alone was fed to rabbits for 53 days. Alessandri (*11*) gave this compound orally and by subcutaneous injection to mice for periods up to 100 days with negative results. The London workers (*15*) obtained with

TABLE 5.—*Bile acids and cholic acids*

[Production of subcutaneous tumors]

Compound	Injection		Mice			Total number of mice with tumors (+) and negative surviving mice (−) after various periods (in months)			
	Dose	Form	Number	Sex	Strain	6 months	9 months	12 months	15 months
	Mg.								
Desoxycholic acid (formula XXII)	50	Crystals	6	M	dba	0+ 6−	0+ 5−	0+ 3−	
	5	Aqueous solution	12	M+F	dba+A	0+ 12−	0+ 12−	0+ 8−	0+ 4−
Cholic acid (formula XXIII)	80	Crystals	14	M	A	0+ 14−	0+ 13−	0+ 10−	0+ 7−
1, 2, 5, 6-Dibenzanthracene-cholic acid.	20	do	25	M+F	A	5+ 13−	8+ 4−		
	6	Aqueous solution	24	M	A+dba	0+ 20−	1+ 14−	1+ 7−	2+ 5−
20-Methylcholanthrene-cholic acid.	10	Crystals	13		A	[1] 10+ 0−			
	5	Aqueous solution	12	M+F	A+dba	3+ 8−	8+ 3−	8+ 1−	
	7.5	do [2]	35	M+F	A	1+ 20−	3+ 9−	5+ 5−	
Acenaphthene-cholic acid (cf. formula XXIV)	60	Crystals	14	M	A	0+ 14−	0+ 14−	0+	

[1] These tumors all developed in 4 months.
[2] Administered intravenously and intraperitoneally.

this compound 1 epithelioma in 26 mice in a skin-painting experiment which lasted for more than 2 years. Ghiron (*16*) stated that desoxycholic acid elicited transplantable subcutaneous fibrosarcomas in a high proportion of the mice and rats injected, but no details are available. The London group (*12, 17*) obtained 1 epithelioma in 100 mice in a skin-painting experiment lasting more than 2 years, and 11 sarcomas in 20 mice following subcutaneous injection of a solution of desoxycholic acid in sesame oil. In view of the physiological importance of this bile acid, it is important to ascertain to what extent the sesame oil contributed to the potency.

Cholic acid (fig. 5, formula XXIII).—Cholic acid, injected without either glycerol or water as lubricant, proved much less toxic than desoxycholic acid. About 10 mg. of the dry crystals was injected (June 28, 1935) without any fatalities. A total of eight such injections was given in the course of a year. Thirteen of the mice lived for 10 months, and six lived for 17 months. The last three survivors were killed after 20 months. No induced tumors were obtained (table 5).

Loeffler (*13*) administered cholic acid and cholesterol orally to mice and rabbits for periods up to 130 days, and cholic acid alone to rabbits for 53 days, without obtaining tumors.

1,2,5,6-Dibenzanthracene-choleic acid.—The addition compound of dibenzanthracene and desoxycholic acid was administered (May 29, 1935) to 53 mice. In the first experiment, the crystalline compound was injected in a dose of about 10 mg.; a second injection was given 2 months later. Ulceration occurred in 4 of the mice during the early part of the experiment.

Tumors [illegible] of the mice in the fifth month [illegible] there were 19 negative survivors. This experiment was terminated in the ninth month by killing the 4 remaining negative mice. Tumors had been induced at the injection site in 8 of the mice (table 5). The striking picture of multiple tumors in the lungs with this compound, mentioned previously (*4*), was noted in 5 of these mice.

In another experiment, this choleic acid was injected as an aqueous solution prepared by dissolving the compound in a buffered phosphate solution in a concentration of 2.5 mg. per cubic centimeter. The final pH was about 8. This was administered (May 29, 1935) to 24 mice in 5 doses of 1.25 mg. each over a period of a year.

The injection produced severe ulceration in many of the mice in a few days; this healed in most cases after a few weeks. Although 14 of the mice survived for 9 months, and 7 lived for a year, tumors were induced at the injection site in only 2 of the mice in this test (table 5). The last 5 survivors were killed at the end of the sixteenth month.

Six of the twelve strain A mice in this experiment showed lungs studded with tumors. In the fifteenth month several tumors in the liver were seen in a strain A male mouse; these may have been spontaneous tumors.

20-Methylcholanthrene-choleic acid.—The analogous compound of methylcholanthrene and desoxycholic acid was administered (June 3, 1935) to 58 mice. In the first experiment 1 dose of about 10 mg. of the solid compound was employed. Ulceration at the site of injection occurred in some of the mice; the tumors in these animals developed around the margins or at the base of these ulcers. The tissues about the injected material became thickened after about a month; the thickened tissues became quite firm before the end of the second month and developed into

well-formed tumors in 6 of the 11 mice early in the third month. Tumors developed in 3 more mice by the end of the third month. When the 2 survivors were killed at the end of 4 months, tumors had been obtained in 10 of the 11 mice.

In the next experiment, a solution was employed which contained 2.5 mg. of the compound per cubic centimeter of water to which just enough dilute NaOH was added to cause solution of the choleic acid. Four injections of 1.25 mg. each were given during the first 6 months. Ulceration occurred in a few of the mice before tumors appeared, as in the preceding experiment. The first two tumors appeared early in the fourth month, another developed in the sixth month, and five more in the seventh month. None of the three negative survivors developed a tumor by the end of a year when the experiment was terminated (table 5).

In the third experiment, this choleic acid was dissolved in a salt solution containing 0.155 M NaCl, 0.005 M KCl and 0.004 M Na_2HPO_4. NaOH was added to effect solution, then the pH was adjusted to 7.8. It was injected intravenously in doses of 0.25 cc. of solutions containing from 1.0 to 5.0 mg. of the choleic acid per cubic centimeter. For the first injection the weakest solution was employed; the concentration was increased in two subsequent injections until it was 5.0 mg. per cubic centimeter. When the intravenous route could no longer be used because of damage to the veins in the tail, the mice were given four intraperitoneal injections of 0.25 cc. of the strongest solution.

During the first 6 months 14 of the mice died. In 2 there were multiple tumors in the lungs. In 1 mouse there was marked congestion of the subcutaneous blood vessels, liver, and lungs; the lungs were congested in 3 other mice. In the fifth month 1 male mouse developed a tumor in the abdomen. During the next 5 months, intraperitoneal tumors developed in 4 other male mice. Lungs studded with tumor nodules were seen in 4 mice. Of the 5 mice negative after a year, 4 survived for 15 months at which time the experiment was terminated. The lungs of these 4 mice all contained numerous tumor nodules, but no tumors at the injection site developed in any of the animals after the tenth month.

Acenaphthene-choleic acid.—The addition compound of acenaphthene (fig. 5, formula XXIV) with choleic acid was injected 7 times in the course of 16 months, in doses which averaged about 9 mg. Survival was excellent in spite of the large amount injected. All of the mice lived for a year, and 10 survived for 15 months. The last 2 mice were killed after 19 months. Ulceration occurred at the injection site in a few of the mice. No induced tumors were obtained (table 5).

MISCELLANEOUS COMPOUNDS

Tests for carcinogenic potency have been completed with a large number of compounds. Many of these have been reported in detail; others have been briefly summarized (2). The experiments with the latter and with 30 additional compounds will be described in a later communication. Carcinogenic activity was exhibited by 6-methylchrysene and 4,9-dimethyl-1,2-benzanthracene in addition to those previously mentioned.

DISCUSSION

Of the chemical carcinogens widely used in many types of experiments in recent years, methylcholanthrene has been considered one of the most interesting. First of all was its production in the laboratory from both cholic acid and desoxycholic acid; this raised the question as to whether

compounds related to methylcholanthrene might be produced in the animal body from precursors like the bile acids and sterols. In the next place, it produced malignant tumors in mice with great rapidity. Furthermore, it was potent in unusually low doses (*18*) and produced unusual types of experimental tumors, such as gliomas (*19*), when previous attempts with other carcinogens had given negative results. From several points of view it was held worth while to examine for carcinogenic potency a considerable number of compounds whose structures were related to methylcholanthrene in one way or another.

The importance of the technique has been discussed in a preceding paper (*6*) and in a recent publication (*12*) from Kennaway's laboratory. The subcutaneous-injection technique has been adopted by the London workers, in studying the new series of compounds reported in this latter paper, for obtaining data to parallel the valuable results obtained with their usual technique of skin painting. However, the medium they employed in the injection experiments was sesame oil, which is open to more serious objection than filtered lard, since the former has been reported (*20*) as occasionally having given rise to induced tumors in mice. We have discontinued the use of the latter material as a vehicle in favor of tricaprylin in recent years for reasons discussed previously. Although it would be of interest to study the action of the constituents of sesame oil itself, and although sesame oil may be of use as a co-carcinogen (*5, 21, 22*), it is difficult to interpret the results of tests of compounds when sesame oil is used as the vehicle.

From the data in table 1 the potencies of the members of this [illegible] related group of compounds in producing subcutaneous tumors in mice were, in our experiments, in the following order of decreasing activity: methylcholanthrene; cholanthrene; 15,20-dimethyl-; 20-ethyl-; 16,20-dimethyl-; 20-*iso*-propyl-; and 20-*t*-butylcholanthrene. These provide still another group of closely related carcinogenic compounds, in which the range of potency is from very high to weak, for studies of the relationship between tumor-producing activity and other properties.

Table 2 indicates that, if carcinogens are formed in the body by ring closure of sterols, bile acids, or related substances, the 3-hydroxyl and 3-methoxyl derivatives of methylcholanthrene probably are not the carcinogens produced, in view of the negative results obtained here and in London with these two compounds. The potencies of the other oxygen-containing derivatives of methylcholanthrene in table 2 do not furnish any support to the hypothesis that oxidation of methylcholanthrene may be a step in the mechanism of tumor production by methylcholanthrene. If any such oxidation occurs (*7*), it probably does not involve the compounds given in table 2.

One of the anthracene derivatives given in table 4 exhibited weak carcinogenic potency. Compound XIX (1, 2-cyclopenteno-5, 10-aceanthrene) is one of the few anthracene derivatives not containing a benzene ring at the 1, 2-position which is active. The London group (*23*) has reported that the 9, 10-dimethyl derivative of anthracene possesses weak activity. While the angular benzene ring of 1, 2-benzanthracene thus is not essential for potency, it apparently is required for conferring a high order of activity on anthracene derivatives.

The reports of Ghiron and of the London group that injection of desoxycholic acid produced sarcomas in rats and mice are of great interest. No information is available here as to the form

of the injected material in Ghiron's experiments, or as regards dosage. The London workers (*17*) injected this bile acid in sesame oil into the right flank of 10 mice of mixed stock, of which 5 lived for more than 6 months. Of these 5, 3 developed spindle-cell tumors at the site of injection within a year. These mice had received 15 injections containing in all 70 mg. of the bile acid in 300 days. In a later experiment, similar injection into 10 C3H mice gave the first sarcoma in about 5 months, the mice having received a total of 28 mg. in 6 injections; sarcomas appeared in 6 additional mice by the end of 7 months. In our experiments 22 mice died shortly after the injection of about 10 mg. of desoxycholic acid moistened with either glycerol or water. When other mice received injections of small amounts of the dry crystals, they tolerated the bile acid much better. During the first 6 months the mice received 4 injections totaling about 22 mg., and during the next 5 months, 4 more injections totaling about 28 mg. Although the number of mice employed was small in this exploratory experiment, 5 of the mice were alive at 9 months, as compared with 5 survivors at 6 months in the first injection experiment in London; moreover, the total amount injected (50 mg.) was approximately the same as that employed in London. This raises the question as to the role played by the sesame oil used as a vehicle in their experiments. Since we had previously not published the details of the experiments with the bile acids, the English workers (*17*) supposed that "a single injection only, in glycerol, was given." Since we gave 8 injections totaling 50 mg. in the course of 11 months, and since 3 mice were still alive and negative at the end of a year, further experiments with desoxycholic acid are required, both with and without sesame oil.

With the crystalline choleic acids of dibenzanthracene and methylcholanthrene, tumor production at the injection site was high and fairly rapid. Subcutaneous injection of a slightly alkaline aqueous solution of these compounds, however, yielded fewer tumors and at a slower rate (table 5). The low and slow production of tumors following the intravenous injection of similar solutions of methylcholanthrene-choleic acid was unexpected. Apart from a very large number of pulmonary tumors, positive results were obtained only after the route of injection was changed from intravenous to intraperitoneal, whereupon tumors were obtained in the peritoneum. No tumors were obtained in the tail at the injection site. Furthermore, the numerous primary tumors throughout the body, which we had expected, were not obtained. Even allowing for the fact that the hydrocarbon part constitutes only a small portion of the weight of these choleic acids, enough was injected, it was thought, to produce tumors in many places. In view of the negative results from this point of view, it may be that a large fraction of the choleic acids, when solutions were injected intravenously, was excreted rapidly in these experiments and so was not available for tumor genesis. The high and rapid production of tumors at the injection site when the crystalline choleic acid was injected was discussed in an earlier paper [9] (*3*).

The pathological lesions reported here have been restricted to tumors induced at the site of injection and to multiple tumors of the lungs when they were unusually numerous. The other patho-

[9] *E. M. H. Green (24, 25) utilized our published and unpublished findings, with the three choleic acids given in table 5, in experiments with tissue culture in vitro. She stated that phenanthrene-choleic acid, which was employed as one of the control compounds, was non-carcinogenic. While this may well prove to be true, phenanthrene-choleic acid has not been [illegible] carcinogenic potency so far as we know.*

logical findings will be reported in a separate communication with Dr. Harold L. Stewart. Mammary-gland tumors have not been included in these tabulations even when they occurred at the injection site. For the present, these are considered as spontaneous in origin because they did not occur in a significant proportion of the mice in any given experiment. Hepatic tumors occurring in C3H mice in these experiments have not been recorded here since they did not occur in a higher proportion of the mice than the spontaneous incidence.[10]

The early development of carcinoma of the breast in a high proportion of the females of the C3H strain and the high incidence of spontaneous hepatoma in C3H males constitute objections to the employment of this strain for long-term assays of carcinogenic potency. However, Shimkin (*27*) considers that the spontaneous hepatomas do not interfere with the use of these mice for subcutaneous testing and recommends the employment of C3H male mice. Since Shear and Leiter[11] have found that there is a sex difference in the response of strain A mice to the carcinogenic action of low doses of 3, 4-benzpyrene, it may be more valuable to employ, for some purposes, strains which permit the use of mice of both sexes. For these various reasons we prefer, as a routine procedure in current experiments, to use strain A mice of both sexes in each test. Of course, if a given material produces such severe ulceration in strain A mice as to interfere with the test, another strain may be employed in that special case.

[10] *Cook and Kennaway* (23, footnote, p. 386), *commenting on an earlier statement of Shear* (2) *that hepatomas were observed in three of the C3H mice and in one strain A mouse in an experiment with a derivative of 3, 4-benzpyrene, stated that since hepatomas occur spontaneously in C3H mice "it is uncertain whether treatment was responsible for all the tumors reported by Shear." The hepatomas which developed in that experiment were probably spontaneous in the case of C3H mice, and possibly spontaneous in the A mouse. These tumors were not stated to have been induced; their occurrence was merely "noted." A further discussion of hepatomas in C3H mice has been given in an earlier paper* (26).

[11] *Shear, M. J., and Leiter, J.: Unpublished experiments, briefly summarized in a previous paper* (18) *and in an abstract* (28).

SUMMARY

Twenty-two compounds related to 20-methylcholanthrene were injected subcutaneously into 475 mice of inbred strains, chiefly strain A. Carcinogenic potency was exhibited by 8 of the 22 compounds.

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THIN-LAYER CHROMATOGRAPHIC SEPARATION OF CHENODEOXYCHOLIC AND DEOXYCHOLIC ACIDS

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SUMMARY

An efficient thin-layer chromatographic system for separation of chenodeoxycholic and deoxycholic acids is proposed.

