THE BILE ACIDS

Chemistry, Physiology, and Metabolism

VOLUME 2: PHYSIOLOGY AND METABOLISM

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VOLUME 2: PHYSIOLOGY AND METABOLISM

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Preface to Volume 2

The first volume in this series was primarily concerned with the physiochemical aspects of bile acids and their salts. In keeping with the original organizational format of this work, Volume 2 focuses our attention on the physiological and metabolic aspects of these substances.

The mechanisms of bile acid biogenesis and the transport of bile salts through the enterohepatic circulation have been clarified by elegant studies in recent years. The metabolism of bile salts in health and disease and its response to hormonal and pharmacological stimulation are also areas of increasing interest. This volume contains an extensive discussion of these aspects of bile acid and bile salt metabolism.

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Chapter 1

MECHANISMS OF BILE ACID BIOSYNTHESIS*

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In this chapter, an attempt is made to summarize present knowledge concerning the mechanisms of the conversion of cholesterol into the primary bile acids. In addition, a section on the formation of bile salts in "primitive" animals is included. Emphasis in discussion and documentation of references will be placed on more recent developments. The early work will be briefly reviewed in the introductory section.

I. INTRODUCTION

The primary bile acids are defined as those formed from cholesterol in the liver. Secondary bile acids are those formed from the primary bile acids through the action of intestinal microorganisms during the enterohepatic circulation of bile acids. The secondary bile acids may be subjected to further structural modifications by liver enzymes. The main primary bile acids in most mammalian species are cholic acid and chenodeoxycholic acid.† Other

*See *Notes Added in Proof*, page 305.

†The following systematic names are given to steroids and bile acids referred to by trivial names: cholestanol, 5α-cholestan-3β-ol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholanoic acid; allodeoxycholic acid, 3α,12α-dihydroxy-5α-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

primary bile acids are α- and β-muricholic acids, present in the mouse and rat and perhaps also in other species, and hyocholic acid, present in the pig. Evidence has been presented that 3α-hydroxy-7-oxo-5β-cholanoic acid, present in the guinea pig and several other species, and lithocholic acid, present in the rat and several other species, can be primary bile acids. Small amounts of other bile acids including 5α-cholanoic acids occur in bile of various species, and many of these bile acids have been shown to be primary bile acids. The origin of bile acids from cholesterol was established in 1943 by Bloch *et al.* (1), who showed that deuterium-labeled cholesterol was transformed into cholic acid in the dog. The conversion of cholesterol into bile acids has since been shown in a number of species, and, in lack of conclusive evidence to the contrary, cholesterol may be considered an obligatory intermediate in the formation of bile acids under normal conditions.

The changes of the cholesterol molecule that occur in its conversion into bile acids include epimerization of the 3β-hydroxyl group, reduction of the Δ^5 double bond, introduction of hydroxyl groups in positions C-7 (chenodeoxycholic acid), C-7 and C-12 (cholic acid), or C-6 and C-7 (α- and β-muricholic acids, hyocholic acid), and transformation of the C_{27} side chain into a C_{24}-carboxylic acid.

Investigations on the quantitative aspects of the conversion of cholesterol into bile acids were conducted in the early 1950s by Bergström and collaborators (2,3) and by Chaikoff and collaborators (4,5), who showed that bile acid formation is a major pathway for the metabolism of cholesterol. The early work on the sequence of reactions in the conversion of cholesterol into cholic acid was carried out mainly by Bergström, Lindstedt, and collaborators (3,6–8). The experimental approach was to synthesize isotopically labeled, hypothetical intermediates, administer these compounds to rats with a biliary fistula, and analyze the labeled products excreted in bile. The results of these studies and those of Chaikoff and collaborators (9,10) indicated that in cholic acid formation the changes in the steroid nucleus preceded the oxidation of the side chain and that introduction of the 7α-hydroxyl group probably was the first step. With respect to intermediates in the formation of cholic acid other than 5-cholestene-3β,7α-diol, the work of Bergström, Lindstedt, and collaborators suggested that 5β-cholestane-3α,7α,12α-triol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid might be normal intermediates.

The conversion of cholesterol into chenodeoxycholic acid was not studied in the same detail as the conversion of cholesterol into cholic acid. It was found that many different C_{27}-steroids were converted into chenodeoxycholic acid in rats with a biliary fistula. It was not possible to deduce a pathway for chenodeoxycholic acid formation that could include all these C_{27}-steroids as intermediates.

The development of present knowledge of the mechanisms of bile acid formation has been possible through a combination of the use of cell-free preparations of liver with the general experimental approach of Bergström, Lindstedt, and collaborators. Metabolites formed in the presence of different subcellular fractions of liver homogenates have been isolated and identified. The compounds have been tested as precursors of bile acids in rats with a biliary fistula. The chemical synthesis of a number of unlabeled as well as isotopically labeled steroids has played an important role in these studies.

II. FORMATION OF CHOLIC ACID

A. Changes in Steroid Nucleus

Early work *in vitro* on the sequence of reactions in the conversion of cholesterol into bile acids was carried out with mitochondrial preparations from rat and mouse liver (11). These preparations were found to catalyze predominantly reactions involving the oxidation of the side chain of C_{27}-steroids. No evidence for the formation of 12α-hydroxylated metabolites was obtained. In 1963, Mendelsohn and Staple (12) reported the conversion of cholesterol into 5β-cholestane-3α,7α-12α-triol in the presence of a 20,000*g* supernatant fluid of rat liver homogenate. This finding provided the first experimental evidence for the long surmised role of 5β-cholestane-3α,7α,12α-triol as an intermediate in the conversion of cholesterol into cholic acid. Subsequent work with this enzyme preparation and subfractions of it has led to the elucidation of the sequences of reactions in the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol (Fig. 1).

1. Conversion of Cholesterol into 5-Cholestene-3β,7α-diol

The first step in the sequence of reactions leading to the formation of 5β-cholestane-3α, 7α, 12α-triol (Fig. 1) is a 7α-hydroxylation of cholesterol yielding 5-cholestene-3β,7α-diol (13–15). The reaction is catalyzed by the microsomal fraction of rat liver homogenate fortified with NADPH (16–18) and involves direct displacement of the 7α-hydrogen by the entering hydroxyl group (19). Boyd *et al.* (20) have reported in preliminary form the solubilization of the 7α-hydroxylase. The 7α-hydroxylation of cholesterol is inhibited by carbon monoxide (20,21) and by antibody against NADPH–cytochrome *c* reductase (21), indicating the participation of NADPH–cytochrome *c* reductase and cytochrome P-450 in the reaction. In contrast to many microsomal hydroxylations, which are stimulated manyfold, phenobarbital administration has been reported to result in no increase or only a small increase of 7α-hydroxylase activity (18,20–22). A fourfold increase has,

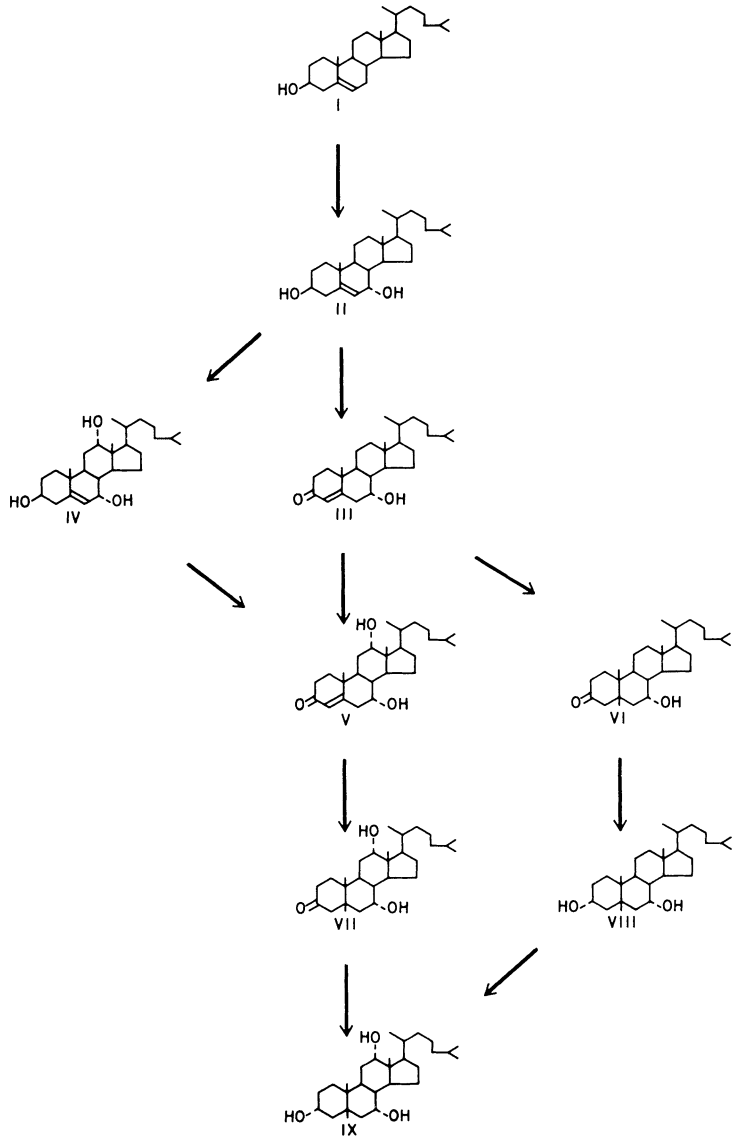

Fig. 1. Conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol. I, Cholesterol; II, 5-cholestene-3β,7α-diol; III, 7α-hydroxy-4-cholesten-3-one; IV, 5-cholestene-3β,7α,12α-triol; V, 7α,12α-dihydroxy-4-cholesten-3-one; VI, 7α-hydroxy-5β-cholestan-3-one; VII, 7α,12α-dihydroxy-5β-cholestan-3-one; VIII, 5β-cholestane-3α,7α-diol; IX, 5β-cholestane-3α7α,12α-triol.

however, been observed by Shefer *et al.* (17). Biliary drainage and cholestyramine feeding lead to a severalfold increase in 7α-hydroxylase activity with no change or reduction in total cytochrome P-450 concentration (17,20,21, 23–25). These observations indicate that either cytochrome P-450 is not rate limiting or a cytochrome P-450 different from that (those) involved in most microsomal hydroxylations participates in the reaction.

The presence of esters of 5-cholestene-3β,7α-diol in rat liver has been shown by Boyd (26), who suggested that 7α-hydroxylation might occur with cholesterol esters rather than cholesterol. It appears, however, that esters of 5-cholestene-3β,7α-diol are formed predominantly by esterification of 5-cholestene-3β,7α-diol. Hutton and Boyd (27) have found that the 100,000*g* supernatant fluid of rat liver homogenate catalyzes the esterification of 5-cholestene-3β,7α-diol, and Katayama and Yamasaki (28) have reported that *in vitro* there is no significant 7α-hydroxylation of cholesterol esters under conditions of efficient 7α-hydroxylation of cholesterol.

The role of the 7α-hydroxylation of cholesterol as a rate-limiting step in the biosynthesis of bile acids will be discussed in Section VI.

2. Conversion of 5-Cholestene-3β,7α-diol into 7α-Hydroxy-4-cholesten-3-one

In its conversion into 5β-cholestane-3α,7α,12α-triol, 5-cholestene-3β,7α-diol is transformed either into 7α-hydroxy-4-cholesten-3-one (15, 29) or 5-cholestene-3β,7α,12α-triol (30). The conversion of 5-cholestene-3β,7α-diol into 7α-hydroxy-4-cholesten-3-one is catalyzed by the microsomal fraction fortified with NAD or NADP (27,31), NAD being several times more active than NADP (31). The enzymatic formation of a Δ^4-3-oxosteroid from a Δ^5-3β-hydroxysteroid is generally assumed to require the participation of two enzymes, a Δ^5-3β-hydroxysteroid dehydrogenase and a Δ^5-3-oxosteroid isomerase. The possibility that the reaction is catalyzed by a single enzyme has not been ruled out (32). The mechanism of the enzymatic conversion of 5-cholestene-3β,7α-diol into 7α-hydroxy-4-cholesten-3-one has been studied by Björkhem (32) with the aid of 5-cholestene-3β,7α-diol labeled with deuterium or tritium in specific positions. The results obtained by Björkhem confirm and extend previous observations by Samuelsson (33) and Gréen and Samuelsson (34) on the conversion of 6-^{3}H- and 4β-^{3}H-cholesterol into bile acids *in vivo*. The rate-limiting step in the conversion of 5-cholestene-3β,7α-diol into 7α-hydroxy-4-cholesten-3-one was found to be oxidation of the 3β-hydroxyl group. This step was coupled with or followed by loss of the 4β-hydrogen, of which a small amount was transferred to the 6β-position. The loss of the 4β-hydrogen did not involve isotope discrimination, whereas significant isotope effects were found for the intramolecular transfer. About 3% of a 4β-tritium and about 12% of a 4β-deuterium were transferred to the 6β-position. The incorporation of isotope from the medium was small

whether the reaction was carried out in deuterated or tritiated water. Since the figures for the intramolecular transfer of a 4β-deuterium and a 4β-tritium make it unlikely that the reaction involves complete intramolecular transfer, it is probable that the low extent of incorporation of isotope from labeled medium can be ascribed to insufficient equilibration between a hydrogen donor on the enzyme and hydrogen in the medium. It may, however, be concluded that the mechanism of isomerization of the Δ^5 double bond in the conversion of 5-cholestene-3β,7α-diol into 7α-hydroxy-4-cholesten-3-one differs from those found for the isomerization of a Δ^5 double bound in steroid hormones catalyzed by adrenal and liver enzymes [no transfer of 4β-hydrogen to the 6β-position (35)] or by an enzyme from *Pseudomonas testosteroni* [complete transfer of the 4β-hydrogen to the 6β-position (36)].

3. 12α-Hydroxylation

In the apparently major pathway for the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol, the step following the formation of 7α-hydroxy-4-cholesten-3-one is a 12α-hydroxylation yielding 7α,12α-dihydroxy-4-cholesten-3-one (Fig. 1). The reaction is catalyzed by the microsomal fraction fortified with NADPH (15,37). The conversion of 5-cholestene-3β,7α-diol into 5-cholestene-3β,7α,12α-triol, which is a reaction in another pathway for the formation of 5β-cholestane-3α,7α,12α-triol, is also catalyzed by the microsomal fraction fortified with NADPH (30,37), as is the 12α-hydroxylation of 5β-cholestane-3α,7α-diol and 7α-hydroxy-5β-cholestan-3-one (37). The rates of 12α-hydroxylation of these C_{27}-steroids differ considerably: the rate with 5-cholestene-3β,7α-diol is about one-tenth and with 5β-cholestane-3α,7α-diol about half of that with 7α-hydroxy-4-cholesten-3-one (37). Einarsson (37) and Suzuki *et al.* (38) have studied some properties of the 12α-hydroxylase system with special reference to the possible participation of electron carriers such as NADPH–cytochrome *c* reductase and cytochrome P-450. The 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was inhibited by cytochrome *c*, indicating that NADPH–cytochrome *c* reductase might be involved. However, no direct evidence for the participation of flavins was obtained. If NADPH–cytochrome *c* reductase participates, it is not rate-limiting, since the activity of this enzyme increases upon treatment with thyroxine whereas the activity of the 12α-hydroxylase decreases (39). Suzuki *et al.* (38) found no inhibition of 12α-hydroxylation by carbon monoxide, whereas Einarsson (37) obtained some inhibition. The 12α-hydroxylase activity was unaffected by methylcholanthrene treatment (40) and lowered by phenobarbital treatment (37,38). These observations indicate that the cytochrome(s) P-450 induced by methylcholanthrene and

phenobarbital does not participate in the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one, at least not as a rate-limiting component.

4. Conversion of 7α,12α-Dihydroxy-4-cholesten-3-one into 5β-Cholestane-3α,7α,12α-triol

7α,12α-Dihydroxy-4-cholesten-3-one, formed by 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one or from 5-cholestene-3β,7α,12α-triol in a reaction catalyzed by the microsomal fraction fortified with NAD (30), is converted into 5β-cholestane-3α,7α,12α-triol by means of the intermediary formation of 7α,12α-dihydroxy-5β-cholestan-3-one (41). The reactions are catalyzed by two soluble enzymes, a Δ^4-3-oxosteroid 5β-reductase and a 3α-hydroxysteroid dehydrogenase. These enzymes have been partially purified from the 100,000*g* supernatant fluid of rat liver homogenate by Berséus (42), After tenfold purification, the Δ^4-3-oxosteroid 5β-reductase preparation catalyzed the reduction of the Δ^4 double bond in a number of Δ^4-3-oxosteroids of the C_{19}-, C_{21}-, C_{24}-, as well as C_{27}-series. NADPH was the required cofactor. Evidence was obtained to indicate the presence of different Δ^4-3-oxosteroid 5β-reductases in the enzyme preparation. A previous study by Tomkins (43) of the enzymatic reduction of the Δ^4 double bond in Δ^4-3-oxosteroids of the C_{19}- and C_{21}-series also indicates that in rat liver there are a number of different Δ^4-3-oxosteroid 5β-reductases with a high degree of substrate specificity. The mechanism of the double bond reduction and the stereochemistry of the hydrogen transfer from NADPH have been studied by Berséus and Björkhem (44) and Björkhem (45) and have been shown to involve transfer of a hydride ion from the A-side of NADPH to the 5β-position of the steroid and the addition of a proton from the medium to the 4α-position. The double bond is thus reduced by means of a *trans* addition of hydrogens.

The 3α-hydroxysteroid dehydrogenase has been purified about 200-fold (42,46). This preparation catalyzes the reduction of the 3-oxo group in 3-oxo-5α- and 3-oxo-5β-steroids of the C_{19}-, C_{21}-, C_{24}-, and C_{27}-series. The rate of reaction is faster with NADPH than with NADH, and 3-oxo-5β-steroids are reduced at a faster rate than the corresponding 3-oxo-5α-steroids. Tomkins (47), Koide (48,49), and Doman and Koide (50) have studied 3α-hydroxysteroid dehydrogenase activity of rat liver with C_{19}- and C_{21}-steroids as substrates and have obtained evidence that at the most only a few 3α-hydroxysteroid dehydrogenases with broad substrate specificity are present. The results of Berséus are in agreement with the work of these authors and indicate that the same 3α-hydroxysteroid dehydrogenase(s) is involved in the biosynthesis of bile acids and in the metabolism of steroid hormones in the liver. It seems probable that the specificity in the conversion of a Δ^4-3-oxosteroid into a 3α-hydroxy-5β-steroid is in the reduction of the double

bond. This reaction is probably the rate-limiting step (41,43,51) and is catalyzed by enzymes that appear to have a high degree of substrate specificity.

The 3α-hydroxysteroid dehydrogenase preparation described by Berséus (42) has been found to catalyze the oxidation of 4-cholestene-3α,7α-diol into 7α-hydroxy-4-cholesten-3-one (52). It is interesting that the reverse reaction proceeds at a rate which is at least ten times slower. Similarly, the rate of oxidation of 5β-cholestane-3α,7α,12α-triol into 7α,12α-dihydroxy-5β-cholestan-3-one, catalyzed by 3α-hydroxysteroid dehydrogenase, is about ten times slower than the rate of reduction (42).

The reduction of a 3-oxosteroid, catalyzed by 3α-hydroxysteroid dehydrogenase from rat liver, has been shown to involve transfer of a hydride ion from the A-side of NADPH to the 3β-position of the steroid (44,52). The oxidation of 4-cholestene-3α,7α-diol into 7α-hydroxy-4-cholesten-3-one, catalyzed by the same enzyme preparation, has also been shown to involve the A-side of NADPH (52). It remains to be established whether or not Δ^4-3α-hydroxysteroid dehydrogenase activity is due to enzyme(s) different from 3α-hydroxysteroid dehydrogenase(s).