INTRODUCTION

Of the major bile acids in man, deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanic acid) are generally more difficult to isolate from each other than from other bile acids. A number of thin-layer chromatographic procedures for bile acids have been suggested[1-23]; but, only a few of them[13-23] achieve some degree of separation of chenodeoxycholic acid from deoxycholic acid. The latter methods include those which use either two-dimensional chromatography[13] or multiple developments in the same or different solvent systems[15]. We propose a solvent system which gives better separation of chenodeoxycholic acid from deoxycholic acid than previously published methods utilizing only a unidirectional single development system.

METHODS

Standard bile acids and their methyl esters were obtained from Applied Science Laboratories, Inc., Calbiochem, and Supelco, Inc. The purity of the standards was tested by thin-layer and gas liquid chromatography. Fecal[8] and bile[24] acids saponified[9] and extracted by previously published methods were also used. Diazomethane was prepared from nitrosomethylurea according to established methods[25]. Methylation of fecal bile acids was done by the method of Subbiah *et al.*[20].

Ceric ammonium sulphate reagent was prepared as follows: Ten grams of ceric ammonium sulphate were powdered and ground into a paste with 17.5 ml of concentrated sulphuric acid and the grinding was continued as 20 ml of distilled water were added carefully on the sides. The resultant mixture was made up to 100 ml with distilled water and filtered if necessary.

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Thin-layer plates (20 × 20 × 0.25 cm) were prepared with silica gel H by Stahl's procedure using Desaga apparatus. For comparison of different solvent systems, commercial plates (20 × 20 × 0.25 cm) obtained in a single pack from Macherey-Nagel and Company, Duren, Germany, were used. The plates were activated in an oven at 110–120° for 2–3 h before use. ACS grade solvents were used as such. Exactly weighed amounts of bile acids and their esters were made into solutions of known concentration in a 2:1 mixture of chloroform and methanol. Desaga jars of identical size were used and they were lined with filter papers. Equal volumes of solvent mixtures (252 ml) were taken in the jars and the chambers were allowed to saturate with solvent vapour for 2 h before use. All runs were done at room temperature (23–25°). The compounds were applied as a thin line 1 cm long at a height of 3 cm from the bottom of the plates using capillary tubes. Ten μg of each compound were applied, allowed to dry, placed in the jar and developed by the ascending technique. When the solvent front was 18 cm from the bottom (15 cm from the origin), the plates were taken out of the jars, dried in hot air and sprayed with ceric ammonium sulphate reagent using Desaga TLC spray apparatus. The plates were dried in the air and then heated for 10 min in an oven at 110–130°. The time required for a run varied with the solvent system used but did not exceed 2 h for a single development.

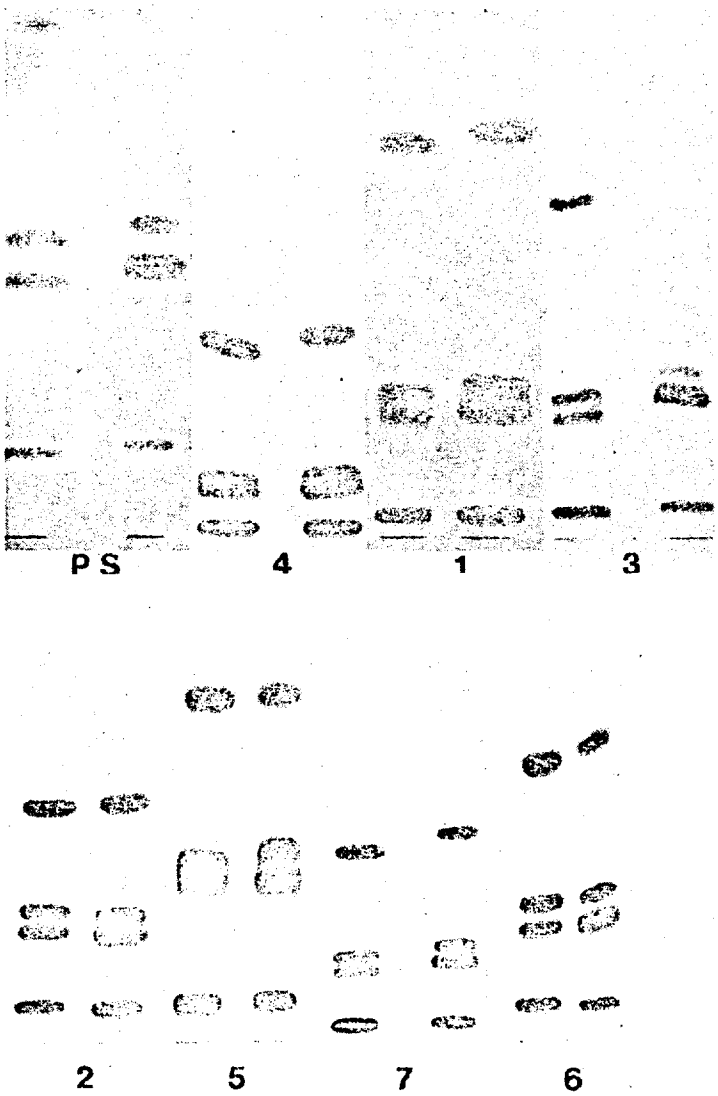
The proposed solvent system for chromatography of free bile acids and their methyl esters was made up of iso-octane–ethyl acetate–acetic acid–*N* butanol (10:5:1.5:1.5). For purposes of comparison of this system with the others published previously, mixtures of bile acids (or their methyl esters) were chromatographed under identical conditions and their mobilities calculated using the position of either the solvent front or lithocholic acid as standards.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the positions of different bile acids on thin-layer plates after development in the 8 solvent systems tested. Resolution of chenodeoxycholic and deoxycholic acids and their esters appears to be best in the proposed system. Correspondingly, the comparison of the R_F values of chenodeoxycholic and deoxycholic acids (Table I) or their methyl esters also showed the maximum difference in their mobility when developed in the proposed solvent system. Using the position of lithocholic acid as a reference point in each system, the differences in the relative mobilities of chenodeoxycholic and deoxycholic acids (or their methyl esters) were equally good in systems 2, 6 and 7. However, the actual mobilities of all bile acids in systems 2, 6 and 7 were considerably less than in our system. The actual separation obtained in a single development was therefore better in our system than in the other systems tested. However, if the thin-layer plates were developed repeatedly in the same solvent system, the separation of the two dihydroxy bile acids obtained by systems 2, 6 and 7 could be as good as that obtained in our system.

The separation of different bile acids present in the feces (Fig. 3A) and in the bile (Fig. 3B) was as good as that obtained with the standards. The bile acids were present as free acids and the fecal bile acids were methylated before chromatography.

Mixtures of chenodeoxycholic and deoxycholic acids in amounts of 1, 2, 5, 10, 25, 50, 100, 250, 500 and 1000 μg of each acid were applied as a 1-cm streak on thin layer plates under identical conditions and developed with the proposed solvent sy-



Figs. 1 and 2. Thin layer chromatograms developed in different solvent systems under identical conditions. The designations of the chromatograms are the same as given in Table I. On the left, from the bottom up, are cholic, chenodeoxycholic, deoxycholic and lithocholic acids and on the right are the methyl esters of the same four bile acids. The thin lines at the bottom are the lines of application.

stem. The same degree of resolution between chenodeoxycholic and deoxycholic acids (and their methyl esters) was obtained when the amount of each acid was up to 250 μg each and with larger amounts the resolution was not nearly as good.

To check the reproducibility of the resolution which the bile acids achieved with the proposed system, the samples of bile acids were chromatographed repeatedly either on commercial or on homemade plates. The expected degree of resolution was invariably achieved.

TABLE I

COMPARISON OF DIFFERENT THIN-LAYER CHROMATOGRAPHIC SYSTEMS FOR SEPARATION OF BILE ACIDS.

The R_F values (as per cent of the distance of solvent front from the origin) and the R_{st} values (as per cent of the distance of lithocholic acid or its ester from the origin) are given. The latter are in brackets and below the R_F values.

No.	*Authors (reference)*	*hR_F and (hR_{st}) Values*									
		C	*CD*	*D*	*L*	*CD—D*	*MC*	*MCD*	*MD*	*ML*	*MCD—MD*
Proposed system (PS)		10	31	37	63	6	11	33	39	65	6
		(16)	(50)	(58)	(100)	(8)	(18)	(52)	(60)	(100)	(8)
1	Hamilton[13]	3	15	17	48	2	3	15	18	50	3
		(6)	(31)	(36)	(100)	(5)	(6)	(31)	(37)	(100)	(6)
2	Subbiah *et al.*[14]	5	18	21	41	3	5	18	21	41	3
		(13)	(44)	(52)	(100)	(8)	(13)	(44)	(52)	(100)	(8)
3	Bruusgaard[15]	3	15	17	41	2	4	17	20	47	3
		(8)	(36)	(42)	(100)	(6)	(9)	(37)	(42)	(100)	(5)
4	Eneroth[16]	1	6	7	24	1	1	6	8	25	2
		(6)	(25)	(30)	(100)	(5)	(6)	(25)	(32)	(100)	(7)
5	Hofmann[17]	6	26	28	57	2	7	27	31	57	4
		(11)	(46)	(49)	(100)	(3)	(12)	(48)	(54)	(100)	(6)
6	Grutt[illegible] *l.*[18]	6	19	23	47	4	7	20	24	48	4
		(13)	(40)	(48)	(100)	(8)	(14)	(42)	(50)	(100)	(8)
7	Stiehl *et al.*[19]	3	11	14	31	3	3	13	15	34	2
		(9)	(36)	(44)	(100)	(8)	(10)	(37)	(45)	(100)	(8)

C = cholic acid; CD = chenodeoxycholic acid; D = deoxycholic acid; L = lithocholic acid; CD—D = the difference in the mobilities of chenodeoxy and deoxycholic acids; M = methyl esters of bile acids.

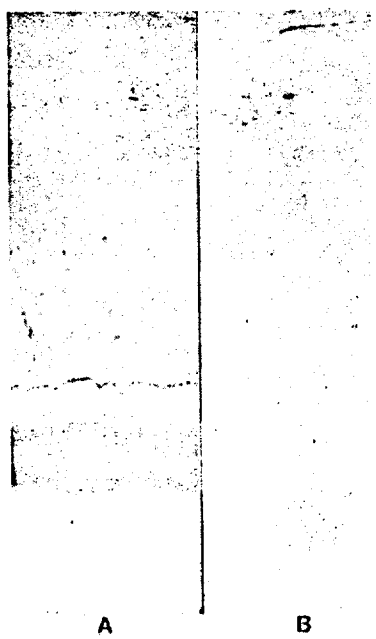


Fig. 3. Thin layer chromatograms developed in the proposed solvent system. On the left is the chromatogram of the methyl esters of fecal bile acids (3A) and on the right are the free bile acids obtained from the bile (3B). The two bands in the upper part of the left and the two spots in the upper part of the right chromatogram are those of chenodeoxycholic and deoxycholic acids (or their methyl esters).

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Induction of gallstones in mice by feeding a cholesterol-cholic acid containing diet[1]

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TEPPERMAN, JAY, FRED T. CALDWELL, AND HELEN M. TEPPERMAN. *Induction of gallstones in mice by feeding a cholesterol-cholic acid containing diet.* Am. J. Physiol. 206(3): 628–634. 1964.—A high incidence of gallstones (90%) was observed during a study of the effect on the serum cholesterol of feeding an "atherogenic" diet to aurothioglucose- (ATG) injected obese and nonobese mice. In a second experiment (without ATG) groups of eight male and eight female mice were examined at monthly intervals after having been started on the stone-inducing diet. Gallstones began to appear after 2 months and the incidence increased to 75% after 8 months. Although there was no significant sex difference in cumulative incidence, stones tended to appear earlier in females. Serum cholesterol values were, however, significantly higher in males during most of the experiment. In a third experiment the following features of the original treatment were singly and systematically excluded from the regimens of groups of 20 male mice: 0.04% thiouracil in drinking water, high fat (30%), cholesterol (1%), cholic acid (0.5%), and low protein (11%). It was found that the combination of cholesterol and cholic acid was necessary and sufficient for stone formation if food intake remained adequate and liver function was unimpaired.

cholesterolemia sex difference and gallstone formation
atherogenic diet and gallstone formation cholelithiasis

SEVERAL YEARS AGO Drachman and Tepperman (11) observed a small but statistically significant elevation of serum cholesterol in aurothioglucose- (ATG) obese mice (3) fed a laboratory chow diet. As a result of this observation an experiment was designed to answer the question: what is the effect of ATG obesity on the serum cholesterol of mice rendered hypercholesterolemic by a cholesterol and cholic acid diet (C-CA) similar to that used by Thomas and Hartroft (20) to produce myocardial infarction in rats?

Accordingly, ATG-obese and ATG-injected nonobese mice were fed a C-CA diet for 9 months. At the time of autopsy of the first mouse a large number of small white objects were seen floating freely in the gall bladder through its transparent wall. On chemical analysis these proved to be practically pure cholesterol. Nine of the ten mice which had received the atherogenic diet showed gallstones.

The serendipitous discovery of a model of a widely prevalent human disease was the point of departure for the studies to be reported here. The natural history of the development of gallstones in male and female mice will be described. Then, a description will be given of experiments in which individual components of the original complex stone-inducing regimen were singly and systematically excluded in an attempt to identify those factors which were chiefly responsible for stone formation.

MATERIALS AND METHODS

Mice were obtained from Albino Farms. Aurothioglucose (generously donated by Schering) was given as a single intraperitoneal injection to 48-hr-fasted animals at a dose of 0.8 mg/g body wt. This is an LD_{50} dose, and of the survivors, only about one-third become obese.

The complete dietary and treatment regimen in the first experiment was as follows: male mice were injected with ATG and were permitted to eat a laboratory chow diet for 3 months. At the end of that time they were fed a high-fat, low-protein, cholesterol- and cholic-acid-containing diet (C-CA) of the following composition by weight: casein, 15.3%; glucose, 51.0%; lard, 30.6%; cholesterol, 1.0%; cholic acid, 0.5%; Nutritional Biochemicals USP no. 14 salt mixture, 1.6%. The per cent protein, carbohydrate, and fat by calories was 11.3, 37.7, and 51.0, respectively. Vitamin supplements in mg/100 g diet were as follows: thiamin, .33; pyridoxine, .33; riboflavine, .42; nicotinic acid, 1.67; pantothenic acid, 1.67; choline chloride, 336; folic acid, .33; vita-

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min B_{12}, .012; biotin, .08; inositol, 80; percomorph oil, .2 ml; alphatocopherol concentrate, .002 ml. The drinking water contained 0.04% thiouracil.

All the diets used in subsequent experiments were single-factor variants of this basic regimen. In the second experiment, ATG was omitted. In the third, in addition to the omission of thiouracil, cholesterol, and cholic acid individually, the low-protein feature was omitted in one group of mice (21% protein) by substituting protein isocalorically for some of the carbohydrate. Similarly in another group (5% fat) some of the fat calories were replaced isocalorically by glucose. In this experiment a prepared multivitamin supplement (Nutritional Biochemicals) was used instead of the vitamin mixture described above.

In the early phases of the study cholesterol analyses of plasma, liver, and gallstones were performed according to the Sperry-Schoenheiner method (19). Beginning with the male-female comparison experiment, the Gans modification (personal communication) of the cholesterol methods of Abell et al. (1) and of Zlatkis et al. (24) was used. There was good general agreement between results obtained by the Sperry and Abell-Zlatkis methods.

The cholesterol concretions found in the gall bladders were of varying sizes and amounts, and it was therefore necessary to devise a standard method of examining gall bladder contents. This method (called the "blotter test") consisted of *a*) removing the gall bladder with the cystic duct occluded by means of forceps and *b*) incising the fundus of the organ so that its contents were permitted to run out on blotting paper. By this technique it was possible to see very small aggregates of crystals with the unaided eye. Any visible stones, no matter how small or how few, were registered as a positive test. Some of the varieties of blotter tests obtained can be seen in Fig. 1.