5. Other Pathways for the Formation of 5β-Cholestane-3α,7α,12α-triol

In the previous sections, the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol by means of the intermediary formation of 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-5β-cholestan-3-one has been discussed. Mention was made also of another pathway for the formation of 5β-cholestane-3α,7α,-12α-triol in which 5-cholestene-3β,7α-diol is 12α-hydroxylated prior to oxidation of the 3β-hydroxyl group. The importance of a pathway involving 5-cholestene-3β,7α,12α-triol as an intermediate has not been conclusively established. It appears, however, that this pathway is less important, quantitatively, than the one involving 7α-hydroxy-4-cholesten-3-one as an intermediate. The main finding to support this contention is the difference in the rate of 12α-hydroxylation by the microsomal fraction fortified with NADPH between 7α-hydroxy-4-cholesten-3-one and 5-cholestene-3β,7α-diol. As mentioned in section II A3, the rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one is about ten times faster than that of 5-cholestene-3β,7α-diol (37). On the other hand, upon incubation of cholesterol with the 20,000*g* supernatant fluid, about equal amounts of 5-cholestene-3β,7α,12α-triol and 7α,12α-dihydroxy-4-cholesten-3-one are formed (30). Since 5-cholestene-3β,7α,12α-triol is converted efficiently into 7α,12α-dihydroxy-4-cholesten-3-one (30), part or all of the 7α,12α-dihydroxy-4-cholesten-3-one may have been formed by means of the intermediary formation of 5-cholestene-3β,7α,12α-triol. An interpretation of these contrasting findings could be that the 12α-hydroxylation of 5-cholestene-3β,7α-diol requires not only the microsomal

fraction fortified with NADPH but also a factor(s) present in the 100,000*g* supernatant fluid. If this were the case, the differences in rate of 12α-hydroxylation between 7α-hydroxy-4-cholesten-3-one and 5-cholestene-3β,7α-diol in the presence of microsomal fraction fortified with NADPH do not permit conclusions concerning the role of a pathway to 5β-cholestane-3α,7α,12α-triol involving 5-cholestene-3β,7α,12α-triol as an intermediate. Another explanation of the data would be that 5-cholestene-3β,7α,12α-triol is formed from cholesterol through an initial 12α-hydroxylation followed by a 7α-hydroxylation. However, this possibility appears excluded. From early work on the metabolism of 5-cholestene-3β,12α-diol in rats and rabbits with a biliary fistula (53,54) as well as from a recent study *in vitro* by Einarsson (55), it can be concluded that 5-cholestene-3β,12α-diol is not an important intermediate in the conversion of cholesterol into cholic acid.

Mendelsohn *et al.* (29) have proposed a pathway for the conversion of 7α-hydroxy-4-cholesten-3-one into 5β-cholestane-3α,7α,12α-triol that includes 4-cholestene-3α,7α-diol and 5β-cholestane-3α,7α-diol as intermediates. These authors (56) have shown the formation of 5β-cholestane-3α,7α-diol from cholesterol in the presence of the 20,000*g* supernatant fluid of rat liver homogenate, and the conversion of 5β-cholestane-3α,7α-diol into 5β-cholestane-3α,7α,12α-triol by the microsomal fraction fortified with NADPH has been reported by Einarsson (37). It is, however, unlikely that 4-cholestene-3α,7α-diol is an intermediate in the conversion of 7α-hydroxy-4-cholesten-3-one into 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol. 7α-Hydroxy-4-cholesten-3-one can be reduced to 4-cholestene-3α,7α-diol by the 100,000*g* supernatant fluid of rat liver homogenate (57,58), but the equilibrium of the reaction is heavily in favor of formation of 7α-hydroxy-4-cholesten-3-one (58). Experiments *in vivo* with 3β-^{3}H,4-^{14}C-4-cholestene-3α,7α-diol and 3β-^{3}H,4-^{14}C-4-cholestene-3α,7α,12α-triol have shown that these compounds are converted into cholic acid by means of the intermediary formation of Δ^4-3-oxosteroids (59,60). The conversion of 7α-hydroxy-4-cholesten-3-one into 5β-cholestane-3α,7α-diol appears to proceed predominantly by means of the intermediary formation of 7α-hydroxy-5β-cholestan-3-one (61) in reactions catalyzed by the soluble Δ^4-3-oxosteroid 5β-reductase and 3α-hydroxysteroid dehydrogenase discussed in Section IIA4. The importance of a pathway from cholesterol to 5β-cholestane-3α,7α,12α-triol with 5β-cholestane-3α,7α-diol as an intermediate is not entirely clear. The rate of 12α-hydroxylation of 5β-cholestane-3α,7α-diol by the microsomal fraction fortified with NADPH is as much as half of that of 7α-hydroxy-4-cholesten-3-one. On the other hand, 5-cholestene-3β,7α,12α-triol and 7α,12α-dihydroxy-4-cholesten-3-one are major metabolites of cholesterol and 5β-cholestane-3α,7α,12α-triol is a minor metabolite of cholesterol in incubations with the 20,000*g* supernatant fluid (15,30), indicating that in a system con-

taining 12α-hydroxylase as well as the soluble enzymes involved in the reduction of the Δ^4 double bond, 12α-hydroxylation occurs more readily with the unsaturated steroids. However, it should be borne in mind that there is evidence that the microsomal fraction inhibits the soluble Δ^4-3-oxosteroid 5β-reductase. The reason for this inhibition is not known, but the same inhibition has been observed in homogenates of guinea pig liver (61) and human liver (62).

6. Pathways Not Leading to Formation of 5β-Cholestane-3α,7α,12α-triol

Two recent investigations on the formation of cholic acid in rats with a biliary fistula indicate the presence of additional pathways for the formation of cholic acid involving other sequences of changes in the steroid nucleus than those discussed above. Nair *et al.* (63) have found that in rats with a biliary fistula labeled 5,7-cholestadien-3β-ol (7-dehydrocholesterol) is converted into cholic acid apparently without the intermediary formation of cholesterol, since hepatic as well as biliary cholesterol was unlabeled. No information is available concerning intermediates in this pathway for cholic acid formation, and 5β-cholestane-3α,7α,12α-triol may or may not be an intermediate. It can, however, be concluded that 12α-hydroxylation must be an early step, since 5,7-cholestadien-3β-ol did not give rise to chenodeoxycholic acid. The contribution to cholic acid formation of a pathway from 5,7-cholestadien-3β-ol is unknown. It should be pointed out that Nair *et al.* (63) obtained evidence to indicate that in intact rats the formation of cholic acid from 5,7-cholestadien-3β-ol occurs with the intermediary formation of cholesterol. Wachtel *et al.* (64) have shown that 5-cholestene-3β,26-diol is converted to a small extent into cholic acid in rats with a biliary fistula. The predominant products are chenodeoxycholic acid and α- and β-muricholic acid (64,65). The conversion of cholesterol into cholic acid by means of a pathway which includes 5-cholestene-3β,26-diol is apparently not of importance, quantitatively, in the rat but may be so in other species, e.g., hamster (64). With respect to intermediates in this pathway, it may be mentioned that mitochondrial preparations from mouse liver have been shown to catalyze the conversion of cholesterol into 5-cholestene-3β,26-diol and the 7α-hydroxylation of 5-cholestene-3β,26-diol (65,66). The stage at which 12α-hydroxylation occurs is not known.

As is apparent from the discussion in the foregoing sections, available data indicate strongly that 5β-cholestane-3α,7α,12α-triol is an obligatory intermediate in the main pathways for the conversion of cholesterol into cholic acid in the rat and is thus the substrate for the 26-hydroxylase, the enzyme system catalyzing the initial reaction in the oxidation of the side chain (Section IIB). It may be mentioned in this connection that the validity of these data is strengthened by the finding that endogenous, prelabeled

cholesterol is metabolized *in vitro* in the same way as is labeled cholesterol added to incubation mixtures as an emulsion or dissolved in acetone (67). However, it is conceivable that the oxidation of the side chain may be initiated at a stage prior to the formation of 5β-cholestane-3α,7α,12α-triol. If this be the case, 7α,12α-dihydroxy-4-cholesten-3-one appears to be one of the few or perhaps the only alternative to 5β-cholestane-3α,7α,12α-triol as substrate for the 26-hydroxylase in cholic acid formation (11,68). It appears desirable to conduct additional experiments on sequences of reactions with an *in vitro* system that catalyzes the complete conversion of cholesterol into cholic acid. Such a system is available, as shown by the work of Mendelsohn *et al.* (69) and Mendelsohn and Mendelsohn (70). These authors have found that under suitable conditions the 20,000*g* supernatant fluid catalyzes the conversion of cholesterol into cholic acid and chenodeoxycholic acid.

7. Sequence of Reactions in Species Other Than Rat

At present, the sequence of changes in the steroid nucleus in cholic acid formation has been examined only in one species other than the rat, *viz.*, man (62). The metabolism of cholesterol and several other C_{27}-steroids in the presence of different subcellular fractions of homogenates of human liver was found to be the same as found previously for the rat, indicating the presence of the same pathways in man as in the rat.

B. Oxidation of Side Chain

The oxidation of the side chain of 5β-cholestane-3α,7α,12α-triol to yield cholic acid or rather cholyl coenzyme A entails an ω-oxidation followed by a β-oxidation (Fig. 2). Early investigations (71,72) showed that the mitochondrial fraction of rat and mouse liver homogenate catalyzed the conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,26-tetrol and, when supplemented with the 100,000*g* supernatant fluid, the further transformation of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (Fig. 2). Suld *et al.* (72) showed that the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid (cholyl coenzyme A), catalyzed by the mitochondrial fraction fortified with the 100,000*g* supernatant fluid, occurs with the release of propionic acid (propionyl coenzyme A).