In some experiments bromsulfalein (BSP) retention was measured by the method of Casals and Olitsky (5). A BSP plasma level above 5 mg/100 ml 30 min after intraperitoneal injection of 0.1 mg BSP/g mouse was arbitrarily selected as evidence of impaired liver function, and the results are reported as per cent of animals per group which show impairment by this criterion.

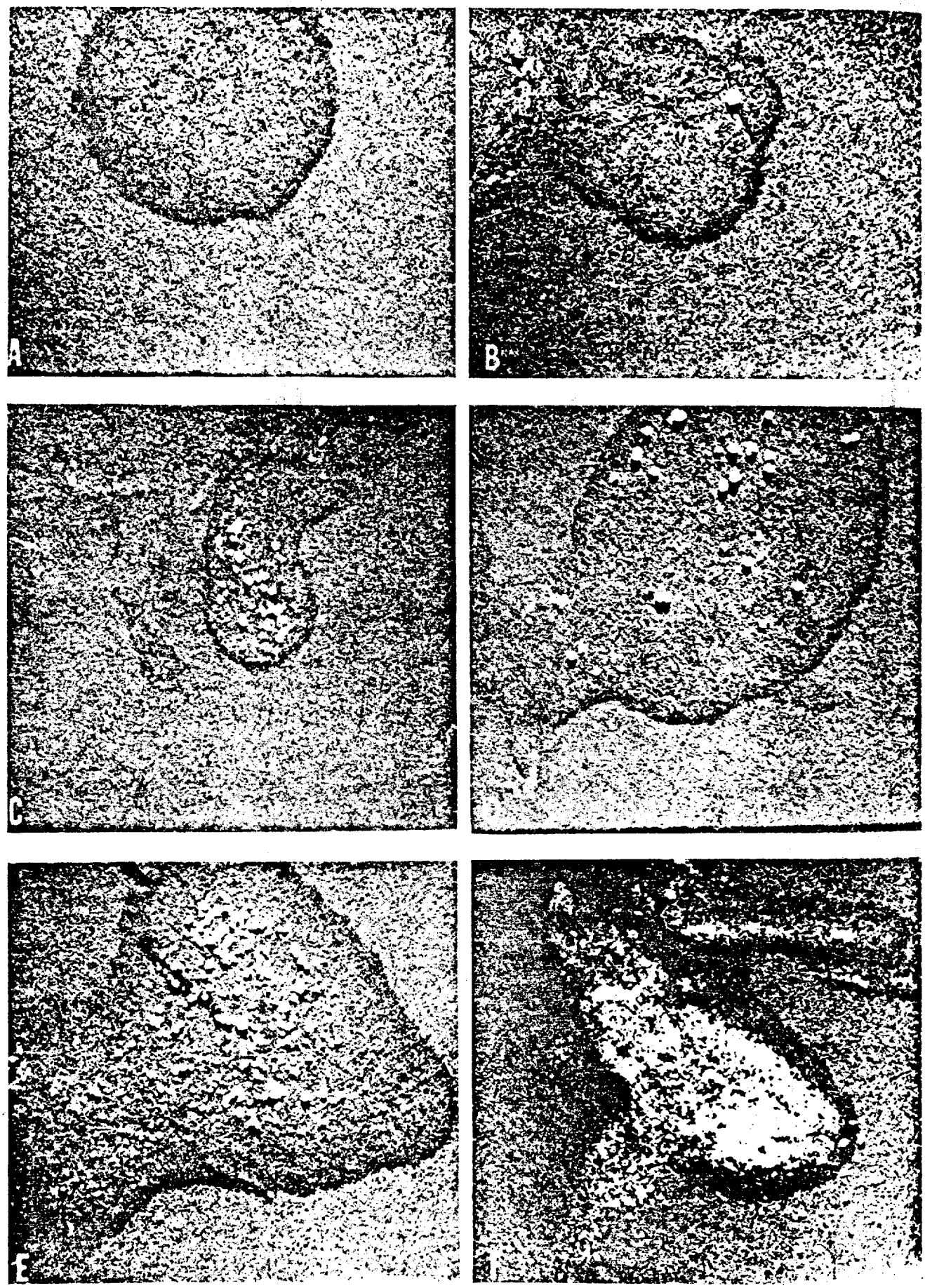
EXPERIMENTAL DESIGN AND RESULTS

A summary of the results of the first experiment is given in Table 1. Since mice with a mean body weight of less than 40 g showed plasma and liver cholesterol values which did not differ significantly from those of the heavier animals either on the chow diet or on the C-CA diet, the two groups have been treated as homogeneous samples for the purpose of this analysis. It will be seen that the mice on the C-CA regimen showed a statistically significant elevation of serum cholesterol (244 mg/100 ml versus 108) and almost a tenfold increase in liver cholesterol (3.2% wet wt. versus .33%). Nine of the ten mice on the C-CA diet contained gallstones. Stones were not found in chow-fed mice, an observation which has been confirmed hundreds of times since. Incidentally, all the C-CA diet mice exhibited grossly fatty livers and three of them had multiple small nodules distributed throughout the hepatic parenchyma. On histologic examination these nodules proved to be primary hepatomata.

A second experiment was designed *a*) to clarify the relationship between hypercholesterolemia and stone formation; *b*) to discover how soon after shifting to the C-CA diet stones begin to appear; and *c*) to determine whether or not there is a readily detectable sex difference in the incidence of gallstones in mice on this stone-inducing regimen. The results of this experiment are summarized graphically in Fig. 2. Groups of male and female mice were killed at monthly intervals after having been placed on the stone-inducing regimen. The presence or absence of stones was established by the blotter test and estimations of serum cholesterol were made. In this experiment there had been no prior ATG injection as there had been in the initial one. It will be seen that a high incidence of stones occurred in mice of both sexes by the end of the 7th month—there was, in fact, no statistically significant difference in cumulative stone incidence between the sexes (chi-square test). There is a tendency for the stones to begin to appear earlier in the females than in the males. The scatter of the serum cholesterol data is such that no statistically significant difference between males and females is detectable from *months 2–5*, but if all the values for males during these months are pooled and compared with similarly pooled data for females, the latter were seen to have significantly lower ($P < 0.01$) serum cholesterol values during this phase of the experiment.

In a third experiment an attempt was made to evaluate the contribution of each of a number of features of the original complex regimen to stone formation by eliminating them singly. Each treatment group consisted of 24 male mice, all pretreated with ATG unless otherwise noted. Twelve were autopsied at the end of 4 months and the survivors were examined at the end of 8 months. Results of BSP retention tests done just prior to autopsy, incidence of gallstones, final body weight, and serum cholesterol were recorded.

The results of the 4-month experiment are shown in Table 2. At first glance it is rather surprising that no stones appeared in the "atherogenic" A + ATG or A − ATG groups of mice in spite of highly elevated serum cholesterol levels. Although the reason for this is not completely known the mice in these groups (especially A + ATG) gained little weight and a considerable proportion of them showed some hair loss and abnormally high BSP retention. In retrospect it was discovered that the choline provided by the commercial vitamin supplement used in this experiment was less than half the amount fed in the original experiment. The A + ATG group were given the C-CA diet only 3 weeks after they had been injected with an LD_{50} dose of ATG in contrast to the mice in the first experiment which were not given the atherogenic regimen until 3 months after ATG treatment.


A
B
C
E

In addition to the lack of correlation between the level of serum cholesterol and the incidence of gallstones, other features of this experiment are worth noting: *a*) stones can occur in the absence of thiouracil from the drinking water (*group 5*); *b*) no stones occur when either cholesterol or cholic acid is omitted from the regimen, although a significant hypercholesterolemia occurs in both of these situations (*groups 6, 7*); *c*) the low dietary protein factor in the original regimen does not contribute to stone formation since many stones appear in the 21% protein group (*group 8*); *d*) the high dietary fat feature of the first experiment is noncontributory to stone formation since many stones were found in the 5% fat group (*group 9*); *e*) those groups (*8, 9*) in which the highest incidence of stones occurred were quite well nourished (see final body weight) and had little evidence of impaired liver function (see BSP retention).

The 8-month experiment (summarized in Table 3) was somewhat marred by the fact that a number of deaths due to undetermined causes occurred in many of the groups. However, the highest incidence of stones again occurred in two groups which had received cholesterol and cholic acid and were well nourished at the time of autopsy.

In one preliminary experiment an attempt was made to ascertain whether the incidence of gallstones would be the same in mice which had been maintained on a stone-inducing regimen for 4 months and then shifted back to a laboratory chow diet. The results, which are shown in Table 4, indicate that mice so treated had a lower incidence of stones than did those of a pooled group consisting of 12 individuals maintained on the diet for 4 months and 6 for 8 months.

Cholesterol analyses were performed on pooled samples of gallstones from four different groups of animals with the following results in per cent of cholesterol: 91.7, 99.2, 89.2, and 96.3, with a mean of 94.1. Analyses were done on pooled samples of gall bladder bile of chow-fed mice and of C-CA-fed mice in which there were no evident stones or crystals. The cholesterol concentration of gall bladder bile was low in chow-fed mice (91 mg/100 ml) and markedly elevated in mice on the cholesterol-cholic acid regimen (550 mg/100 ml).

Representative samples of precipitated cholesterol and concretions of various sizes were examined under a microscope and photographed by reflected light. Some typical specimens are shown in Fig. 3. Many different kinds of crystal aggregates were identified: flat individual crystals and thin bundles of flat crystals (*A*, *B*); larger, ball-shaped objects which clearly show the projecting edges of the flat crystals of which they are composed (*C*); more firmly packed spheres in which less prominent, but still clearly identifiable, crystal edges can be seen (*D*); slightly larger objects which resemble miniature mothballs and show some degree of faceting (*E*); and, finally, still larger irregularly shaped stones (about 0.5 mm in diameter) which appear to be composed of several of the ball-like structures previously described. Many specimens of each of these types were seen. In some mice only one type of concretion was found, i.e., predominantly loose crystalline or predominantly firm stones. In other individual animals examples could be found of all the types enumerated above. Commonly, single crystals of cholesterol could be seen next to stones as in Fig. 3*C*.

Histologic examination revealed some thickening of the gall bladder wall in the mice on the gallstone-inducing diet. Both muscularis and mucosa were involved, and, in addition, there was a slight infiltration of the submucosal zone with leukocytes. The surface of the mucosa did not appear to be grossly abnormal and there was no evidence of cholesterol crystal deposition in it.

FIG. 1. The blotter test. *A* represents a negative test. *B-F* illustrate the variety of form and amount of concretions found in individual gall bladders. A millimeter scale is shown in each frame.

DISCUSSION

Dietary induction of gallstones has been reported in the hamster (9), the guinea pig (15), in ground squirrels and prairie dogs (17), and in the rabbit (2, 16). In Patey's study (16), trauma and infection of the gall bladder were used in conjunction with cholesterol feeding. The stones in Bevan's and Mosbach's rabbits (2) contained large amounts of bile acids, but little cholesterol. In the experiments of Patton et al. (17) a 50% egg-yolk diet was fed and the stones were 70% cholesterol. In Okey's experiments (15) the stones were predominantly calcium phosphate, but contained some cholesterol. In the series of experiments done by Christensen and Dam (6) a low-fat, high-sucrose diet was used and the cholesterol content of the stones in various experiments was reported as 50–94%. In these experiments the composition of the salt mixture was an important contributory factor, since stones could be prevented by altering the proportions of certain salts in the diet. Most pertinent to this report is the fact that Christensen, Prange, and Dam (8) were unable to induce the formation of stones in mice with the same regimen that had been used successfully in the hamster.

The authors are unaware of any previous description of gallstone formation in mice due to a modification of the diet. Since there have been so few studies of experimental gallstones the mouse model affords an opportunity to learn additional facts about the conditions of cholesterol precipitation in bile and stone formation.

The comparison of the incidence of stones in male and female mice reported here was undertaken because of the higher incidence of cholelithiasis that has been reported in the female (4, 13, 21, 23). Apparently (13) the sex difference in incidence in the human is evident even in unmarried individuals which suggests that a factor in addition to pregnancy is operative. Although no significant difference in cumulative incidence of stones was observed between the sexes, female mice did

tend to show stones earlier than did males, and they also tended to have lower serum cholesterol values. It is possible that a contributory mechanism to the serum cholesterol-lowering effect of estrogens may be an increase in the rate of secretion of cholesterol into the biliary tract.

The factorial analysis of the original stone-inducing regimen suggests that the combination of cholesterol and cholic acid in the diet is necessary and sufficient for stone formation. The elimination of other potential contributory factors (thiouracil, low-protein diet, high-fat diet) did not prevent gallstone formation. The highest incidence of stones occurred in those mice which gained weight satisfactorily and showed no obvious impairment of liver function as measured by the BSP test. In a number of groups there was a striking elevation of serum cholesterol without stone formation.

In recent years a number of observers (i.e., 12, 14) have discussed the problem of spontaneous disappearance of gallstones in man and Christensen and Dam (7) have stated that gallstones disappear when hamsters are shifted from a stone-inducing regimen to a control diet. The preliminary experiment reported here is consistent with the idea that established stones may in fact disappear, but it cannot be inferred from our data that actual dissolution of stones occurred as a result of the transfer from the C-CA diet to a chow diet. Many of the "positive blotter tests" revealed extremely small aggregates of crystals which could easily have passed through the common duct into the gastrointestinal tract. However, an increase in the cholesterol-holding capacity of the bile may have occurred as a result of the change to a chow diet, and previously precipitated cholesterol concretions may have been redissolved. Solution of human gallstones in dog bile has repeatedly been reported (e.g. 10, 22).

The authors are aware of the fact that the juxtaposition of photographs in Fig. 3 to suggest an evolutionary sequence in the development of "mature" stones constitutes a hypothesis rather than a definitive description. The mechanism involved in the clumping of flat crystals into plates and clusters is unknown to us. The apparent erosion of the protruding crystal edges may be due to a sort of ball-mill effect of the stones on one another.

TABLE 1. *Plasma cholesterol, liver cholesterol, and gallstone incidence in mice on the C-CA diet-thiouracil regimen for 9 months*

Diet	No. of Mice	Mean Body Wt.	Plasma Cholesterol, mg/100 ml	Liver Cholesterol, % Wet Wt.	Gallstone Incidence
Chow	4	32.5	108	.33	0/9
	5	49.6	±SE 11.4	±SE 0.01	
C-CA	5	31.6	244*	3.20*	9/10†
	5	51.6	±17.4	±.42	

* $P < 0.01$ (t test). † $P < 0.02$ (chi square with Yates correction).

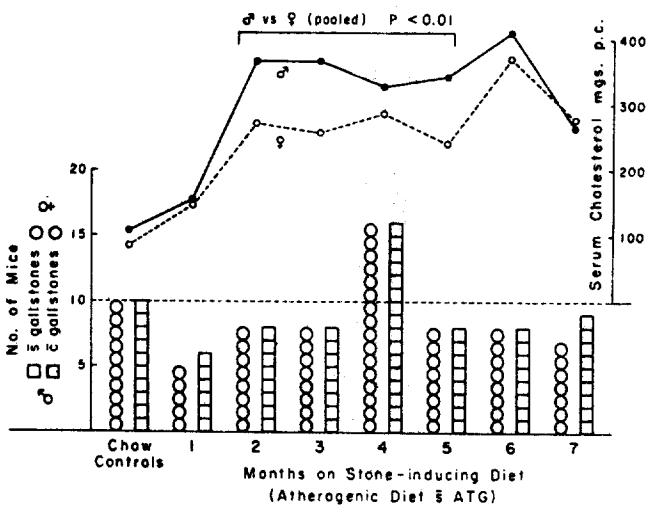


FIG. 2. Gallstone incidence and plasma cholesterol of male and female mice fed a C-CA diet for 1–7 months.