1. 26-Hydroxylation

The 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol may be assumed to be stereospecific. Berséus (73) and Mitropoulos and Myant (74) have shown that the 26-hydroxylation of cholesterol, catalyzed by mitochondrial preparations from rat liver, is stereospecific. Mendelsohn and Mendelsohn

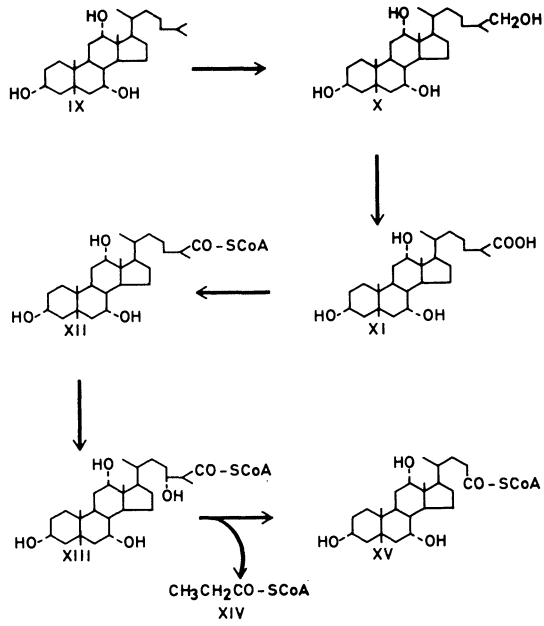

Fig. 2. Conversion of 5β-cholestane-3α,7α,12α-triol into cholyl coenzyme A. IX, 5β-Cholestane-3α,7α,12α-triol; X, 5β-cholestane-3α,7α,12α,26-tetrol; XI, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; XII, 3α,7α,12α-trihydroxy-5β-cholestanoyl coenzyme A; XIII, 3α,7α,12α,24α-tetrahydroxy-5β-cholestanoyl coenzyme A; XIV, propionyl coenzyme A; XV, cholyl coenzyme A.

(75) have found that cholesterol is converted preferentially into 25D-3α,7α,-12α-trihydroxy-5β-cholestanoic acid by the 20,000g supernatant fluid. When 3α,7α,12α-trihydroxy-5β-cholestanoic acid occurs in bile, it is usually present as only one of the two 25-isomers (75–77). Okuda and Hoshita (78) have studied the oxidation of 5β-cholestane-3α,7α,12α-triol by different subcellular fractions of rat liver homogenate. These authors found that the 26-hydroxylase activity was present only in mitochondria and that the activity was stimulated by the addition of boiled liver extract or NADPH. In contrast, Cronholm and Johansson (79) have reported recently that the microsomal fraction of rat liver homogenate fortified with NADPH exhibits considerably greater 26-hydroxylase activity than the mitochondrial fraction. The microsomal 26-hydroxylation was inhibited by carbon monoxide, indicating the participation of a cytochrome P-450. Further studies are required to assess the significance of microsomal versus mitochondrial 26-hydroxylation in bile

acid formation. It should be mentioned that in addition to 26-hydroxylation the microsomal fraction fortified with NADPH catalyzed 23ξ-, 24α-, 24β-, and 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol. These hydroxylations are not likely to play a role in the conversion of 5β-cholestane-3α,7α,12α-triol into cholic acid (79), and their biological significance, if any, remains to be established.

2. Conversion of 5β-Cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-Trihydroxy-5β-cholestanoic Acid

The conversion of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid occurs by means of the intermediary formation of the aldehyde, 3α,7α,12α-trihydroxy-5β-cholestan-26-al (Fig. 2), and is catalyzed by the microsomal or soluble fraction of rat liver homogenate fortified with NAD, the soluble fraction showing the highest activity (80–82). Staple (83), Okuda and Takigawa (84), and Okuda *et al.* (85) have described the partial purification of the soluble alcohol and aldehyde dehydrogenases, and Okuda and Takigawa (84) have suggested that the enzymes are identical with ethanol dehydrogenase and acetaldehyde dehydrogenase, respectively. Waller *et al.* (86) have shown that 5β-cholestane-3α,7α,12α,26-tetrol is a substrate for horse liver alcohol dehydrogenase. The product was not identified but was probably the 26-aldehyde. In this connection, it may be mentioned that Dean and Whitehouse (87) have found that there is a mitochondrial dehydrogenase system catalyzing the conversion of 5-cholestene-3β,26-diol into 3β-hydroxy-5-cholestenoic acid. Ethanol dehydrogenase is inactive in this reaction (86,87).

3. Conversion of 3α,7α,12α-Trihydroxy-5β-cholestanoic Acid into Cholic Acid

The conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid (cholyl coenzyme A) occurs by means of a β-oxidation with release of propionic acid (propionyl coenzyme A) (Fig. 2). Although no definite experimental evidence is available, it may be assumed that the reactions involve the coenzyme-A esters of the steroid acids. Only one of the intermediates in the β-oxidation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid has been isolated. Masui and Staple (88,89) have shown that the mitochondrial fraction of rat liver homogenate supplemented with the 100,000*g* supernatant fluid catalyzes the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into one of the two C-24 epimers of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. It is probable that the C-24 isomer formed is the 24α-hydroxy compound (89,90). The transformation of 3α,7α,12α,24α-tetrahydroxy-5β-cholestanoic acid into cholic acid is catalyzed by the 100,000*g* supernatant fluid of rat liver homogenate (89). No studies have yet been carried out to compare the enzymes catalyzing β-oxidation of 3α,7α,12α-trihydroxy-5β-

cholestanoic acid with those catalyzing β-oxidation of long-chain fatty acids. An investigation by Dean and Whitehouse (91) suggests that the oxidation of 3β-hydroxy-5-cholestenoic acid into propionic acid is catalyzed by enzymes different from those catalyzing β-oxidation of fatty acids and present only in liver tissue.

4. Formation of Acetone

Several years ago, Whitehouse *et al.* (92,93) reported that under certain conditions mitochondrial preparations from rat liver catalyzed the formation of labeled acetone from 26-^{14}C-cholesterol. It was shown that the acetone resulted from cleavage of the C-24–C-25 bond. In analogy with the formation of isocaproic aldehyde and pregnenolone from cholesterol in endocrine tissues, it appears probable that the immediate precursor of acetone is a 24,25-dihydroxy compound. The natural occurrence or formation of a 24,25-dihydroxy-C_{27}-steroid has not been reported. The conversion of cholesterol into 5-cholestene-3β,25-diol has been observed with mitochondrial preparations from mouse liver (66) as well as with the 20,000*g* supernatant fluid of rat liver homogenate (70). It should be mentioned that part of the 5-cholestene-3β,25-diol may have been formed by autoxidation of cholesterol (70). The microsomal fraction of rat liver homogenate has been found to catalyze hydroxylation of 5β-cholestane-3α,7α,12α-triol at C-24 as well as C-25 (79). These cholestanetetrols, i.e., 5β-cholestane-3α,7α,12α,24α-tetrol, 5β-cholestane-3α,7α,12α,24β-tetrol, and 5β-cholestane-3α,7α,12α,25-tetrol, are converted into cholic acid in rats with a biliary fistula much less efficiently than either 5β-cholestane-3α,7α,12α-triol or 5β-cholestane-3α,7α,12α,26-tetrol (79,94,95). These findings as well as those discussed in the previous sections indicate that oxidation of the side chain by means of acetone formation is of little importance, quantitatively. The same opinion has been recently expressed by Staple (96).

III. FORMATION OF CHENODEOXYCHOLIC ACID

The structural changes involved in the conversion of cholesterol into chenodeoxycholic acid are the same as those in the formation of cholic acid with the exception that no 12α-hydroxyl group is introduced. It has been shown that the mechanisms of conversion of the Δ^5-3β-hydroxy configuration of cholesterol into the 3α,7α-dihydroxy-5β configuration of chenodeoxycholic acid are the same as those in the formation of cholic acid. Similarly, the mechanisms of oxidation of the side chain are the same for chenodeoxycholic acid and cholic acid. Whereas it is now possible to formulate a few probable sequences for these events in cholic acid formation, available information

concerning the biosynthesis of chenodeoxycholic acid is compatible with a number of different pathways, differing only with respect to the stage of nuclear transformations at which oxidation of the side chain is initiated.

In the early studies of the metabolism of cholesterol and other C_{27}-steroids in the presence of mitochondrial preparations from rat and mouse liver, several metabolites were isolated that could be intermediates in the biosynthesis of chenodeoxycholic acid (11). Cholesterol, 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol were found to be converted into the corresponding 26-hydroxy derivatives by mitochondrial preparations. In addition, these preparations were shown to catalyze the 7α-hydroxylation of 5-cholestene-3β,26-diol and the oxidation of 5-cholestene-3β,7α-diol into 7α-hydroxy-4-cholesten-3-one. All the 26-hydroxy compounds were found to be transformed predominantly into chenodeoxy-

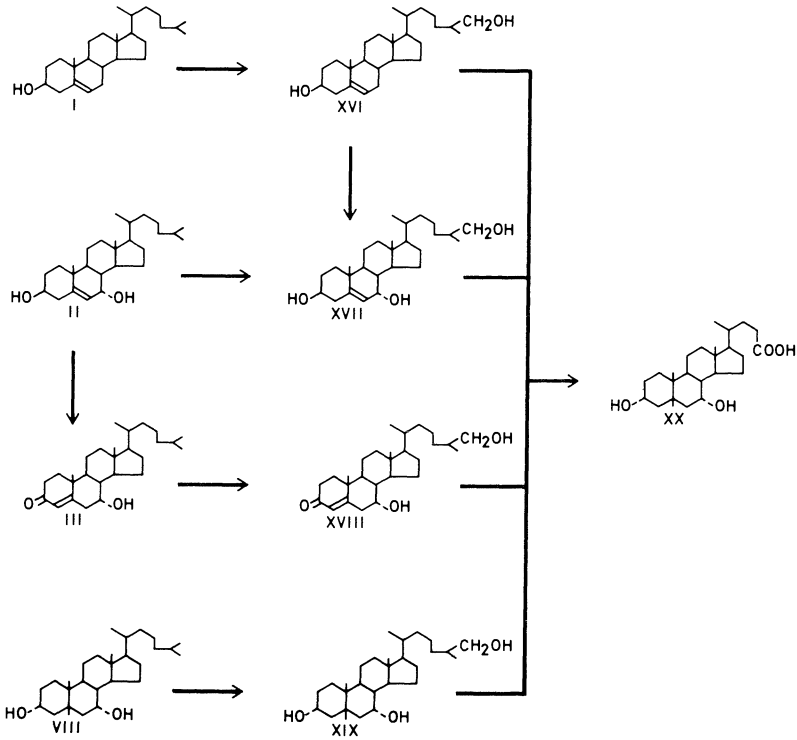

Fig. 3. Formation of chenodeoxycholic acid: some reactions catalyzed by mitochondrial preparations. I, Cholesterol; II, 5-cholestene-3β,7α-diol; III,7α-hydroxy-4-cholesten-3-one; VIII, 5β-cholestane-3α,7α-diol; XVI, 5-cholestene-3β,26-diol; XVII, 5-cholestene-3β,7α,26-triol; XVIII, 7α,26-dihydroxy-4-cholesten-3-one; XIX, 5β-cholestane-3α,7α,26-triol; XX, chenodeoxycholic acid.

cholic acid and its metabolites, α- and β-muricholic acid, in rats with a biliary fistula (11,64). The results of the studies with mitochondrial preparations allow formulation of several pathways for the conversion of cholesterol into chenodeoxycholic acid (Fig. 3). However, the multitude of pathways, differing only with respect to the stage of 26-hydroxylation, could reflect a low degree of specificity of the mitochondrial 26-hydroxylase system. In fact, it has been shown that mitochondrial preparations catalyze ω-oxidation of a number of different mono-oxygenated C_{27}-steroids that are not intermediates in the biosynthesis of chenodeoxycholic acid and cholic acid (11).

As discussed in Section IIA, more recent investigations have shown that the early reactions in the biosynthesis of cholic acid are catalyzed by microsomal and soluble enzymes. It appears that there is a pathway for chenodeoxycholic acid formation which involves the same intermediates as those formed in the early steps in cholic acid formation. The experimental evidence comes from a study of the metabolism of cholesterol and other C_{27}-steroids in homogenates of liver from a strain of guinea pigs that have chenodeoxycholic acid as their main primary bile acid (61). Microsomal and soluble enzymes were shown to catalyze the conversion of cholesterol into 5β-cholestane-3α,7α-diol by means of the intermediary formation of 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 7α-hydroxy-5β-cholestan-3-one (Fig. 4). The subcellular localization and cofactor requirements of the enzymes concerned were found to be the same as those in rat liver (*cf.* Section IIA1,2,4,5). Although no definite experimental evidence is yet available, it is tempting to suggest that in these guinea pigs 5β-cholestane-3α,7α-diol is the main substrate for the 26-hydroxylase system.

With respect to the oxidation of the side chain in chenodeoxycholic acid formation, it may be inferred from the early studies with mitochondrial preparations that it involves an ω-oxidation followed by a β-oxidation (*cf.* Section IIB). More direct evidence has been presented by Dean and Whitehouse (87,91), who showed that mitochondrial preparations from rat liver catalyze the oxidation of 5-cholestene-3β,26-diol into 3β-hydroxy-5-cholestenoic acid and the formation of propionic acid from 3β-hydroxy-5-cholestenoic acid. Mitropoulos and Myant (97) have shown that mitochondrial preparations from rat liver catalyze the conversion of cholesterol into 5-cholestene-3β,26-diol, 3β-hydroxy-5-cholestenoic acid, 3β-hydroxy-5-cholenoic acid, lithocholic acid, and chenodeoxycholic acid (Fig. 5). Additional evidence for a pathway to chenodeoxycholic acid involving the successive, intermediary formation of above-mentioned compounds is provided by the finding that 3β-hydroxy-5-cholenoic acid is converted into lithocholic acid and chenodeoxycholic acid by mitochondrial preparations (98).

The importance, quantitatively, of the different pathways for chenodeoxycholic acid formation has not been fully established. Several lines of evi-

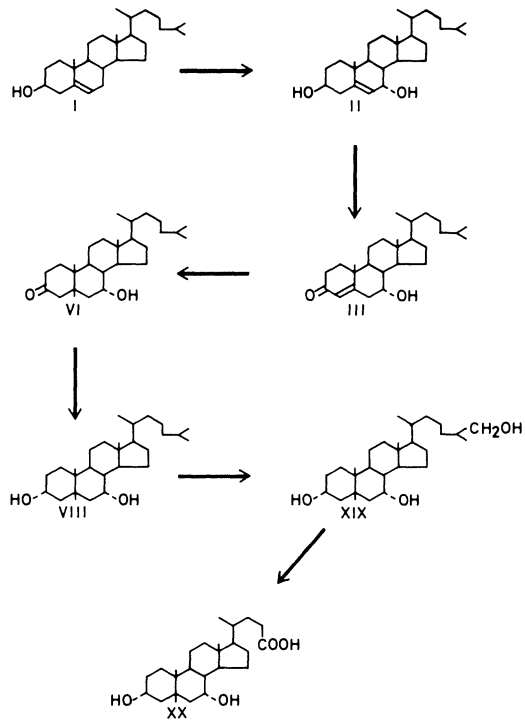

Fig. 4. Formation of chenodeoxycholic acid: some reactions catalyzed by homogenates of liver from guinea pigs having chenodeoxycholic acid as the main primary bile acid. I, Cholesterol; II, 5-cholestene-3β,7α-diol; III, 7α-hydroxy-4-cholesten-3-one; VI, 7α-hydroxy-5β-cholestan-3-one; VIII, 5β-cholestane-3α,7α-diol; XIX, 5β-cholestane-3α,7α,26-triol; XX, chenodeoxycholic acid.

dence indicate that the pathway involving 5-cholestene-3β,26-diol, 3β-hydroxy-5-cholenoic acid, and lithocholic acid as intermediates is a minor one. Mendelsohn and Mendelsohn (70) have found that 5-cholestene-3β,26-diol is not an intermediate in the conversion of cholesterol into chenodeoxycholic acid catalyzed by the 20,000*g* supernatant fluid of rat liver homogenate. In several species including man and rabbit that have chenodeoxycholic acid as a major bile acid, lithocholic acid has been found to be metabolized by the liver to a very small extent or not at all (99–101). These findings lead to the conclusion that in major pathway(s) for chenodeoxycholic acid formation at least the 7α-hydroxyl group is introduced prior to initiation of

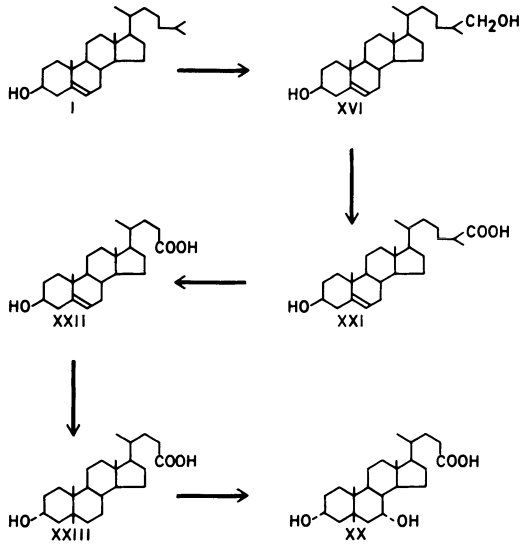

Fig. 5. Conversion of cholesterol into chenodeoxycholic acid by means of the intermediary formation of lithocholic acid. I, Cholesterol; XVI, 5-cholestene-3β,26-diol; XX, chenodeoxycholic acid; XXI, 3β-hydroxy-5-cholestenoic acid; XXII, 3β-hydroxy-5-cholenoic acid; XXIII, lithocholic acid.

side-chain oxidation. Whether or not the nuclear changes are completed before introduction of the 26-hydroxyl group remains to be established.

IV. FORMATION OF OTHER PRIMARY BILE ACIDS

Chenodeoxycholic acid is converted into α-muricholic acid (3α,6β,7α-trihydroxy-5β-cholanoic acid) and β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) in the mouse and the rat and probably also in man (68, 102, Chapter 11 in this volume). α-Muricholic acid is a precursor of β-muricholic acid in a reaction involving the intermediary formation of the 7-oxo compound (Chapter 11 in this volume). In the rat, β-muricholic acid has been shown to be formed also from 3α,7β-dihydroxy-5β-cholanoic acid, which is a minor metabolite of chenodeoxycholic acid, and from 3α,6β-dihydroxy-5β-cholanoic acid, which is a metabolite of lithocholic acid (Chapter 11 in this volume). The microsomal 6β-hydroxylase system in rat liver catalyzing the conversion of (tauro)chenodeoxycholic acid into (tauro)α-muricholic acid has been studied by Hsia and collaborators (103–105), who

have shown that cytochrome P-450 participates in the reaction but probably not as a rate-limiting component. Chenodeoxycholic acid is the precursor of hyocholic acid in the pig (Chapter 11 in this volume). Whereas chenodeoxycholic acid is not converted into cholic acid in mammals, the reaction has been shown to occur in some lower animals, *viz.*, the python (106), the eel (107), and the cod (108).

Deoxycholic acid is predominantly if not exclusively a secondary bile acid formed from cholic acid (Chapter 11 in this volume). Deoxycholic acid has been shown to be formed from cholest-5-ene-3β,12α-diol in rats and rabbits with a biliary fistula (53,54). However, only minute amounts of cholest-5-ene-3β,12α-diol are formed from cholesterol in the presence of the 20,000*g* supernatant fluid of rat liver homogenate (55). Rosenfeld and Hellman (109) have shown that 5β-cholestan-3α-ol is converted efficiently into deoxycholic acid in man, but there is at present no evidence that 5β-cholestan-3α-ol is formed in the liver.