TABLE 2. *4-Month experiment**

Group No.	Regimen	Gallstone Incidence	Final Body Wt.	Plasma Cholesterol, mg/100 ml	BSP >5 mg/100 ml % of Group
1	Chow + ATG	0/12	38±.49	151±14.6	0
2	A + ATG	0/12	17±1.1	424±86	66
3	Chow − ATG	0/12	38±.55	141±29	0
4	A − ATG	0/12	24±1.87	373±53	36
5	A − thiouracil	4/12	29±.87	316±26	55
6	A − cholesterol	0/12	17±1.1	229±28	0
7	A − cholic acid	0/12	31±.84	217±32	25
8	A − low protein†	10/12	35±.38	323±27	0
9	A − high fat‡	11/12	29±1.7	574±85	0

Values are means ± SEM. * Effect of systematically eliminating single features of the original gallstone-inducing regimen on stone incidence, final body weight, plasma cholesterol, and liver function. † 21% protein. ‡ 5% fat.

TABLE 3. *8-Month experiment*

Group	Regimen	Gallstone Incidence	Final Body Wt.	Plasma Cholesterol, mg/100 ml
1	Chow + ATG	0/10	39±1.6	139±7.6
2	Chow − ATG	0/10	37±.9	125±5
3	A + ATG	2/5	24±1.8	270±60
4	A − cholesterol	0/5	24±1.1	329±35
5	A − cholic acid	1/10	24±.7	351±39
6	A − thiouracil	8/12	28±1.4	418±45
7	A − low protein	5/6	31±2.1	275±53

Footnotes same as in Table 2.

TABLE 4. *Effect of shift from C-CA to chow diet on plasma cholesterol level and gallstone incidence*

Group	No. of Mice	Plasma Cholesterol, mg/100 ml	Gallstone Incidence	%
C-CA1	18	306	15/18	83
C-CA, C2	9	90	4/9	44*

C-CA = 21% protein, 1% cholesterol, 0.5% cholic acid 1:12 on C-CA diet for 4 months plus 6 on C-CA diet for 8 months, Incidence of stones was the same (83%) in both of these subgroups. 2:9 on C-CA diet for 4 months followed by chow diet for 4 months. * $Chi^2 = 4.35$. $P < .05$ but $> .02$.

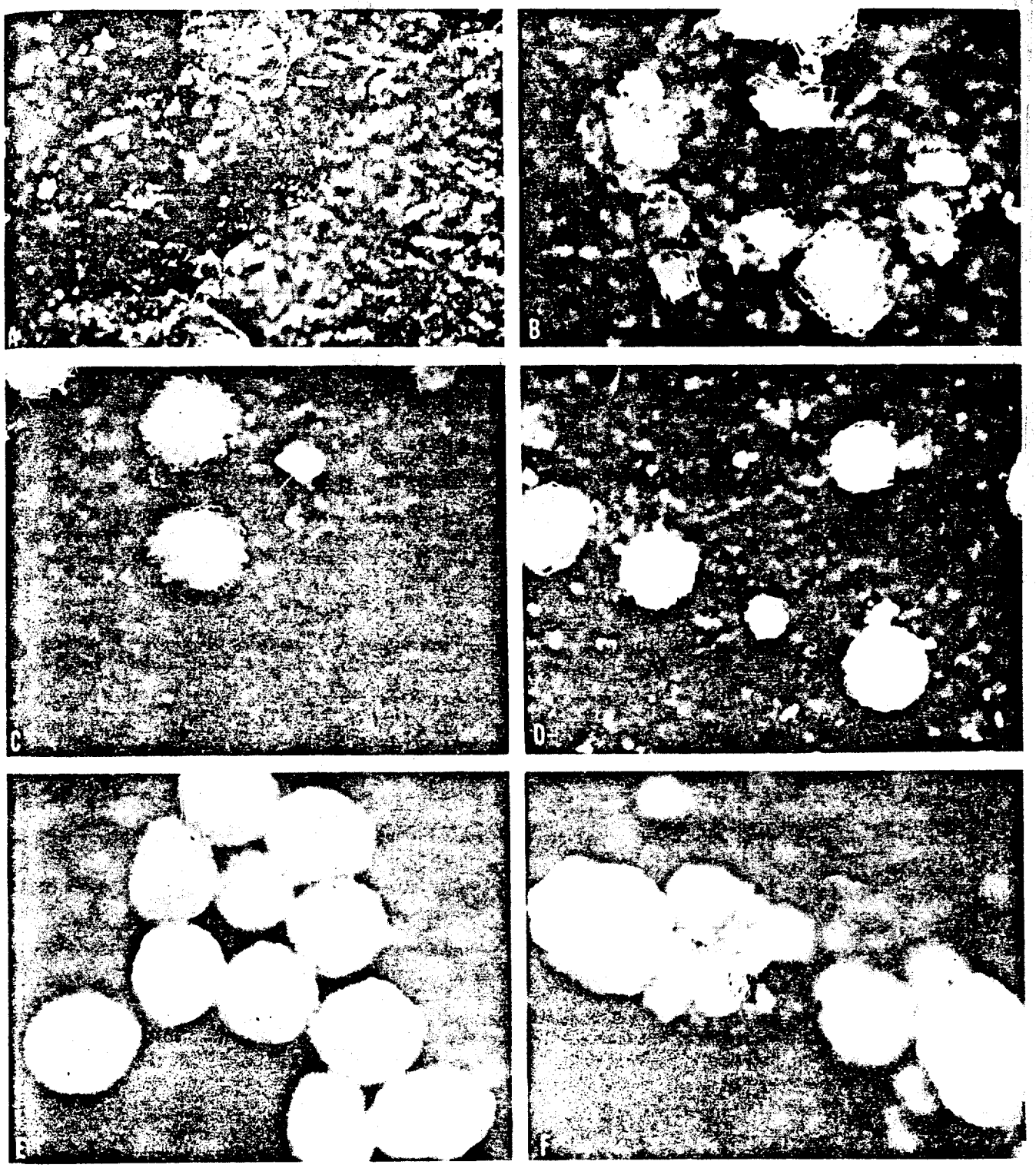


FIG. 3. Varieties of cholesterol concretions found. ×50.

The physicochemical conditions for stone-formation in the bile cannot be inferred from the data presented. Precipitation of cholesterol is clearly associated with a high concentration of that sterol in gall-bladder bile. The role of bile flow rate in the establishment of high gall bladder bile cholesterol concentration is the subject of a companion report (18).

The authors are grateful to Drs. Barbara Rosenberg and John Prior for preparing and examining histological specimens.

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Growth of Rats Fed Bile Salts, Urea and Chlortetracycline.* (30440)

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Bile acids are secreted into the gastrointestinal lumen in conjugated form and may undergo hydrolysis by microorganisms but not by endogenously produced enzymes(1,2). Feces of germfree and antibiotic treated animals have been found to contain no detectable deoxycholic acid(3,4), often the most prominent bile acid in feces of animals and man(5,6). *In vitro* incubation with deoxycholic acid causes marked damage to the intestinal villi(7) and extensive histological changes in the small intestinal mucosa of mice have been reported following 2 days of feeding on a diet containing 2% sodium deoxycholate(8). Microorganisms in the intestine liberate ammonia by urea hydrolysis and strains known to hydrolyze bile acids also produce urease(9,10). Ammonia production in the gastrointestinal tract by microorganisms may lead to toxic manifestations and is reduced with oral administration of antibacterial substances(11). The effects of varying the type or quantity of bile acids in the diet of growing animals with antibiotics in the diet have been studied(12,13). However, the authors have not found published data for growing animals following simultaneous feeding of bile salts, urea and antibacterial substances.

Methods. Weanling male rats of the Holtzman strain having initial weights of 48-60 g were fed and housed in individual cages with wire floors. Their purified basal diet calculated to meet the dietary requirements of growing albino rats(14) contained the following ingredients in per cent: vitamin free casein 14; sucrose 75; stripped lard† 5; glucose monohydrate 1.8075; dl methionine 0.18; minerals 3‡; vitamins in glucose 1§; antioxidant 0.0125.‖ Additions were made

* Supported in part by funds from the Herman Frasch Foundation, New York.

† Most volatile fraction from prime steam lard obtained by molecular distillation. Contains less than 5 ug of tocopherols per g of fat and fulfills fatty acid requirements of the rat. Distillation Products Industries, Rochester, N. Y.

‡ Minerals per kg of diet: $Ca(PO_4)_2$ 11.1 g; $Ca(H_2PO_4)_2$ H_2O 11.1 g; $KHCO_3$ 4.67 g; $MgCO_3$ 1.42 g; NaCl 834 g; Na_2HPO_4 549 mg; $MnSO_4$ H_2O 156 mg; $FeSO_4$ $7H_2O$ 126 mg; $ZnCO_3$ 23 ug; $CuSO_4$ 5HO 20 ug; KIO_3 253 mg; Na_2SeO_3 87.8 ug.

§ Crystalline vitamins provided per kg of diet: thiamine HCl 1.25 mg; riboflavin 2.5 mg; pyridoxine HCl 1.2 mg; niacinamide 15 mg; calcium pantothenate 8 mg; choline dihydrogen citrate 1582 mg; vit. B_{12} 5 ug; vit. A 2000 I.U.; vit E 60 I.U.

‖ Santoquin, Monsanto Chemical Co., St. Louis, Mo.

TABLE I. Gain in Weight and Efficiency of Feed Utilization for Rats Fed a Basal Diet Supplemented with Bile Salts or 100 mg/kg of Chlortetracycline (6 Animals per Treatment Group).

Treatment groups	28 Day wt gain (g)*	Gain/feed ratio†
1. Basal	103.5	.359
2. " + CTC 100 mg/kg	92.7	.356
3. " + .1% deoxycholate	96.8	.355
4. " + .5% "	53.1	.263
5. " + 1.0% "	No survivors after 5 days	
6. " + .1% dehydrocholate	95.3	.370
7. " + .5% "	77.0	.328
8. " + .5% dehydrocholate + chlortetracycline (100 mg/kg)	78.1	.316
9. " + .1% cholate	104.0	.373
10. " + 2.0% "	No survivors after 21 days	
11. " + .1% taurocholate	112.0	.364
12. " + 2.0% "	12.0	.072
13. " + 2.0% taurocholate + CTC (100 mg/kg)	14.8	.087
14. Basal	97.7	.371
15. " + .5% ox bile	71.2	.330
16. " + 1.0% " "	49.7	.251

* Avg of 6 animals per group. Error mean square for growth 230.6.
† Error mean square for gain/feed 0.0014.

to the total basal diet unless they exceeded 1% whereupon they were made at the expense of sucrose. With the exception of ox bile all additives were of reagent or USP grade. All animals were provided with distilled water and their assigned diets *ad libitum* for the experimental period of 28 days. Weekly weights and feed consumption were recorded. A factorial experiment described below followed preliminary experiments in which ox bile and sodium salts of bile acids were tested for differential toxicity (Table I). Groups 1 through 13 of the preliminary experiments were started simultaneously, whereas 14 through 16 were started on a subsequent date.

The factorial experiment was a 4 × 2 × 2 arrangement with 16 treatment groups of 5 animals. Four concentrations of sodium deoxycholate as a percentage of the diet were used: 0, 0.1, 0.3, and 0.5. Each concentration of deoxycholate was tested with or without 1% urea and with or without 100 mg/kg of chlortetracycline¶ singly or in combination. Data obtained were treated by analysis of variance.

Results. Sodium deoxycholate proved to be more toxic than ox bile or the other bile salts as shown by growth depression and mortality (Table I). Sodium cholate (0.1%) caused no depression in growth and the results suggested that growth might be increased in the presence of 0.1% sodium taurocholate. All animals receiving 1.0% of deoxycholate died in 5 days or less but all animals fed 2.0% taurocholate survived the entire 28 days although their average gain was only 12 g. Addition of chlortetracycline failed to counteract the depression in growth produced by 2.0% taurocholate. All of the animals receiving 2.0% sodium cholate died between the 7th and 21st days. Animals that died regardless of treatment showed severe diarrhea. Diarrhea was also noted in the 28 day survivors of the groups to which 0.5% or more of ox bile or any bile salt were fed. At death or at 28 days animals fed 0.5% deoxycholate, 0.5% dehydrocholate, 0.5% cholate, 0.5% ox bile, or 1.0% taurocholate showed hyperemia of the distal small intestine. Group 8, fed 0.5% dehydrocholate plus chlortetracycline, had enlargement and increased weight of the ceca plus contents.

The average gains in body weight of the 78 animals surviving 28 days in the factorial experiment were reduced when deoxycholate was added to the diet and the depression approached linearity when plotted as a function of deoxycholate concentration. Each 0.1% of sodium deoxycholate added reduced gain in body weight by an increment of about 10% below the average of 108 g for all groups receiving no deoxycholate. The differences between concentrations of deoxycholate were highly significant ($P<0.001$). The lowest concentration of deoxycholate (0.1%) also depressed feed intake but at higher concentrations growth depression was greater than could be attributed to reduced feed intake alone. Data on the conversion of feed to gain in body weight paralleled the differences in growth.

In the preliminary experiments (Table I) and in the factorial experiment (Table II) chlortetracycline led to a depression of growth in animals fed the basal diet without

¶ Gift of American Cyanamid Co., Princeton, N. J.

TABLE II. Gain in Body Weight (GMS) and Efficiency of Feed Utilization for Male Weanling Rats Fed Sodium Deoxycholate, 1% Urea and 100 mg/kg Chlortetracycline for 4 Weeks.*

Deoxycholate, %	Without chlortetracycline		With chlortetracycline		Avg by deoxycholate concentration
	Without urea	With urea	Without urea	With urea	
0	114.0	118.2	98.4	101.8	108.1
G/F†	.385	.400	.375	.342	.375
.1	98.2	90.4	90.0	90.4	92.3
G/F	.352	.347	.323	.336	.340
.3	82.8	71.2	75.6	59.2	72.2
G/F	.311	.289	.294	.275	.292
.5	50.0‡	48.2	43.8	81.2‡	55.8
G/F	.226	.245	.219	.322	.253
Mean gain	86.2	82.0	77.0	83.2	
Mean G/F	.318	.320	.303	.319	

Means of 8 groups with and without chlortetracycline and with and without urea regardless of deoxycholate concentration

	Without chlortetracycline	With chlortetracycline
Gain (8 groups)	84.1	80.1
G/F (8 ")	.319	.311
	Without urea	With urea
Gain (8 ")	81.6	82.6
G/F	.311	.319

* Each value is an avg for 5 animals unless otherwise indicated. Error mean square for growth 173.9.
† Ratio of gain to feed consumed. Error mean square 0.0013.
‡ 4 animals.

other additives. Growth depression has been recorded several times in this laboratory with rats fed chlortetracycline in this basal diet. All groups receiving chlortetracycline regardless of the concentration of deoxycholate showed a greater depression in growth when urea was absent. The group fed urea alone showed the greatest growth but when urea was added in the presence of deoxycholate growth depression was also greater than that of the corresponding concentration of deoxycholate alone. Statistical analysis demonstrated a highly significant 2-way interaction between deoxycholate and urea ($P<0.005$) and between deoxycholate and chlortetracycline ($P<0.01$). Likewise there was a significant 3-way interaction among groups fed deoxycholate, chlortetracycline and urea ($P<0.05$). These interactions became apparent (Table II) when the mean of 81.6 for 8 groups receiving no urea is compared to 82.6, the mean for all 8 groups receiving urea. Urea appeared to increase growth for all 8 groups in contrast to the apparent growth depression when only urea was added in the presence of deoxycholate. Similarly the mean of 84.1 for 8 groups fed no chlortetracycline was slightly higher than 80.1 for the groups fed chlortetracycline. The individual effects of chlortetracycline and urea when added in the presence of deoxycholate were masked in these averages which include data where chlortetracycline and urea were added in combination. Their interactions were revealed by statistical analysis to be highly significant.

Discussion. The basal diet was compounded to meet the published requirements of the albino rat(14). It was employed despite its inability to produce maximum growth.** Highly purified ingredients were used to facilitate possible future comparisons involving changes in concentrations of specific nutrients. The NRC tabulation does not include components which are often included in purified diets of rats and it is recognized that the results of this study might have been altered with their inclusion since the effects of antibacterial agents on nutrition of substances like folic acid, biotin, and vit. K are well known for the rat(14).

** R. G. Warner, unpublished data.

The apparent toxicity as indicated by growth depression of urea when added in the presence of deoxycholate gives evidence that bile acids and urea both produced endogenously by warm blooded species may provide the gastrointestinal microflora with substrates which permit production of substances having ability to depress nutrient utilization and growth of the host. It has been speculated that bacterial products of bile acid hydrolysis lead to malabsorption and the entity known as the blind loop syndrome(7). Work of others has suggested that antibiotics counteract the growth depression by non-conjugated bile acids because microbial production of products having growth depressing properties is inhibited (12,13). However, lithocholic acid has been found to decrease growth equally in germfree or conventional chicks(15). The evidence indicates that urea is converted *in vivo* into ammonia and CO_2 by microbial urease(s) (11), but the products of bile acid hydrolysis are more varied and complex(1,2). Thus the products and mechanisms responsible for growth depression in the present studies are uncertain. Evidence has been presented that ureolytic activity in the gastrointestinal lumen is reduced concurrently with increased growth following urease immunity(16,17). Urea hydrolysis and bile acid hydrolysis are carried out by extracellular microbial enzymes(18). It is therefore possible that bile acid hydrolysis may also be controlled by immune mechanisms induced by appropriate antigens.

Summary. Addition of ox bile and bile salts to a purified diet produced differential effects on growth in rats. Deoxycholate produced growth depression ($P<0.001$) at all concentrations. Growth was reduced further by 1% urea or 100 mg/kg of chlortetracycline. Addition of urea with chlortetracycline tended to abolish growth depression produced by either alone.

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Effects of Egg Oil, Cholesterol, Cholic Acid and Choline upon Plasma and Liver Lipids[1]

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ABSTRACT Young adult female rats of the Wistar strain were fed diets containing 27% egg oil or a mixture of butter fat and olive oil with the same iodine number as the egg oil but with different fatty acid content. The other variables were 0.1 or 0.3% choline chloride, 1.0% cholic acid, and 1.29% cholesterol — the level of cholesterol present in the egg oil. The experiments were of 5 to 10 weeks' duration. Rats fed the rations containing the mixture of fats with cholesterol and either level of choline had plasma and liver cholesterol and liver lipid levels that were significantly augmented over those receiving the egg oil. Cholic acid affected an increase in the cholesterol and lipid values of the rats receiving both the fat mixture with cholesterol and the egg oil. The liver lipids and cholesterol decreased when the higher level of choline was fed in conjunction with the mixture of fats with cholesterol and cholic acid. The liver lipids and cholesterol were threefold and twelve-fold, respectively, greater for the rats receiving egg oil than for those fed the fat mixture without cholesterol or cholic acid. Groups given the fat mixture with cholesterol and cholic acid had far higher values than did any of the other groups.

A number of investigators have suggested that certain natural fats contain substance(s) other than cholesterol that increase serum cholesterol. Messinger and co-workers (1) reported that the elevation of serum cholesterol in human subjects fed diets containing egg yolk powder or cream was greater than could be accounted for by the cholesterol contained therein. They concluded, "there is a substance as yet unidentified in egg yolk which elevates the cholesterol level of human serums." Mayer and co-workers (2) failed to note demonstrable change in the plasma cholesterol level when 800 mg of cholesterol/day were given in the form of egg yolk. This observation is in agreement with the results of Keys et al. (3) and Hildreth and co-workers (4). Mayer and co-workers (2) have pointed out that the lack of agreement between the work carried out in their laboratory and that of Messinger et al. (1) could be accounted for by the higher level of egg yolk powder, and the cholesterol contained therein, fed by Messinger et al. The saturated fatty acids in egg yolk could also contribute to the hypercholesterolemia. The cholesterol consumed by Messinger's subjects was at least 4 times that of Mayer's. Bronte-Stewart et al. (5) confirmed the observations of Messinger, giving 10 eggs/day — an amount which supplied essentially the same quantity of cholesterol as received by Messinger's subjects. Portman (6), in a review on factors influencing serum cholesterol levels states: "It appears that the form in which dietary cholesterol occurs is of importance; for example, egg yolk appears to have greater hypercholesteremic activity than does an equivalent amount of crystalline cholesterol. This latter observation may be related to the nonsterol portion of the egg yolk, however. The nature of the basic ration in which the sterol is included may also be of importance."

Wells and Bronte-Stewart (7), working with a single human subject, have recently reported that the cholesterogenic entity in egg yolk resided in the acetone-soluble fraction. Neither the unsaponifiable nor the saponifiable fractions gave an increase in serum cholesterol when fed separately. The 2 fractions were active when fed together but a delay of 6 hours in feeding one or the other nullified this effect. A fat of like iodine number with added cholesterol gave similar effects.

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Rowsell et al. (8) fed diets to swine in which 33% of the calories was replaced by egg yolk or by butter fat. Controls received the non-supplemented basal diet. The swine fed egg yolk had approximately 6 times as much aortic atherosclerosis as did the controls, and the butter-fed swine 3 times as much. The group fed egg yolk showed a significant increase in serum cholesterol and phospholipid levels. The swine fed butter fat showed only a slight increase in serum lipid levels.

Hegsted et al. (9) studied the effects of the interaction of cholesterol, cholic acid, and the type of fat on serum cholesterol and vascular sudanophilia in the rat, using 3 levels of cholic acid and 3 levels of cholesterol in all of their combinations. They found that cholic acid had a relatively greater effect than did cholesterol in producing an increase in serum cholesterol. Dose-level responses appeared to indicate an interaction of cholesterol and cholic acid.

The effect of choline on serum cholesterol was studied by Mayfield and Roehm (10) who observed that female rats receiving no choline for 18 days had serum cholesterol values that were essentially the same as those of rats fed varying levels of choline ranging from 30 to 350 mg/100 g diet. Male rats maintained with a choline-free diet had significantly lower serum cholesterol than those receiving the rations supplemented with choline. However, the male and female rats receiving the lower levels of choline — zero, 30 or 60 mg/100 g diet — had lower serum cholesterol when compared with those receiving the higher levels of choline — 100, 250 or 350 mg/100 g diet.

Olson et al. (11) observed hypocholesterolemia, hypolipemia and hypobetalipoproteinemia in rats fed diets deficient in choline and choline-precursors. These effects were prevented by 0.3% choline in the diet and partially prevented by rations containing 18% casein. The level of dietary fat ranging from 6 to 42% and including butter fat, corn oil and lard did not modify the effects of choline upon serum lipids.

The present study deals with the effects of egg oil and of a fat mixture upon plasma cholesterol and liver total lipids and cholesterol of young adult female rats.

MATERIALS AND METHODS

Diets. The purified diets contained 18% casein, 4% salts (Hegsted (12)), optimal vitamins,[2] dextrose and 27% fat (table 1). The level of fat corresponded to the fat content of the average American diet (13). The rations contained either egg oil or a mixture of fats of like iodine number and with or without added cholesterol or cholic acid or both. Choline chloride was fed at 2 levels.

The egg oil was extracted from baker's dried whole egg powder with diethyl ether for 16 hours in a Soxhlet extractor. The residual ether was distilled off; the brown viscous oil that remained was free of peroxides. It was stored at 4°C. The egg oil was analyzed by gas-liquid chromatography for individual fatty acids using a column packing of diethylene glycol succinate on chromosorb W in the Barber-Colman gas chromatograph. The fatty acid compostion of the egg oil is shown in table 1. The iodine number as determined by the method of Hanus (14) was 72.

The fat mixture was composed of a 21:6 ratio of olive oil-butter fat. The butter fat was prepared from unsalted butter by heating until molten and centrifuged at 800 rpm for 15 minutes to remove the water and milk solids. The butter fat was frozen until used. A single batch of olive oil stored at 4°C was used. The iodine number for the butter fat was 32 and the olive oil was 85; the resulting mixture of olive oil and butter fat had an iodine number of 73. Although the iodine number indicated a like degree of unsaturation when compared with the egg oil, the content and proportion of individual fatty acids were not the same. The primary difference was in the presence of short-chain fatty acids in the olive oil-butter fat mixture as contrasted with their absence from the egg oil. A higher content of 16:0, 16:1, and 18:2 fatty acids was present in the egg oil.

The fat mixture was fed with and without cholesterol. The fat mixture with added cholesterol contained a total of

[2] Vitamins through the courtesy of Merck, Sharp and Dohme Research Laboratories, Inc., Rahway, New Jersey, and Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Diet composition

	g/100 g		Iodine no.
Casein	24	Egg oil	72
Fat	27	Olive oil	85
Salts [1]	4	Butter fat	32
Dextrose to	100	Fat mixture	73

Fatty acid composition

Fatty acids	Egg oil	Fat mixture
	%	%
4:0 [2]	—	0.32
6:0	—	0.03
8:0	—	0.51
10:0	—	0.89
10:1	—	0.16
12:0	—	1.31
14:0	0.03	3.39
14:1	—	0.48
16:0	29.50	14.34
16:1	3.18	1.60
18:0	9.62	6.12
18:1	47.90	62.88
18:2	9.55	6.61
19:0	—	1.36
Polyunsaturated	9.55	6.61
Monounsaturated	51.08	65.12
Saturated	39.42	28.02

Vitamin addendum

	mg/100 g
Thiamine	1
Riboflavin	2
Pyridoxine·HCl	1
Ca pantothenate	10
Niacinamide	10
Inositol	5
p-Aminobenzoic acid	30
Biotin	0.05
Folic acid	0.2
α-Tocopherol	14.2
Vitamin B_{12}	0.01
Menadione	14.2
	IU/100 g
Vitamin A	150
Vitamin D	15

[1] Hegsted et al. (12).
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.

1.29% cholesterol which corresponded to the cholesterol content of the egg oil as determined by the Sperry-Webb method (15). This quantity was provided by the butter fat containing 0.02% cholesterol and by 1.27% of added cholesterol.

As bile acids are known to increase the absorption of cholesterol, 1% cholic acid was added to some of the diets. Choline chloride was fed as 0.1 or 0.3% of the rations.

The dietary groups were as follows: 1) egg oil with 0.1% choline; 2) egg oil with 0.3% choline; 3) fat mixture with 0.1% choline; 4) fat mixture with 0.3% choline; 5) fat mixture, cholesterol and cholic acid with 0.1% choline; 6) fat mixture, cholesterol and cholic acid with 0.3% choline; 7) fat mixture and cholesterol with 0.1% choline; 8) fat mixture and cholesterol with 0.3% choline; 9) fat mixture and cholic acid with 0.1% choline; 10) fat mixture and cholic acid with 0.3% choline; 11) egg oil and cholic acid with 0.3% choline; and 12) egg oil with 0.3% choline.

This study was carried out in 3 experiments. Experiment 1 (groups 1–6) compared the effects of egg oil with the mixture of fat with and without added cholesterol and cholic acid. Experiment 2 (groups 7–10) compared the individual effects of cholesterol and cholic acid fed with the fat mixture. Experiment 3 (groups 11 and 12) studied the effects of cholic acid upon the absorption of cholesterol as contained in egg oil.

Animals. Young adult female rats of the Wistar strain were used. Groups of 10 rats each were used in experiment 1. The animals averaged 214 g in weight. They were maintained with their respective diets for 10 weeks. Groups of 3 rats each were used in experiment 2. The rats averaged 240 g in weight and were fed the diets for 8 weeks. Groups of 5 rats each were used in experiment 3. They averaged 214 g in weight and the duration of the experiment was 5 weeks.

The rats in all groups were housed in individual cages in an air conditioned room kept at 21.8°C. The diets were fed

ad libitum and food consumption was recorded 3 times weekly. The rats were weighed semi-weekly.

The animals were killed after the period stated by an intraperitoneal injection of pentobarbital sodium.[3] The blood was taken from the deeply anesthetized animals by heart puncture and the livers were removed immediately. The livers were wiped with tissue and weighed. A piece of liver, approximately 2 cm^3 was placed in 30% formalin for histological studies. The remainder was wrapped in Saran Wrap,[4] frozen and stored until used for biochemical determinations.

Methods. Plasma cholesterol was determined by the method of Sperry-Webb (15). The frozen livers were brought to room temperature and ground in a mortar with sodium sulfate. The total lipids were extracted from an aliquot of the wet liver with petroleum ether. An aliquot of the petroleum ether fraction was used for the cholesterol determination (15).

RESULTS AND DISCUSSION

Experiment 1. The food consumption averaged the same for all groups, 12 g daily. The rats receiving the egg oil (groups 1 and 2) made somewhat greater weight gains than those fed the fat mixtures with or without any variants (groups 3–6). The animals made gains expected for females of their age and did not vary appreciably from group to group. All data for experiment 1 are shown in table 2.

The majority of the rats receiving cholesterol and cholic acid with either level of choline (groups 5 and 6) had bloody noses and tails. Several of the animals in these groups excreted urine of gelatinous consistency. The visceral fat in some of these rats was not white and translucent, as in normal animals, but rather brown and opaque in appearance. Despite rough fur and the abnormalities mentioned, the rats were otherwise normal in appearance and activity.

The plasma cholesterol of the rats fed the egg oil diets (groups 1 and 2) was not affected by tripling the choline in their rations. The cholesterol was higher for the animals receiving the egg oil ($P < 0.01$) than for those fed the fat mixture without added cholesterol and cholic acid (groups 3 and 4). The increased choline produced a significant lowering of the cholesterol in the latter groups ($P < 0.05$). The rats receiving the fat mixture with cholic acid and cholesterol (groups 5 and 6) had extremely high levels of plasma cholesterol: namely, a fivefold increase over groups 1 to 4, inclusive ($P < 0.01$). Increasing the choline from 0.1% to 0.3% was without effect upon the plasma cholesterol of groups 5 and 6.

The liver weights were greater for groups 1 and 2 than for groups 3 and 4 ($P < 0.01$). The liver weights of groups 5 and 6 were almost twice those of the other groups. Increasing the choline had no effect.

The total liver lipids and the liver cholesterol were several times higher ($P < 0.01$) when cholesterol and cholic acid were added to the diets containing the fat mixture (groups 5 and 6) than for any other groups. This observation held when calculated on both milligrams per gram and total liver bases. A significant decrease in the liver cholesterol ($P < 0.02$) was produced by the higher level of choline (group 6). The livers from the animals fed diets containing the fat mixture (groups 3 and 4) were of low lipid content and were practically devoid of cholesterol. The liver lipids and cholesterol for the rats fed egg oil (groups 1 and 2) were much higher on both the milligrams per gram and grams per liver bases ($P < 0.01$) than for the corresponding groups given the fat mixture (groups 3 and 4). In the egg oil groups, the increased choline produced a significant decrease in the total lipids ($P < 0.02$), but not in the cholesterol.

Experiment 2. The food consumption averaged the same for all groups — 14 g/day. The rats fed the diets containing cholesterol were somewhat heavier than were those fed cholic acid. One rat in each of the groups receiving cholesterol (groups 7 and 8) had a bloody nose and rough fur. All animals fed the like diet but with cholic acid appeared normal. Data for experiment 2 are given in table 3.

The plasma cholesterol showed almost a threefold increase when cholesterol was

[3] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.
[4] Dow Chemical Company, Midland, Michigan.

TABLE 2

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg/100 ml*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 1, 10 weeks [1]							
1 Egg oil; 0.1% choline	312	102 ± 2.4 [2]	11.9 ± 0.75	207 ± 11.5	2.50 ± 0.19	43.4 ± 2.3	0.48 ± 0.05
2 Egg oil; 0.3% choline	298	104 ± 4.3	11.3 ± 0.35	166 ± 8.5	1.95 ± 0.16	40.6 ± 2.4	0.47 ± 0.04
3 Fat mixture; 0.1% choline	301	88 ± 7.5	8.9 ± 0.35	66 ± 3.8	0.57 ± 0.03	3.1 ± 0.3	0.03 ± 0.00
4 Fat mixture; 0.3% choline	286	66 ± 4.8	7.8 ± 0.71	64 ± 2.0	0.51 ± 0.02	4.2 ± 0.4	0.03 ± 0.00
5 Fat mixture; 0.1% choline cholesterol; cholic acid	292	471 ± 60.9	20.6 ± 0.71	355 ± 9.0	7.30 ± 0.37	164.4 ± 5.4	3.57 ± 0.13
6 Fat mixture; 0.3% choline cholesterol; cholic acid	290	547 ± 57.6	20.0 ± 0.71	344 ± 9.8	6.90 ± 0.40	143.6 ± 4.8	2.66 ± 0.20

[1] Each group consisted of 10 female rats with an average initial weight of 214 g.
[2] SD of mean.