In the guinea pig, 3α-hydroxy-7-oxo-5β-cholanoic acid is partly a primary bile acid and partly a secondary bile acid formed from chenodeoxycholic acid (Chapter 11 in this volume) The pathway for the formation of 3α-hydroxy-7-oxo-5β-cholanoic acid in the liver has not been established. It might be mentioned that 3β-hydroxy-5-cholesten-7-one is not a precursor of this acid (110).

Lithocholic acid, formed through the reactions described by Mitropoulos and Myant (*cf.* Fig. 5 and Section III), is by definition a primary bile acid. Lithocholic acid is predominantly a secondary bile acid formed from chenodeoxycholic acid (Chapter 11 in this volume). Lithocholic acid is transformed by rat liver into 3α,6β-dihydroxy-5β-cholanoic acid, chenodeoxycholic acid, and α- and β-muricholic acids (Chapter 11 in this volume).

Allocholanoic acids (5α-cholanoic acids) are found mainly in lower animals (68,76), but small amounts of allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid), allodeoxycholic acid (3α,12α-dihydroxy-5α-cholanoic acid), and probably also allochenodeoxycholic acid (3α,7α-dihydroxy-5α-cholanoic acid) may be present in bile and feces of mammals (68,76,102). Karavolas *et al.* (111) and Ziller *et al.* (112) have shown that cholestanol is converted into allocholic acid and allochenodeoxycholic acid in rats with a biliary fistula. The conversion of cholestanol into allocholic acid has also been shown in the rabbit (113). Allodeoxycholic acid is a secondary bile acid, formed from allocholic acid (113,114) and deoxycholic acid (115,116). The early steps in the sequence of reactions from cholestanol to allocholic acid (Fig. 6) have been the subject of two recent investigations. Shefer *et al.* (17) have shown that the microsomal fraction of rat liver homogenate fortified with NADPH catalyzes 7α-hydroxylation of cholestanol. Björkhem and Gustafsson (117) have compared the rates of 7α-hydroxylation of cholestanol,

5α-cholestan-3α-ol, and 5α-cholestan-3-one by the 20,000*g* supernatant fluid of rat liver homogenate and found that 7α-hydroxylation occurs at an appreciable rate only with cholestanol. 5α-Cholestane-3β,7α-diol has been shown to be oxidized to 7α-hydroxy-5α-cholestan-3-one by the microsomal fraction fortified with NAD (117). Reduction of 7α-hydroxy-5α-cholestan-3-one to 5α-cholestane-3α,7α-diol is catalyzed by the 100,000*g* supernatant fluid (117). 5α-Cholestane-3α,7α-diol was found to be hydroxylated in the 12α-position at a much faster rate than either 5α-cholestane-3β,7α-diol or

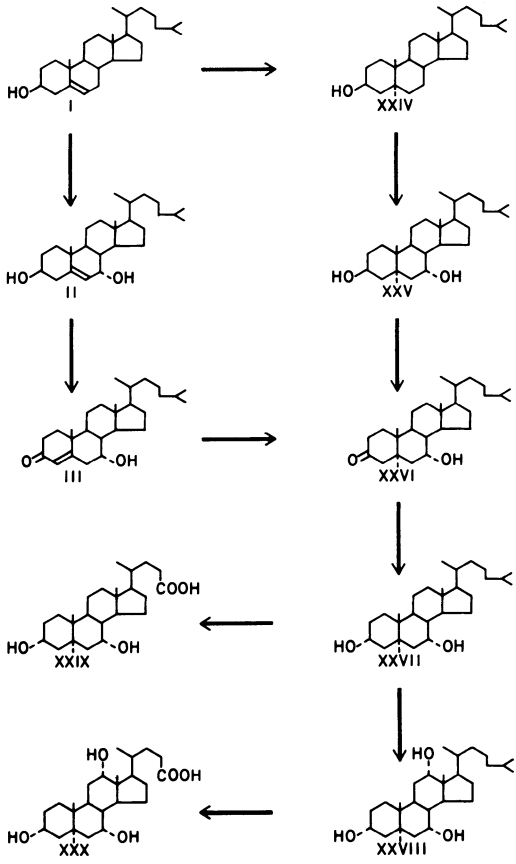

Fig. 6. Conversion of cholesterol into allocholanoic acids. I, Cholesterol; II, 5-cholestene-3β,7α-diol; III, 7α-hydroxy-4-cholesten-3-one; XXIV, cholestanol; XXV, 5α-cholestane-3β,7α-diol; XXVI, 7α-hydroxy-5α-cholestan-3-one; XXVII, 5α-cholestane-3α,7α-diol; XXVIII, 5α-cholestane-3α,7α,12α-triol; XXIX, allochenodeoxycholic acid; XXX, allocholic acid.

7α-hydroxy-5α-cholestan-3-one (117). Björkhem and Einarsson (118) have presented evidence that in the rat allocholanoic acids may be formed from cholesterol without the intermediary formation of cholestanol. The microsomal fraction of rat liver homogenate fortified with NADPH was found to catalyze the conversion of 7α-hydroxy-4-cholesten-3-one into 7α-hydroxy-5α-cholestan-3-one and of 7α,12α-dihydroxy-4-cholesten-3-one into 7α,12α-dihydroxy-5α-cholestan-3-one (*cf.* Fig. 6). The presence of similar pathways in animals that have allocholanoic acids or 5α-sterols as major bile salts has been shown by Hoshita (119–121) and Hoshita *et al.* (122). These investigations will be discussed in Section VII. Björkhem (123) has studied the mechanism of the conversion of 7α-hydroxy-4-cholesten-3-one into 7α-hydroxy-5α-cholestan-3-one. The reaction was found to involve transfer of a hydride ion from the B-side of NADPH to the 5α-position of the steroid and addition of a proton to the 4-position. Although the stereochemistry of the addition of the proton could not be unequivocally established, the results indicate a nonstereospecific addition or an addition to the 4α-position. The reduction of the double bond may thus be either nonstereospecific or a *cis* addition of hydrogens (4α and 5α). As discussed in Section IIA4, the reduction of the Δ^4 double bond in the formation of a 5β-steroid is a *trans* addition.

V. CONJUGATION OF BILE ACIDS

Bile acids occur in bile as conjugates with glycine or taurine. Conjugation with glycine has been observed only in mammals (76). The ratio between glycine- and taurine-conjugated bile acids in bile differs with species of mammal (76) and is influenced by diet, vitamins, and hormones (11,102). Early work by Bremer and Gloor (124–126), Murray and Siperstein (127, 128), and Elliott (129,130) showed that conjugation of bile acids was catalyzed by the microsomal fraction of rat liver homogenate. Evidence was obtained to indicate that the reaction involved the intermediary formation of the coenzyme-A ester of the bile acids. Scherstén (131) and Scherstén *et al.* (132) have studied the conjugation of bile acids in homogenates of human liver and have obtained evidence to indicate that the coupling of the coenzyme-A ester of the bile acid with the amino acid is catalyzed predominantly by enzymes localized in the lysosomal fraction. As discussed in Sections IIB and III, β-oxidation of the side chain in the biosynthesis of cholic acid and chenodeoxycholic acid leads to the formation of the coenzyme-A esters. The microsomal system catalyzing conversion of bile acids into coenzyme-A esters is required for conjugation of the free bile acids that are formed in the intestinal tract, absorbed from the intestine, and transported to the liver with the portal blood.

Palmer (133) has isolated labeled glycolithocholic acid 3-sulfate and taurolithocholic acid 3-sulfate from human bile after oral administration of labeled lithocholic acid. No further information concerning sulfate esters of bile acids is available at present.

The formation of ornithine-conjugated bile acids has been shown in guinea pigs treated with a capsular polysaccharide from *Klebsiella pneumoniae* or subjected to hepatic injury of unspecific nature (134). Ornithocholanoic acids have also been detected in bile from patients infected with *K. pneumoniae* (135). Gordon *et al.* (136) have presented evidence for the presence of ornithocholanoic acids in a human subject free of liver and gallbladder disease.

VI. REGULATION OF BILE ACID FORMATION

Continuous drainage of bile through a biliary fistula leads to large increases in the rate of biosynthesis of bile acids (102). In the rat, the increase may be ten- to fifteenfold (102). Feeding cholestyramine, an anion exchange resin that binds bile acids, also leads to increases in the rate of biosynthesis of bile acids (137–140). Evidence that the formation of bile acids is regulated by the amount of bile acids returned to the liver with the portal blood was first presented in a study of the effect of infusion of taurochenodeoxycholic acid on the biosynthesis of cholic acid in rats with a biliary fistula (141). Intraduodenal infusion of 5–15 mg of taurochenodeoxycholic acid per hour resulted in a very marked reduction in the rate of cholic acid synthesis. Beher *et al.* (142–144) concluded from a series of investigations concerning the effect of feeding bile acids on biosynthesis and catabolism of cholesterol that the formation of bile acids is regulated by a double feedback mechanism involving an inhibitory effect of bile acids on the conversion of cholesterol into bile acids followed by an inhibition of the biosynthesis of cholesterol by accumulated cholesterol. Inhibition of biosynthesis of bile acids by feeding bile acids has also been observed in man (145). The concept that bile acid formation is regulated homeostatically by bile acids was challenged by Lee *et al.* (146), who found that the biosynthesis of bile acids in rats with a biliary fistula was not inhibited by infusion of taurocholic acid under conditions when infusion of taurochenodeoxycholic acid caused inhibition. It was suggested (146,147) that the effect observed with taurochenodeoxycholic acid might not be related to the physiological mechanism of regulation of bile acid formation but reflect the fact that taurochenodeoxycholic acid is a much more potent inhibitor of electron transport and coupled phosphorylation than taurocholic acid (148). Tauro- and glycochenodeoxycholic acids constitute about 15–20% of the bile acids in rat bile and tauro- and glyco-

cholic acids 50–60% (149). Chenodeoxycholic acid and its conjugates account for even less of the bile acids in portal blood (150). A detailed study by Wilson *et al.* (151) on the effect of infusion of taurocholic acid on the biosynthesis of bile acids in rats with a biliary fistula showed, in agreement with the results of Lee *et al.* (146), that no repression of bile acid synthesis was obtained even with infusion of about 35 mg of taurocholic acid per hour. Recently, Shefer *et al.* (152) have found that infusion of taurocholic acid into rats with a biliary fistula does inhibit biosynthesis of cholic acid and chenodeoxycholic acid and have concluded that the formation of bile acids is controlled homeostatically by the amount of bile acids returned to the liver. To obtain inhibition, a quantity of 10 mg or more of taurocholic acid per 100 g rat had to be infused hourly. Shefer *et al.* (152) ascribe the negative results obtained by Lee *et al.* (146) and Wilson *et al.* (151) to the use of too low concentrations of taurocholic acid. The concentrations used by these authors reflect previous underestimates of the size of the bile acid pool and of the number of times per day the pool circulates. Shefer *et al.* (152) have estimated the pool of taurocholic acid to be about 15 mg per 100 g rat and the number of circulations per day to be 10–13.