TABLE 3

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg %*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 2, 8 weeks [1]							
7 Fat mixture; 0.1% choline cholesterol	302	273 ± 9.5 [2]	9.7 ± 0.72	329 ± 0.7	3.43 ± 0.02	113.9 ± 5.9	1.19 ± 0.01
8 Fat mixture; 0.3% choline cholesterol	288	297 ± 32.2	13.0 ± 1.6	294 ± 4.5	4.26 ± 0.36	63.9 ± 17.3	0.77 ± 0.05
9 Fat mixture; 0.1% choline cholic acid	265	95 ± 8.7	9.3 ± 0.14	120 ± 17.5	1.10 ± 0.04	17.6 ± 4.5	0.16 ± 0.02
10 Fat mixture; 0.3% choline cholic acid	272	103 ± 15.2	9.7 ± 0.61	91 ± 11.5	0.86 ± 0.06	13.0 ± 4.6	0.12 ± 0.01
Experiment 3, 5 weeks [3]							
11 Egg oil; 0.3% choline cholic acid	264	209 ± 31.3	16.3 ± 0.46	185 ± 7.5	3.02 ± 0.17	50.3 ± 1.9	0.82 ± 0.03
12 Egg oil; 0.3% choline	287	123 ± 8.8	14.1 ± 0.76	170 ± 8.5	2.38 ± 0.14	25.3 ± 5.5	0.39 ± 0.04

[1] Each group consisted of 3 female rats with an average initial weight of 240 g.
[2] SD of mean.
[3] Each group consisted of 5 female rats with an average initial weight of 214 g.

in the rations (groups 7 and 8) than when cholic acid-containing diets were fed (groups 9 and 10), ($P < 0.01$). The plasma cholesterol was not affected by the choline intakes.

The liver weights appeared to be increased by the higher level of choline in the presence of cholesterol (groups 7 and 8); however, this increment was not significant because of the variance and small number of animals in these groups. Increased choline with cholic acid was without effect (groups 9 and 10).

The total liver lipids and cholesterol followed the same pattern as did the plasma cholesterol in that a threefold increase was noted for the rats fed cholesterol (groups 7 and 8) over those receiving cholic acid (groups 9 and 10). The values for these latter groups (groups 9 and 10) were lower than for any other groups in experiments 1 and 2 except those receiving only the fat mixture (groups 3 and 4). Increasing the choline in the presence of cholesterol produced a significant rise ($P < 0.05$) in the lipids on a gram per liver basis, but not on a milligram per gram basis (groups 7 and 8). The cholesterol did not follow this pattern on a total weight basis; in fact, a lower value was noted. Due to the variance in the small number of observations for groups 9 and 10 the choline appeared to be without significance.

The fat mixture with cholesterol produced a two- to threefold increase in plasma and liver cholesterol and liver total lipids over that observed with the egg oil in experiment 1. The modest increase in food intakes in experiment 2 would not appear to account for this difference.

Experiment 3. The food consumption for the 2 groups in this experiment was higher than in experiment 1 in which egg oil was fed even though the initial weights were the same; the food intake being approximately 20 g/day as compared with 12 g in experiment 1. The weight gains were somewhat less for the animals receiving cholic acid than for those without it (groups 11 and 12). Both groups were in good condition throughout the 5-week experiment. The data for experiment 3 are shown in table 3.

Cholic acid produced an increase of approximately 50% in plasma ($P < 0.05$) and liver cholesterol ($P < 0.01$) and a 20% increment in liver lipids based on grams per liver ($P < 0.02$) over the group not receiving cholic acid. These observations suggest the need for a study of egg lipids as influenced by cholic acid in other species including man.

HISTOLOGY — (Experiments 1 and 2)

The livers from the animals receiving cholesterol and cholic acid (groups 5 and 6) were brownish yellow in color and had a butter-like consistency.

Histological examination revealed that 7 out of the 10 animals receiving the diets containing egg oil (groups 1 and 2) had needle-like crystals, presumably cholesterol, in the cytoplasm. The nuclei of the peri-central zone was atrophic and the cells appeared somewhat atrophied. Increasing the level of choline (group 2) appeared to have little or no effect upon the livers. The values for the liver cholesterol were the same for these groups, an observation noted in experiment 1. Rats that were fed cholesterol and cholic acid with the fat mixture (groups 5 and 6) showed massive fatty depositions with innumerable crystals in the parenchymal cells. The liver cords were somewhat distorted due to the great increase in cell size; in fact, the cell appeared to be completely replaced by fat with little evidence of cytoplasmic remnants. The nuclei were atrophic. Increasing the choline in the diet appeared to have little effect, although slightly less lipid and crystalline deposition was noted.

When cholesterol alone was given in the diet containing 0.1% choline (group 7) a massive crystalline deposition with atrophy of the nuclei was noted. These changes were not as marked as in group 8 in which 0.3% choline was given. The livers of the rats fed the fat mixture without cholesterol and cholic acid (groups 3 and 4) showed only mild fatty deposits which were primarily periportal. Increasing the choline was without effect upon these livers. When cholic acid was given with the fat mixture with either level of choline (groups 9 and 10) the fatty deposits were the least observed for any of

the groups. There were few crystals and only a small number of periportal hepatic parenchymal cells. The choline level was without effect.

The livers from experiment 3 were not examined.

The feeding of egg oil with or without cholic acid resulted in lower plasma and liver cholesterol values in rats than did a mixture of fats with the same level of cholesterol present in egg oil. The results appear to indicate that factors, or a balance of factors, present in egg oil tended to lower the cholesterol levels that would have been expected from the feeding of cholesterol as contained in the egg oil. This observation may hold only for the Wistar rat and would not necessarily be in conflict with the observations of Messinger et al. (11) and Bronte-Stewart et al. (5) who used human subjects. Furthermore, egg oil was fed in our experiments; Messinger used egg yolk powder and Bronte-Stewart fed fresh eggs.

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Absorption of bile salts from the small intestine in vivo[1,2]

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WEINER, I. M., AND LEON LACK. *Absorption of bile salts from the small intestine in vivo.* Am. J. Physiol. 202(1): 155–157. 1962.—The absorption of bile salts from the small intestine of the guinea pig was studied in vivo by determining the biliary excretion of bile salts injected into isolated regions of the small bowel. Taurocholate, glycocholate, and cholate appeared in bile in significant amounts only when they were introduced into the distal part of the small intestine (ileum), indicating that there is little, if any, absorption by diffusion through the proximal mucosa. Allowing animals to eat a relatively high fat diet before the experiments did not alter the pattern of intestinal absorption of bile salts. The results confirm previous in vitro studies on the active transport of bile salts by the ileum.

IT HAS BEEN SHOWN with the everted gut sac technique that the small intestines of rats and guinea pigs actively transport cholic acid bile salts from the mucosal to the serosal side. This activity is located in the ileum and appears to be completely absent from the first half of the small intestine (1). Two questions have arisen: first, the extent to which this discrete localization of function exists in intact animals, and second, the degree to which bile salts might cross the mucosal wall by passive diffusion in the absence of a specific transport system.

Earlier workers showed that there is a gradient of bile salt absorption along the small intestine, absorption being greatest in the ileum (2, 3). This was recently confirmed by Baker and Searle (4), who found that absorption was four times greater in the ileum than in the duodenum. In all of these studies absorption was estimated by the differences between the amounts of bile salts originally added to and those recoverable from intestinal loops after an interval of time. Although attempts were made to eliminate errors due to tissue binding, unequivocal evidence that this factor can be ignored is not available. For this reason we have employed another experimental technique to study this question. Instead of measuring the disappearance of bile salts from intestinal loops we have measured the appearance in the bile of cholic acid bile salts that were originally placed in the intestinal loops. This enables estimation of net movement across the mucosal cell, permitting accurate estimation of any diffusion process in those segments previously shown to lack the active transport system. The animals employed were young guinea pigs since cholic acid derivatives are absent in immature members of this species (5). The natural bile salts of these animals are not detected by the Pettenkoffer reactions. Since bile salts are intimately related to fat digestion and absorption, the effect of feeding a high fat diet immediately prior to the experiment was also studied.

We are able to conclude that the proximal half of the small intestine plays a negligible role in the enterohepatic circulation of bile salts and that transmucosal bile salt and fat absorption appear to be independent functions.

METHODS

In most experiments female guinea pigs of the Hartley strain weighing 200–400 g were fasted for 24 hr. In a few experiments when the effect of feeding was studied, the animals were deprived of food for 5 hr during the day before the experiment and then allowed to eat ad libitum a regular guinea pig diet, Purina (guinea pig chow) supplemented with sufficient olive oil to bring the fat content to 10% by weight. Each animal was anesthetized with ether (in the case of fed animals, a tracheal cannula inserted), the abdomen opened, the cystic duct tied off, and a plastic tube inserted into the common bile duct. The abdomen was closed with clamps, and bile was collected for 30 min. The abdomen was then reopened, and a solution of bile salt was introduced into a tied-off intestinal segment. Care was taken in tying ligatures not to compromise the intestinal blood flow. The abdomen was closed with sutures, and bile was collected for six 30-min periods. Light ether anesthesia was maintained throughout the experiment, at

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[2] A preliminary report was made at the April 1961 meeting of the Federation of American Societies for Experimental Biology.

TABLE 1. *Absorption of simultaneously administered taurocholate and p-aminobenzoate from the small intestine*

Guinea Pig No.	Wt., g	Segment	Length of Segment, cm	Length of Total Small Intestine, cm	Taurocholate Recovered in Bile		p-Aminobenzoate Remaining in Sac	
					mg	% Administered dose	mg	% Administered dose
1	215	Prox.	63	140	.36	6*	.56	9†
2	225	Prox.	48	110	.22	3.7*	1.7	28
3	205	Dist.	51	127	3.76	63	1.6	27
4	200	Dist.	36	140	5.19	87	1.93	33

* These values are within the analytical error, see text.
† Control recovery (zero time), 87%.

the end of which the animal was sacrificed. The lengths of the intestine and tied-off segment were measured. In no case was necrosis of the intestine observed. In each case the bile salt introduced into the intestinal segment was contained in 3 ml isotonic sodium chloride solution containing potassium phosphate buffer .01 M, pH of approximately 7.0. The compounds studied were cholic acid (Fisher Scientific), taurocholic acid (Pfanstiehl) purified as described previously (1), and glycocholic acid (K & K Laboratories).

Bile was analyzed for cholic acid and its derivatives by the modified Pettenkoffer reaction of Irvin et al. (6). Two-tenths of a milliliter of bile was diluted to 1 ml and used for analysis.

In four cases *p*-aminobenzoic acid was included in the bile salt solution. The amount of this substance remaining in the small intestine was estimated by the method of Bratton and Marshall (7) after the entire intestinal segment and its contents were homogenized and deproteinized with 3% trichloroacetic acid.

RESULTS

The left side of Fig. 1 gives the results obtained when 6 mg of taurocholate was introduced into proximal and distal segments. By proximal segments we mean a length of small intestine beginning just distal to th point of entrance of the bile duct and including 26–55% of the entire length of the small intestine. The dista segments included the most distal 15–40% of the smal intestine.

In all cases the results are given as cumulative excre tion of cholic acid derivatives in the bile expressed a per cent of the amount placed in the intestine. The values were corrected for the nonspecific absorbance o control bile obtained from each animal. Control experi ments in which no cholic acid derivatives were admin istered indicated that the concentration of the nonspecifi blank remained fairly constant during the 3½-h experimental period. The maximal cumulative erro attributable to variations in the blank corresponds t less than 10% of the 6-mg loads introduced into th experimental animals.

As can be seen from Fig. 1, taurocholate placed in th distal segments is rapidly excreted in the bile. Whe comparable amounts were placed in the proxima segments, the rate of excretion in the bile fell within th error attributable to variations in the blank.

In order to minimize the error due to the nonspecifi chromogen a series of experiments were made with large dose, 30 mg, of taurocholic acid. The results a shown in the right side of Fig. 1 demonstrate tha recovery from the proximal segments never exceede 3.3% of the administered load (average 2.2%). Thi small amount of material appearing in the bile ma well be due to slight trauma to the mucosa caused b preparative manipulations. The biliary excretion c taurocholic acid placed in the most distal region of th small intestine averaged 55%.

The presence of food (10% fat diet) in the gastro intestinal tract had no detectable effect on the absorptio of taurocholic acid. It should be noted that in all of th fed animals chyle was readily observed in the mesenteri lymphatics at the time of operation and at the end of th experiment.

In order to determine whether the present method would permit the detection of very small amounts c cholic acid derivatives leaving the small intestine, tw

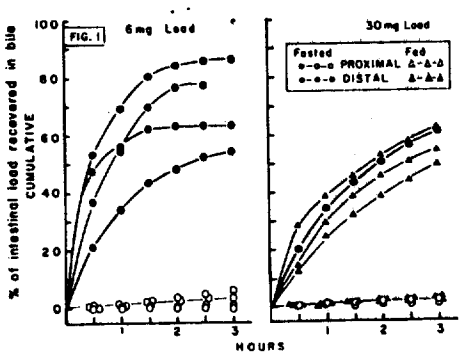

FIG. 1. Recovery in bile of taurocholate placed in small bowel segments of bile fistula guinea pigs. Each *curve* represents an experiment on one animal. The *open circles* have been displaced slightly along the time axis in order to enable depiction of each point.

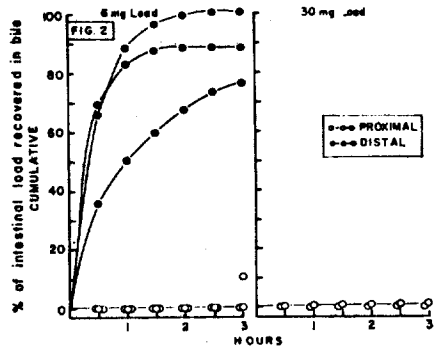

FIG. 2. Recovery in bile of glycocholate placed in small bow segments of bile fistula guinea pigs. See legend to Fig. 1.

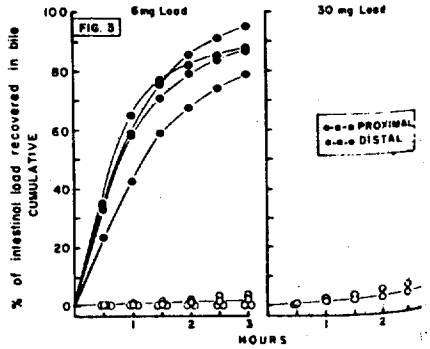

FIG. 3. Recovery in bile of cholate placed in small bowel se ments of bile fistula guinea pigs. See legend to Fig. 1.

guinea pigs were given 0.6 mg of taurocholic acid in distal segments. Under these conditions 52 and 85% were recovered in the bile. This was easily detected since these small amounts appeared entirely during the first collection period.

In four animals *p*-aminobenzoic acid (6 mg) was included, along with taurocholate, in the fluid of the intestinal segments. This was done in order to demonstrate that the blood supply to the proximal segments was adequate. Table 1 demonstrates that during the 3-hr experiment *p*-aminobenzoic acid was removed from the proximal as well as the distal segments. At the same time only the bile salt present in the distal segments appeared in the bile.

The data depicted in Figs. 2 and 3 demonstrated that taurocholic and cholic acid are handled in a manner strictly similar to taurocholic acid.