The results of Shefer *et al.* (152) support the conclusion by Lee *et al.* (146) that taurochenodeoxycholic acid has an effect on bile acid formation in rats with a biliary fistula not entirely related to the physiological regulation of bile acid formation, since the concentration of taurochenodeoxycholic acid needed to repress bile acid biosynthesis is less than half of that of taurocholic acid.

The major rate-determining step in the biosynthesis of bile acids appears to be the 7α-hydroxylation of cholesterol. The rate of this reaction is increased manyfold by biliary drainage and cholestyramine feeding (17,18,20,21,23,24). Several other reactions in the biosynthesis and metabolism of bile acids are unaffected or only moderately stimulated by biliary drainage and cholestyramine feeding (23,24). The half-life time of the 7α-hydroxylase in rats with a biliary fistula has been estimated to be about 2–3 hr (153), and a preliminary report (154) indicates the same short half-life time for the 7α-hydroxylase in intact rats. Further evidence for the role of the 7α-hydroxylase as a rate-determining step has been presented by Shefer *et al.* (155), who have found that infusion of taurocholic acid does not affect the rate of conversion of cholest-5-ene-3β,7α-diol into bile acids in rats with a biliary fistula.

The rate of biosynthesis of cholesterol in the liver is increased several-fold by biliary drainage and cholestyramine feeding (23,138,139,156,157). The major rate-determining step in the biosynthesis of cholesterol is the reduction of hydroxymethylglutaryl coenzyme A (158–160).

The homeostatic regulation of bile acid formation could involve action of bile acids on one or both of the rate-determining reactions in the conver-

sion of acetate into bile acids. Experiments with addition of bile acids *in vitro* to liver slices and subcellular preparations of liver homogenate have yielded differing results. Siperstein (161) and Dietschy (unpublished work cited in 162) found no inhibition of cholesterol synthesis by addition of bile acids to liver slices, whereas Seitz and von Brand (163) and Fimognari and Rodwell (164) obtained inhibition in liver slices and liver homogenates. The possibility that bile acids may exert nonspecific effects on systems *in vitro* has been pointed out by several authors (165,166). Shefer *et al.* (17) found inhibition of the 7α-hydroxylation of cholesterol *in vitro* by the addition of bile acids but did not consider these results conclusive in view of the possibility of a nonspecific effect of bile acids. Experiments *in vivo* have led to differing conclusions. Weis and Dietschy (162) have concluded from a detailed study of the effect of biliary drainage on the biosynthesis of cholesterol in the liver that bile acids do not control hepatic cholesterogenesis directly but affect it indirectly by virtue of their role in the absorption of cholesterol. The increase in hepatic cholesterogenesis observed upon biliary drainage would be due to defective absorption of cholesterol from the intestine. On the other hand, Back *et al.* (167) have presented evidence that bile acids have a direct influence on hepatic cholesterogenesis. These authors studied the effect of addition of cholic acid or cholesterol to the diet on the biosynthesis of cholesterol in rat liver and found that feeding cholic acid resulted in a more rapid inhibition of cholesterol biosynthesis than feeding cholesterol. Cholic acid was suggested to affect cholesterogenesis by influencing the biosynthesis of hydroxymethylglutaryl–coenzyme A reductase. A similar mechanism has been proposed by Dietschy (168) for the effect of bile acids on the biosynthesis of cholesterol in the intestine. In view of the results of Shefer *et al.* (152), it is conceivable that the concentrations of taurocholic acid used by Weis and Dietschy (162) for infusion into the rats with a biliary fistula were too low to cause repression of cholesterol and bile acid biosynthesis. If it is accepted that bile acids have a direct influence on hepatic cholesterogenesis, the mechanism of the stimulatory effect on hepatic cholesterogenesis by ligation of the bile duct (162,169) remains to be explained.

Myant and Eder (156) found that the increase in biosynthesis of cholesterol elicited by biliary drainage preceded the increase in biosynthesis of bile acids. This finding and those of Back *et al.* (167) appear to disprove the double feedback mechanism suggested by Beher *et al.* (142–144). However, it is not thereby excluded that bile acids may influence both the biosynthesis of cholesterol and the conversion of cholesterol into bile acids in the liver. Shefer *et al.* (155) have found that infusion of taurocholic acid into rats with a biliary fistula leads to inhibition not only of the conversion of labeled acetate into bile acids but also of the conversion of labeled mevalonate and cholesterol. These results indicate that the homeostatic regulation of bile

acid formation involves effects of bile acids on hydroxymethylglutaryl–coenzyme A reductase as well as on 7α-hydroxylase.

Another aspect of regulation of bile acid formation is the mechanisms by which the composition of bile acids in bile is regulated. Of particular interest is the regulation of the ratio of cholic acid to chenodeoxycholic acid, since chenodeoxycholic acid is not a precursor of cholic acid in mammals. There are large variations in the ratio of cholic acid to chenodeoxycholic acid among species but considerably fewer variations among animals of the same species. It is not possible at present to state definitely at which stage the pathways for biosynthesis of cholic acid and chenodeoxycholic acid separate, but it is tempting to suggest that, at least in the rat, this occurs when 7α-hydroxy-4-cholesten-3-one has been formed. This suggestion is based on some observations concerning the effects of thyroid hormones on the formation of bile acids in the rat. In rats with a biliary fistula as well as in intact rats, administration of thyroid hormones or D-tri-iodothyronine leads to a reversal of the normal ratio of about 3:1 between cholic acid and chenodeoxycholic acid (170–173). The rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one is slower in hyperthyroid rats than in euthyroid rats (39). The rate of oxidation of the C_{27} side chain, measured by the formation of propionic acid from cholesterol (174) or by the hydroxylation of the side chain of 5β-cholestane-3α,7α,12α-triol (175), is faster in hyperthyroid rats than in euthyroid rats. The reversal of the ratio of cholic acid to chenodeoxycholic acid induced by thyroid hormones could be due to the combination of an inhibition of 12α-hydroxylation and a stimulation of side-chain oxidation. As discussed in section IIA3,5, 7α-hydroxy-4-cholesten-3-one is the substrate for the 12α-hydroxylase in the apparently major pathway for the conversion of cholesterol into cholic acid, and an inhibition of the 12α-hydroxylation might therefore be expected to affect primarily the metabolism of 7α-hydroxy-4-cholesten-3-one.

VII. FORMATION OF BILE SALTS IN "PRIMITIVE" ANIMALS

Bile salts with a steroid structure appear to be confined to vertebrates (76). In some evolutionarily more primitive vertebrates, the major bile salts are sulfate esters of polyhydroxy C_{27}- and C_{26}-steroids and/or taurine-conjugated C_{27}-steroid acids. In other "primitive" vertebrates, C_{24} bile acids, usually cholic acid and/or allocholic acid, or mixtures of "primitive" bile salts (bile alcohols and C_{27} bile acids) and "modern" bile salts (C_{24} bile acids) occur. Most of the work concerning the structure and occurrence of "primitive" bile salts has been carried out in the laboratories of G. A. D. Haslewood and T. Kazuno, and Haslewood and collaborators have accumu-

lated a wealth of information concerning the composition of bile salts in different species. Haslewood (76,176) and Hoshita and Kazuno (177) have summarized recently the work on structure and occurrence of bile salts and have discussed bile salt structure from the aspect of evolution. In this chapter, discussion will be limited to investigations that have dealt with sequences of reactions in the formation of bile salts in "primitive" animals. In a number of instances, it has been shown that cholesterol is the precursor of these bile salts. Reference to these investigations will be found in the review of Hoshita and Kazuno (177).