DISCUSSION

The in vitro studies, previously cited, demonstrated that the active transport of bile salts is limited to the distal portion of the small intestine. The present studies were undertaken to determine whether or not the proximal small bowel participates to a significant extent in the enterohepatic circulation of bile salts by allowing the passive diffusion of these compounds into the portal blood. The data demonstrate that any such passive component is exceedingly small if it exists at all.

The finding that bile salt absorption is virtually absent in the proximal intestine is at variance with earlier reports (2–4). Baker and Searle (4) in their studies on the rat found that the relatively low absorption from the proximal intestine was about 1.5 μM cholate/cm/hr. This would correspond to approximately 15 mg cholate/hr transported under the conditions of the present experiments. The most likely explanation for this discrepancy is the gradual binding of the bile salts by intestinal contents and mucosa during the experiments. In vitro studies in rats as well as guinea pigs indicated that in both species the proximal gut is impermeable to bile salts (1); consequently it seems unlikely that the in vivo behavior of these species is considerably different.

Even in the case of unconjugated cholic acid the absorption from the proximal gut was minimal. This is noteworthy because cholic acid by virtue of its relatively high solubility in lipid solvents and high pKa (8) would be expected to be the most diffusible of the compounds studied.

The presence of dietary constituents including lipid did not increase the removal of taurocholate from the proximal gut. If the same phenomenon occurs in normal animals without anesthesia it is of interest in terms of the mechanism of fat absorption. Since fat is absorbed from the proximal small intestine (9, 10) while bile salts are not, it seems unlikely that fat absorption occurs by the transmucosal transport of an emulsified particle containing both substances.

In order to retain the hypothesis of the particulate absorption of a lipid-bile salt emulsion one would have to postulate that this particle is destroyed within the mucosal cell, the bile salt then being free to leave the cell, but only in the direction of the intestinal lumen.

Studies on unanesthetized, iliectomized bile fistula animals are in progress.

The authors are indebted to Mabel Smith and Mary Jane Miller for expert technical assistance.

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Bile Acids in Blood: Methods and Their Application to Studies of Experimental Atherosclerosis in Monkeys[1]

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Introduction

The bile acids are implicated in the problem of hypercholesteremia and experimental atherosclerosis in several ways. It has been contended by Byers *et al.* (1) that in rats an elevation of serum bile acid levels by any of several methods is associated with hypercholesteremia. It was reported by Friedman *et al.* (2) that hypercholesteremia in human subjects of various clinical descriptions was associated with increased levels of bile acids in the blood. Those workers concluded that the cholate accumulation in serum was the cause of the hypercholesteremia and that this effect was accomplished by interference with hepatic extraction of cholesterol from the blood, possibly through a detergent effect of cholate. Frantz *et al.* (3) have recently proposed that the hypercholesteremia in rats associated with bile duct ligation or administration of Triton[4] results from an increased hepatic synthesis of cholesterol. Rats, which are notably resistant to the hypercholesteremic effects of cholesterol feeding, can be made less so by cholate feeding (4). In associated work we have shown (5) that cholesterol fed to bile-cannulated rats will cause a significant increase of bile acid production, presumably a consequence of increased conversion of cholesterol to bile

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[2] Postdoctoral fellow of the Life Insurance Medical Research Fund.

[3] Established investigator of the American Heart Association.

[4] Wetting agent, Rohm & Haas Co.

acids. Such a transformation has been demonstrated by Bloch *et al.* (6).

We have established that Cebus monkeys can be made to respond to cholesterol feeding with hypercholesteremia, hyperlipoproteinemia, and atherosclerosis if they are deprived of the essential sulfur-containing amino acids at the same time (7). It was then of interest to determine whether significant alterations of bile acid metabolism occurred in these monkeys and also to evaluate the response of monkeys to cholate feeding.

We have evaluated several of the spectrophotometric methods for bile acid determination (8–11) as well as the chromatographic procedure of Wootton (12) which utilizes a silica gel column to separate the methyl esters of the bile acids. The chromatographic separation was not satisfactory on the necessary micro scale because of an apparent partial alteration of the compounds, particularly cholic acid, during the elution. This alteration was evidenced by a progressive movement of the cholic acid to less polar regions with repeated chromatograms of the same material. A procedure has been developed which represents a combination of several spectrophotometric methods. This has been adapted to both blood and bile, the principal difference for the two materials being the preliminary solvent preparation of the extracts for spectroscopy. The bile methods will be described in another publication (5).

This procedure has been applied to the blood serum of normal and hypercholesteremic human subjects and to normal and atherosclerotic monkeys some of which were fed cholate in addition to cholesterol. This method failed to show elevated cholic or deoxycholic acids in either hypercholesteremic human subjects or monkeys. There was an increase in the levels of the material designated chenodeoxycholic acid in hypercholesteremic serum. Cholate feeding in monkeys produced significant increases of the serum cholesterol. The hypercholesteremia produced by cholate feeding appeared to be as atherogenic as that produced by organic sulfur deficiency.

Experimental

Reference Compounds

The reference compounds, their melting points after recrystallization, and their sources were as follows: cholic acid, m.p. = 194–196°C. (Nutritional Biochemicals Corp.); taurocholic acid, m.p. = 125°C.;[5] glycocholic acid, m.p. = 162°C.; and

[5] The method of Bergström for synthesis of taurolithocholic acid was adapted for taurocholic acid. A very small yield of uniform-sized but amorphous crystals

TABLE I

The Molar Extinction Coefficients (ϵ_λ) for Several Bile Acids According to State of Conjugation and Reaction

The condition of the tests are described in the text

Compound	Reaction			
	Pettenkofer 620 mμ	Szalkowski 700 mμ	"Minibeck"	
			310 mμ	390 mμ
Cholic acid	9.2×10^3	6.24×10^2	3.3×10^3	2.4×10^4
Taurocholic acid	9.2×10^3	—	3.3×10^3	2.4×10^4
Glycocholic acid	9.2×10^3	—	3.3×10^3	2.4×10^4
Chenodeoxycholic	7.8×10^2	4.35×10^2	8.7×10^3	2.1×10^3
Deoxycholic	7.8×10^2	1.00×10^4	9.4×10^3	2.1×10^3
Lithocholic	—	8.80×10^1	5.3×10^3	1.2×10^3
Taurodeoxycholic	3.6×10^2	1.25×10^4	7.4×10^3	2.4×10^3

taurodeoxycholic acid, m.p. = 141–144°C. (synthesized by the method of Bergström *et al.*, 13); deoxycholic acid, m.p. = 172–176°C. (Eastern Chemical Corporation); chenodeoxycholic, m.p. = 188–189°C.; and lithocholic acid, m.p. = 137–140°C. (gifts from Professor L. F. Fieser).

The spectrophotometry was accomplished in a Beckman model DU spectrophotometer. Silica cuvettes with a 1-cm. light path were used for materials measured below 400 mμ.

The human blood was collected in the course of other studies from subjects who were hypercholesteremic and who were classified after a physical examination designed to reveal the presence of acute or chronic disease.

The preliminary preparation of serum for spectrophotometry was essentially that of Josephson (9) which consisted of the precipitation of the serum proteins in the presence of barium and extraction of interfering lipides from the sodium salts of the bile acids with ethyl acetate until the ethyl acetate washings gave a negative Liebermann-Burchard reaction for cholesterol. The next step was acidification of the bile salt solution to pH 1.0 with H_2SO_4. The resulting bile acids were dissolved in 25 ml. of ethanol and separated from the $BaSO_4$ precipitate.

Spectrophotometry

The cholic acid content of blood serum was determined on aliquots of the ethanolic extract (dried at 60°C. in a nitrogen atmosphere) to which the Pettenkofer reaction as adapted by Irwin, Johnston, and Kopala (10) was applied. Molar extinction coefficients of the different bile acids treated in this manner are shown in Table I.

Deoxycholic acid was determined with the Szalkowski reaction (11). Chenode-

was obtained by crystallization from 2 ml. of ethanol, 10 ml. of ethyl acetate and 5 ml. of ethyl ether. The crystals melted sharply at 125°C.

oxycholic acid contributes to this measurement, but the influence is small (Table I) and no correction has been made. The correction for the contribution of cholic acid was based on the result obtained with the Pettenkofer reaction.

Chenodeoxycholic acid was determined by an adaption of the method described by Minibeck (8) for cholic acid. The absorption spectra of the different bile acids with this reagent are shown in Fig. 1. The maximal absorption for chenodeoxycholic acid was at 310 mμ and the molar extinction coefficients for the various bile acids at this wavelength are shown in Table I. Corrections for the cholic acid and deoxycholic interference at 310 mμ were subtracted before calculation of the chenodeoxycholic acid.

All of the bile acids have been calculated as unconjugated bile acids. This was possible, since the molar extinction coefficients of the unconjugated and conjugated bile acids considered were identical or similar with all of the color reactions used (Table I). With these methods recoveries of 95–98% were obtained with mixtures of the several bile acids.

Preparation of Animals

The procedures used for the production of hypercholesteremia and atherosclerosis in Cebus monkeys have been described (7). Nine monkeys of various ages and including both sexes were observed for 4 weeks while maintained on a complete purified diet (840—Table II) without added cholesterol. The animals were then fed the cholesterol- and cholate-containing diets of Table II according to

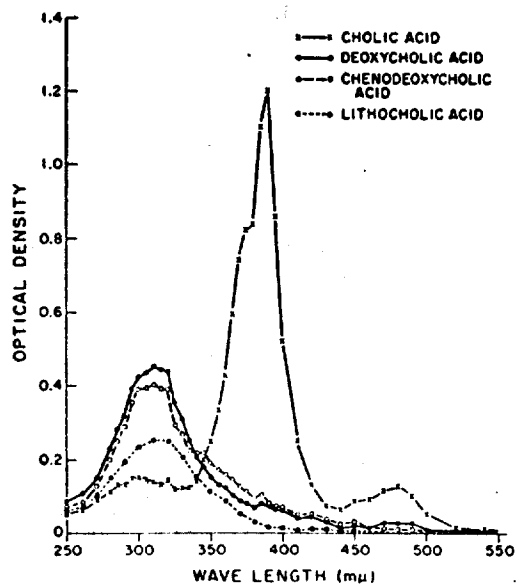

FIG. 1. The absorption spectra of several bile acids in the reagent of Minibeck (8)—H_2SO_4:HAc = 9:1 (*v/v*).

TABLE II

The Composition of Diets

(per cent)

Each animal received a daily portion of water-soluble vitamins in 20% ethanol which supplied thiamine·HCl 1.0 mg., riboflavin 1.0 mg., pyridoxine·HCl 1.0 mg., niacin 4.9 mg., calcium pantothenate 3.0 mg., folocin 0.1 mg., biotin 0.02 mg., and ascorbic acid 25.0 mg.

One per cent cholic acid was added to 825 cF + cholic acid and 820 αF + cholic acid diets at the expense of sucrose.

Thirty milligrams of cystine daily was added to the 820 αF + cystine diet at the expense of sucrose.

Diet	825 cF	820αF	840
Casein	25.0	—	25.0
α-Protein[a]	—	20.0	—
Sucrose	48.3	53.3	60.3
Corn oil (Mazola)	15.0	15.0	8.0
Cod liver oil	2.0	2.0	2.0
Cholesterol[b]	5.0	5.0	—
Choline	0.5	0.5	0.5
p-Aminobenzoic acid	0.1	0.1	0.1
Inositol	0.1	0.1	0.1
Salt mix (22)	4.0	4.0	4.0

[a] α-Protein is a derivative of soybeans prepared by the Glidden Company, Chicago, Illinois, and was furnished for this work through the courtesy of Mr. W. M. Bain.

[b] Crystalline cholesterol was generously supplied by Armour and Co., Chicago, Illinois.

the arrangement in Table IV. The animals were weighed weekly, and they were bled at 2–4-week intervals throughout the experiment for the measurement of serum total cholesterol by the method of Abell *et al.* (14) and β-lipoproteins by the method of Gofman *et al.* (15). After 52 weeks of this feeding procedure, the animals were anesthetized with ether, exsanguinated through a cardiac puncture, and tissues were removed for histological studies. The extent of atherosclerosis was graded by the gross and microscopic appearance of the fresh and stained material.[4]

Eight other monkeys were also bled for cholesterol and bile acid measurements. Three of these were control animals which had received diet 825cF (Table II) for 25 months and were not hypercholesteremic. The remaining five animals had received diet 820αF supplemented with very small amounts (30 mg.) of cystine daily for 6 months, and these animals were hypercholesteremic.

[4] Experiments of S. B. Andrus. To be published.

TABLE III

A Comparison of the Levels of Cholesterol and Bile Acids in the Blood Serum of Normal and Hypercholesteremic Human Subjects

Group	Number	Bile acids (mg. %)			Total cholesterol mg. %
		Cholic	Deoxycholic	Chenodeoxycholic	
Normal	7	0.86 ± 0.06[a]	0.35 ± 0.07	6.5 ± 0.9	234 ± 16.5
Hypercholesteremic	6	0.69 ± 0.30	0.28 ± 0.03	13.0 ± 1.1	388 ± 20.7
t =		0.48	0.92	4.6	5.8
P =		0.5	0.1–0.5	0.01	0.01

[a] Values given are means and standard errors of the mean.

Results

Bile Acids in Normal and Hypercholesteremic Human Sera

The values for serum cholesterols and bile acids in seven normal and six hypercholesteremic human subjects are shown in Table III. The hypercholesteremic patients had a mean cholesterol value of 388 mg. % compared to 234 mg. % for the normal controls ($t = 5.8$, $P < 0.01$). There was, however, no significant difference in the values obtained for cholic or deoxycholic acids in the sera. The hypercholesteremic subjects had mean values for the reading designated chenodeoxycholic acid of 13.0 mg. % compared to 6.5 mg. % for the normal controls ($t = 4.6$, $P < 0.01$).

The Effects of Cholate and Cholesterol Feeding on Monkeys

The results of the feeding experiment with monkeys are shown in Fig. 2 which illustrates the data of five representative animals. The addition of sodium cholate to the diets produced diarrhea initially, and this was associated with weight loss particularly in the animals fed diet 820αF. One of the animals died after 16 days of the regimen. The addition of sodium cholate to the casein diet (825cF) quickly produced increased serum levels of cholesterol and β-lipoproteins which were similar in degree and character to that produced by the α-protein diets which are low in essential sulfur compounds. We have previously shown that diet 825cF without cholate will produce only a small increase of serum lipide levels. The effect of cholate feeding was to make the response of monkeys fed diets made with casein similar to that of animals fed diets low in organic sulfur compounds.

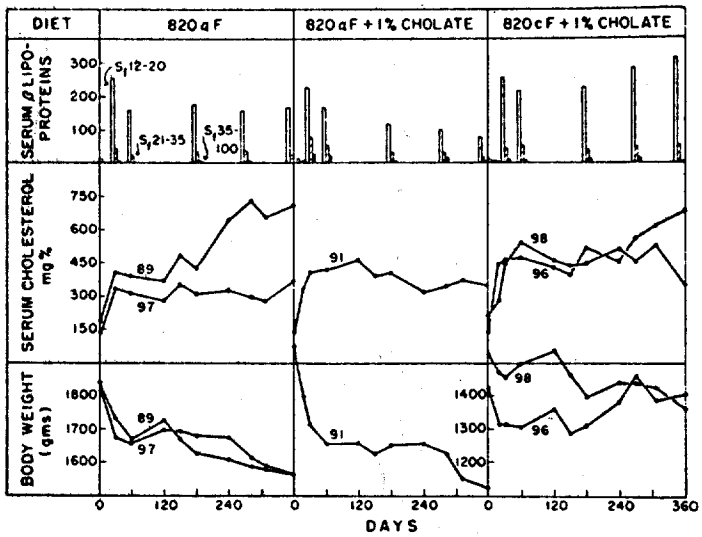

FIG. 2. The cholesterol and lipoprotein response of representative Cebus monkeys on three dietary regimens (Table II).

The bile acid and cholesterol levels in the blood of these animals as well as those of monkeys in other similar experiments are summarized in Table IV according to the dietary regimen. These data indicated a remarkable constancy of the serum cholic and deoxycholic acid levels regardless of the dietary treatment. There was no evidence that the degree of cholesteremia was related to the level of these two bile acids. The fraction called chenodeoxycholic acid was the highest. The monkeys fed the 820 αF diet had higher chenodeoxycholic acid levels than did those fed 825cF ($t = 3.6$, $P < 0.01$). Similarly, the value for monkeys fed 825cF + cholic acid was greater than that of the group fed 825cF ($t = 2.3$, $P = 0.10$). The measurement of chenodeoxycholic acids, though highly reliable in the measurement of bile acids in bile, is probably less reliable when applied to serum, since the concentration of bile acids is about 1/5000 of that in bile, and the concentration of potentially interfering chromogens is greater than in bile.

The examination of the vascular tissues of the monkeys fed the 820αF, 825cF + cholic, and 820αF + cholic diets revealed significant atherosclerosis in all the animals. The degree of aortic involvement was at least as great in the monkeys fed the diet made with casein and supplemented with 1% cholic acid as was that found in the animals fed the diets prepared with α-protein. Monkey No. 91, which had lost a large

TABLE IV

The Cholesterol and Bile Acid Content of Blood Sera of Monkeys Fed Different Diets

Diet	Number of monkeys	Number of determinations	Serum bile acids (mg. %)			Total serum cholesterol mg. %
			Cholic	Deoxycholic	Chenodeoxycholic	
825 cF	3	3	0.55 ± 0.30[a]	0.24	3.3 ± 0.21	173 ± 19.4
820 αF + cystine	5	5	0.53 ± 0.08	—	3.2 ± 0.79	361 ± 43.3
820 αF	6	12	0.70 ± 0.08	0.20 ± 0.08	7.5 ± 1.13	395 ± 46.1
825 cF + cholic acid	2	4	1.87 ± 2.82	0.20 ± 0.14	9.5 ± 2.72	529 ± 88.0
825 αF + cholic acid	1	2	1.09 ± 0.77	0.22 ± 0.20	5.4 ± 3.3	358 ± 23.2

[a] All values given are means and standard errors of the mean.

amount of weight because of the cathartic action of the cholate coupled with the inadequate protein, showed the least disease. This finding was no doubt related to the somewhat lower lipide levels in life.

Discussion

While procedures have been published for the separation of mixtures of bile acids by countercurrent distribution (16), reversed phase (17) and normal phase (12) chromatography, and paper chromatography (18), these have been applied to animal bile which is a much easier problem because of the abundance of the acids in this material. There has been no successful, quantitative separation of the bile acids in blood. Our experience has indicated that the necessary microprocedure must be based upon spectrophotometric procedures after preliminary treatment of the crude solvent extracts.

The data obtained with the present methods for chenodeoxycholic acid appeared disproportionately high and variable. Although Wootton (12) had reported high levels of chenodeoxycholic acid in human bile using column chromatography methods, the present observation of high levels in human and monkey serum must be considered tentative until a more precise method for determination of chenodeoxycholic acid is available.

The values found for cholic acid are in good agreement with those

previously reported (8–10). Kier (19) found deoxycholic acid levels of 25–52 mg.%. Our experience with Kier's method indicates that substances other than deoxycholic acid were interfering in the reaction to give excessively high values.

The question of the state of conjugation of bile acids in the blood was avoided in the present work by choosing a series of reactions which did not appear to discriminate between conjugated and unconjugated acids and expressing all the results as free acids. We have recently obtained data with rats using radio-labeled materials which suggests that conjugated bile acids are partially resorbed into the portal circulation and re-excreted without enteric hydrolysis (20). It appeared reasonable to expect that at least a part of the bile acids in the peripheral circulation may be conjugated.

The contention of Friedman and Byers that hypercholesteremia in humans is invariably associated with hypercholatemia was not substantiated by our findings. The series of normal patients of those workers showed an average serum cholesterol of 228 mg.% and a serum sodium cholate of 5.3 mg.%. This corresponds to our normal series with a mean serum cholesterol of 234 mg.% and a mean serum cholic acid concentration of 0.86 mg.%. One series of patients of Friedman *et al.* showed a mean serum cholesterol of 388 mg.% and a mean serum cholate of 13.0 mg.%, compared to our series of hypercholesteremic patients showing a mean serum cholesterol concentration of 388 mg.% and a serum cholic acid concentration of 0.69 mg.%. We observed a mean value of total bile acids in normal humans of 7.7 mg.%, and in the hypercholesteremic patients the mean value was 14.0 mg.%. Friedman conceded that his "cholate reading" might include other bile acids. The present data did assure us that neither cholic nor deoxycholic acids were increased in the hypercholesteremic blood sera measured.

The experiment relating hypercholesteremia in monkeys to the level of serum bile acids presented findings similar to those described for the human material. Elevations of serum cholesterol were not associated with elevated serum cholic acid or deoxycholic acid values (even when cholic acid was fed) but were associated with higher values for "chenodeoxycholic acid."

The addition of 1% cholic acid to the diets of animals fed casein diets led to the same degree of cholesteremia and atherosclerosis as did the deprivation of organic sulfur compounds. Since White (21) has shown that growing rats fed diets which are marginal in cystine content can

be made sulfur-deficient by cholic acid feeding, presumably by causing an excess demand for taurine, the response of these monkeys may represent a sulfur deficiency produced by a mechanism other than simple dietary deprivation.

Summary

1. A method for assay of blood sera for cholic, deoxycholic, and "chenodeoxycholic" acids was described.

2. The values for cholic acid and for deoxycholic acid in human hypercholesteremic sera did not differ statistically from the values for these bile acids in normal sera. The value for the measurement designated as "chenodeoxycholic acid" was significantly elevated in human hypercholesteremic sera.

3. Hypercholesteremia and hyper-β-lipoproteinemia were produced in Cebus monkeys by feeding diets containing α-protein with and without added sodium cholate and in monkeys fed casein diets with added cholate. The monkeys fed α-protein and those fed casein with added sodium cholate had levels of cholic acid and deoxycholic acid in the sera which were not elevated above the levels in normal cholesteremic monkeys fed a casein diet without added sodium cholate. The measurement designated "chenodeoxycholic acid" was, however, elevated in the hypercholesteremic monkeys.

4. An evaluation of the vascular lesions revealed significant aortic atherosclerosis in all hypercholesteremic animals. The degree of involvement was, at least, as great in the animals fed casein and cholic acid as in those animals fed α-protein.

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Yamasaki, K., K. Shimizu, and K. Kimijima: ON THE LETHAL DOSES OF SEVERAL BILE ACIDS. Sogo Igaku, Vol. 17, No. 5., pp. 361-363, 1960.

I. INTRODUCTION

There are many papers discussing the pharmacological or physiological action of bile acids /1/. The authors /2/ recently discovered that deoxycholic acid or aphocholic acid notably depresses blood cholesterol level in rat. Depression of cholesterol level also occurred when bile acids were orally administered to human. Thus, there is a strong indication that bile acids are potential cholesterol reducing agents.

Deoxycholic acid and apocholic acids are known to possess powerful hemolytic action as compared to other bile acids, particularly cholic acid /3/. Consequently, their toxicity is assumed to be stronger. The lethal dose of deoxycholic acid is 0.015 g/kg for rabbit by the intravenous route, and 0.1 g/kg for guinea pig by the intraperitoneal route, the values being 1.5 - 3 times higher than those of cholic acid /1/.

Since no investigation in this area has yet been conducted, the authors recently studied the toxicity of these acids when administered orally as cholesterol reducing agents, and the cause of death due to the acids.

II. EXPERIMENTAL PROCEDURE

Animals: White mice, male and female, weighing 18 - 28 g (mice S of the National Genetic Research Institute).

Mode of Administration: The drug was injected into the stomach through an oral sonde. The solution was so adjusted that the amount injected be between 0.2 and 0.5 cc. An attempt was made to inject the solution by the hypodermic route, but the site of injection developed necrosis, leaving a blackish brown coloration for several days. Thus, the solution was administered by the oral route only.

Drugs: Five types of bile acids were employed: cholic acid, linolic choleic acid, deoxycholic acid, apocholic acid, and linolic apocholic acid. They were used in the form of an aqueous solution of sodium salt, and the maximum concentration was 4/10 mol.

Computation of lethal dose: Ld_{50} was calculated by Litchfield and Wilcoxon's method /4/, and 95% confidence limit was also obtained.

III. EXPERIMENTAL RESULTS

1. General Symptoms and Time up to Death following Oral Administration

The general symptoms were observed after the bile acids were administered to groups of mice, each comprising 5 to 8, at varying doses. There was no qualitative difference in the symptoms caused by the acids, the symptoms being basically the same in all groups. Immediately after the injection, some mice began to run restlessly in the cage (perhaps due to local stimulation ?) which subsequently subsided. Demunition or reduction of automatic movement was a typical reaction after the initial hyperactivity. The animals crouched quietly and appeared weak. Since no loss of mobility or reflex inhibition was present, the effects of these acids are obviously different from those of anesthesia.

These symptoms appeared in 10 minutes when the dose was large, but generally began 1 or 2 hours after the injection. A greater proportion

of the animals indicated the symptoms 4 hours later. The animals which survived returned to normal in 6 hours at the earliest or in the following day (20 hours later) in some instances.

In fatal cases, the symptoms continued progressively. The animals fell on the side spontaneously or remained without motion then died. No spasm was observed. The time up to death varied considerably, from 15 minutes to approximately 1 hour in the case of large doses. At intermediate doses, the time to death varied widely from 2 to 24 hours, or 48 hours after injection (Table 1).

TABLE 1. MORTALITY IN ORAL ADMINISTRATION OF BILE ACIDS (MORTALITY BY TIME)

a 胆汁酸	b 用量 mg/kg (cc/10g)	c 例数	d 经过时间 2	4	6	24	43
Cholic acid	408 (1/10M 0.1)	5	0	0	0	0	0
	612 (〃 0.15)	5	0	0	0	0	0
	816 (2/10M 0.1)	8	0	0		2	2
	1224 (〃 0.15)	8	2	2	2	2	2
	1632 (4/10M 0.1)	8	1	3	3	3	3
	2448 (〃 0.15)	5	5				
Lithocholic acid	428 (1/10M 0.1)	5	0	0	0	0	0
	642 (〃 0.15)	5	1	1	3	3	3
	856 (2/10M 0.1)	8	0	1		6	6
	1274 (〃 0.15)	8	2	3	3	4	5
	1712 (4/10M 0.1)	8	3	3	5	5	5
	2568 (〃 0.15)	5	3	3	4	4	4
Deoxycholic acid	392 (1/10M 0.1)	5	0	0	0	0	0
	588 (〃 0.15)	5	1	1	2	2	3
	784 (2/10M 0.1)	8	0	0		2	3
	1176 (〃 0.15)	8	3	4	5	5	5
	1568 (4/10M 0.1)	8	1	2	2	3	4
	2352 (〃 0.15)	5	3	3	4	4	4
Apocholic acid	390 (1/10M 0.1)	5	0	0	0	0	1
	585 (〃 0.15)	5	0	1	1	1	1
	780 (2/10M 0.1)	8	0	0		3	4
	1170 (〃 0.15)	8	1	4	4	4	4
	1560 (4/10M 0.1)	8	0	2	2	4	5
	2340 (〃 0.15)	5	3	4	4	5	
Lithocholic apocholic acid	436 (1/10M 0.1)	5	0	0	0	0	0
	644 (〃 0.15)	5	0	0	0	0	1
	852 (2/10M 0.15)	8	0	0		1	3
	1278 (〃 0.15)	8	1	1	2	3	3
	1704 (4/10M 0.1)	8	2	2	3	3	3
	2556 (〃 0.15)	5	2	2	2	3	4
	3408 (〃 0.2)	5	5				

Keys:

a, bile acid

b, dose

c, No. of subjects

d, time in hours

2. Determination of Lethal Dose

LD_{50} in Table 2 were obtained from the data given in Table 1. The mortality as of 48 hours after the injection were used as the basis of computation. These numerical values are generally within a narrow range, without significant difference between the maximum and the minimum.

3. Blood Pressure and Respiration in Rabbit

In order to provide data for the determination of the cause of death, Na salt of deoxycholic acid (DC), representative of bile acids, was injected to the femoral vein of urethane-anesthetized rabbits while their blood pressure and respiration were recorded on a kymograph, and changes were observed.

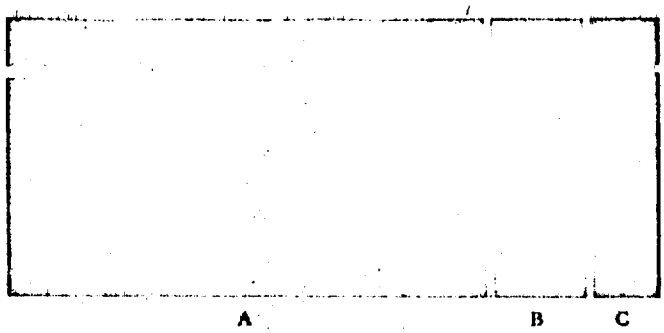

Figure 1. Effects of Deoxycholic Acid on the Respiration and Blood pressure of Rabbit.

A: DC, 5 mg/kg i.v. B: 30 minutes later
C: 45 minutes later

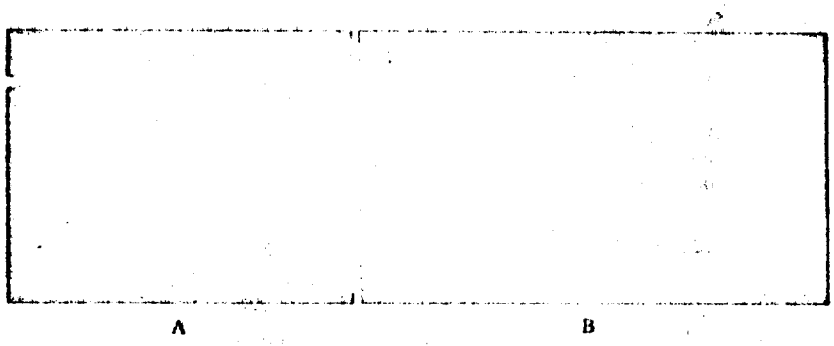

Figure 2. Respiration and Blood Pressure before and after Bilateral Vagotomy

A: DC, 3 mg/kg i.v. B: DC, 3 mg/kg i.v. after bilateral vagotomy

TABLE 2. LD_{50} OF BILE ACIDS BY ORAL ADMINISTRATION

a. 胆汁酸	LD_{50} (g/kg) (Mol)	b. 95% 信賴限界 (g/kg)
Cholic acid	1.52 (0.00372)	1.13~2.04
Lithocholic acid	0.84 (0.00196)	0.59~1.19
Deoxycholic acid	1.06 (0.00270)	0.74~1.53
Apocholic acid	1.07 (0.00374)	0.79~1.44
Lithocholic apocholic acid	1.31 (0.00308)	0.95~1.81

Keys: a, bile acid; b, 95% confidence limit

As shown in Figure 1, the blood pressure indicated sudden depression (approximately by 50 mmHg) in the reaction to DC at 3 - 5 mg/kg, accompanied by brachycardia and transitory suppression of respiration. The blood pressure recovered rapidly, returning to the original level in several minutes, but it subsequently rose to a level higher than original and remained at that level (+10 - 30 mmHg) for a long period of time. Since the sudden depression of blood pressure was also noted in the animals subjected to bilateral vagotomy, as shown in Figure 2, it is not likely to be of reflex. Intravenous injection of DC at 10 - 15 mg/kg caused considerable depression in blood pressure and subsequent death of the animals.

From these experimental results, the death due to the intravenous

injection of DC is attributed to the termination of cardiac function.

IV. CONCLUSION

The LD_{50} of bile acids administered to mice by the oral route was approximately 1 g/kg in all cases. The time up to death varied over a wide range from 1 hour to 48 hours. The death is attributed to cardiac intoxication.

Notes

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