A. Changes in Steroid Nucleus

The "primitive" bile salts so far isolated have, with a few exceptions, a steroid nucleus carrying hydroxyl groups in the 3α-, 7α-, and 12α-positions. The conversion of the Δ^5-3β-hydroxy configuration of cholesterol into the 3α,7α,12α-trihydroxy-5α or 3α,7α,12α-trihydroxy-5β configuration of "primitive" bile salts has as yet been studied only in the carp. The major bile salt in the carp is 5α-cholestane-3α,7α,12α,26,27-pentol (5α-cyprinol). Carp bile also contains 5α-cholestane-3α,7α,12α,26-tetrol (27-deoxy-5α-cyprinol) and cholic acid. Hoshita (119) has shown that *in vivo* cholestanol as well as 5-cholestene-3β,7α-diol is converted into 27-deoxy-5α-cyprinol and 5α-cyprinol. 5-Cholestene-3β,7α-diol was found to be a more efficient precursor than cholestanol, indicating that 5-cholestene-3β,7α-diol is an intermediate in the major pathway for the formation of the 5α bile alcohols. Hoshita (120) has also shown that in the conversion of cholesterol into 27-deoxy-5α-cyprinol and 5α-cyprinol the Δ^5 double bond of cholesterol is isomerized to a Δ^4 double bond before being reduced. However, this finding does not permit conclusions concerning the importance, quantitatively, of the different pathways for the formation of the 5α bile alcohols, since the conversion of cholesterol into cholestanol probably involves the intermediary formation of 4-cholesten-3-one. Studies with homogenates of carp liver have provided additional evidence that the major pathway for the conversion of cholesterol into 5α bile alcohols does not involve cholestanol as an intermediate (121, 178). 7α,12α-Dihydroxy-4-cholesten-3-one was found to be converted into 5α-cholestane-3α,7α,12α-triol by the microsomal fraction fortified with NADPH, and, as discussed below, 5α-cholestane-3α,7α,12α-triol was shown to be the precursor of 27-deoxy-5α-cyprinol and 5α-cyprinol. It may be expected that the conversion of cholesterol into 7α,12α,-dihydroxy-4-cholesten-3-one occurs by the same pathways as found in the rat (Section IIA). The conversion of 7α,12α-dihydroxy-4-cholesten-3-one into 5α-cholestane-3α,7α,12α-triol by means of the intermediary formation of 7α,12α-dihydroxy-5α-cholestan-3-one has also been shown in the lizard *Iguana iguana,* a species

that has allocholic acid as the main bile acid (122). The reactions were catalyzed by the microsomal fraction of liver homogenate fortified with NADPH.

B. Oxidation of Side Chain

The formation of 27-deoxy-5α-cyprinol and 5α-cyprinol in the carp has been shown by Hoshita (121) and Masui *et al.* (178) to involve 26(27)-hydroxylation of 5α-cholestane-3α,7α,12α-triol, catalyzed by the microsomal fraction fortified with NADPH, followed by 26(27)-hydroxylation of 27-deoxy-5α-cyprinol, catalyzed by the mitochondrial fraction fortified with NADPH.

The main bile alcohol in toads is 5β-cholestane-3α,7α,12α,25,26-pentol (5β-bufol). Enomoto (179) has shown that *in vivo* 5β-cholestane-3α,7α,12α-triol is converted into 5β-bufol. *In vitro,* 5β-cholestane-3α,7α,12α-triol was converted into 5β-cholestane-3α,7α,12α,26-tetrol but not 5β-bufol (180). It appears probable, however, that 5β-cholestane-3α,7α,12α,26-tetrol is the immediate precursor of 5β-bufol, as suggested by Enomoto (180) and Hoshita and Kazuno (177).

Major bile salts in the bullfrog, *Rana catesbiana,* are 27-nor-5β-cholestane-3α,7α,12α,24,26-pentol (5β-ranol) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Betsuki (181,182) has shown that 5β-cholestane-3α,7α,12α-triol and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid are converted into 5β-ranol and 26-deoxy-5β-ranol. The following sequence of reactions in the formation of 5β-ranol was proposed: 5β-cholestane-3α,7α,12α-triol ⟶ 3α,7α,12α-trihydroxy-5β-cholestanoic acid ⟶ 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid ⟶ 27-nor-5β-cholestane-3α,7α,12α,24-tetrol (26-deoxy-5β-ranol) ⟶ 27-nor-5β-cholestane-3α,7α,12α,24,26-pentol (5β-ranol).

The information so far available concerning biosynthesis of bile salts in "primitive" animals indicates that the sequences of reactions involved in the transformations of the steroid nucleus as well as of the side chain are the same as or very similar to those found in mammals.

REFERENCES

1. K. Bloch, B. N. Berg, and D. Rittenberg, *J. Biol. Chem.* **149,** 511 (1943).
2. S. Bergström, *Proc. Roy. Physiog. Soc. Lund* **22,** No. 16 (1952).
3. S. Bergström, *Rec. Chem. Progr. (Kresge-Hooker Sci. Lib.)* **16,** 63 (1955).
4. I. L. Chaikoff, M. D. Siperstein, W. G. Dauben, H. L. Bradlow, J. F. Eastham, G. M. Tomkins, J. B. Meier, R. W. Chen, S. Hotta, and P. A. Srere, *J. Biol. Chem.* **194,** 413 (1952).
5. M. D. Siperstein and I. L. Chaikoff, *J. Biol. Chem.* **198,** 93 (1952).
6. S. Bergström and S. Lindstedt, *Biochim. Biophys. Acta* **19,** 556 (1956).

7. R. J. Bridgwater and S. Lindstedt, *Acta Chem. Scand.* **11,** 409 (1957).
8. S. Lindstedt, *Acta Chem. Scand.* **11,** 417 (1957).
9. F. M. Harold, M. E. Jayko, and I. L. Chaikoff, *J. Biol. Chem.* **216,** 439 (1955).
10. F. M. Harold, D. D. Chapman, and I. L. Chaikoff, *J. Biol. Chem.* **224,** 609 (1957).
11. H. Danielsson, *Advan. Lipid Res.* **1,** 335 (1963).
12. D. Mendelsohn and E. Staple, *Biochemistry* **2,** 577 (1963).
13. H. Danielsson and K. Einarsson, *Acta Chem. Scand.* **18,** 831 (1964).
14. D. Mendelsohn, L. Mendelsohn, and E. Staple, *Biochim. Biophys. Acta* **97,** 379 (1965).
15. H. Danielsson and K. Einarsson, *J. Biol. Chem.* **241,** 1449 (1966).
16. J. R. Mitton and G. S. Boyd, *Biochem. J.* **103,** 17P (1967).
17. S. Shefer, S. Hauser, and E. H. Mosbach, *J. Lipid Res.* **9,** 328 (1968).
18. G. Johansson, *Europ. J. Biochem.* **21,** 68 (1971).
19. S. Bergström, S. Lindstedt, B. Samuelsson, E. J. Corey, and G. A. Gregoriou, *J. Am. Chem. Soc.* **80,** 2337 (1958).
20. G. S. Boyd, N. A. Scholan, and J. R. Mitton, *Advan. Exptl. Med. Biol.* **4,** 443 (1969).
21. F. Wada, K. Hirata, K. Nakano, and Y. Sakamoto, *J. Biochem. (Tokyo)* **66,** 699 (1969).
22. K. Einarsson and G. Johansson, *Europ. J. Biochem.* **6,** 293 (1968).
23. H. Danielsson, K. Einarsson, and G. Johansson, *Europ. J. Biochem.* **2,** 44 (1967).
24. G. Johansson, *Europ. J. Biochem.* **17,** 292 (1970)
25. W. Voigt, E. P. Fernandez, and S. L. Hsia, *Proc. Soc. Exptl. Biol. Med.* **133,** 1158 (1970).
26. G. S. Boyd, *Fed. Proc.* **21,** Suppl. 11, 86 (1962).
27. H. R. B. Hutton and G. S. Boyd, *Biochim. Biophys. Acta* **116,** 336 (1966).
28. K. Katayama and K. Yamasaki, *Yonago Acta Med.* **12,** 103 (1968).
29. D. Mendelsohn, L. Mendelsohn, and E. Staple, *Biochemistry* **5,** 1286 (1966).
30. O. Berséus, H. Danielsson, and K. Einarsson, *J. Biol. Chem.* **242,** 1211 (1967).
31. O. Berséus and K. Einarsson, *Acta Chem. Scand.* **21,** 1105 (1967).
32. I. Björkhem, *Europ. J. Biochem.* **8,** 337 (1969).
33. B. Samuelsson, *J. Biol. Chem.* **234,** 2852 (1959).
34. K. Gréen and B. Samuelsson, *J. Biol. Chem.* **239,** 2804 (1964).
35. D. K. Fukushima, H. L. Bradlow, T. Yamauchi, A. Yagi, and D. Koerner, *Steroids* **11,** 541 (1968).
36. S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.* **87,** 3228 (1965).
37. K. Einarsson, *Europ. J. Biochem.* **5,** 101 (1968).
38. M. Suzuki, K. A. Mitropoulos, and N. B. Myant, *Biochem. Biophys. Res. Commun.* **30,** 516 (1968).
39. K. A. Mitropoulos, M. Suzuki, N. B. Myant, and H. Danielsson, *FEBS Letters* **1,** 13 (1968).
40. G. Johansson, *Biochem. Pharmacol.* **19,** 2817 (1970).
41. O. Berséus, H. Danielsson, and A. Kallner, *J. Biol. Chem.* **240,** 2369 (1965).
42. O. Berséus, *Europ. J. Biochem.* **2,** 493 (1967).
43. G. M. Tomkins, *J. Biol. Chem.* **225,** 13 (1957).
44. O. Berséus and I. Björkhem, *Europ. J. Biochem.* **2,** 503 (1967).
45. I. Björkhem, *Europ. J. Biochem.* **7,** 413 (1969).
46. O. Berséus, *Opuscula Med.,* Suppl. VI (1967).
47. G. M. Tomkins, *J. Biol. Chem.* **218,** 437 (1957).
48. S. S. Koide, *Biochim. Biophys. Acta* **110,** 189 (1965).
49. S. S. Koide, *Steroids* **6,** 123 (1965).

50. E. Doman and S. S. Koide, *Biochim. Biophys. Acta* **128,** 209 (1966).
51. G. M. Tomkins, R. F. Bakemeier, and A. N. Weinberg, *in* "Proceedings of Fifth International Congress of Biochemistry," Vol. VII, p. 405, Pergamon Press, London (1963).
52. I. Björkhem and H. Danielsson, *Europ. J. Biochem.* **12,** 80 (1970).
53. H. Danielsson, *Ark. Kemi* **17,** 381 (1961).
54. H. Danielsson, *Acta Chem. Scand.* **16,** 1534 (1962).
55. K. Einarsson, *Europ. J. Biochem.* **6,** 299 (1968).
56. D. Mendelsohn, L. Mendelsohn, and E. Staple, *Biochemistry* **4,** 441 (1965).
57. H. R. B. Hutton and G. S. Boyd, *Biochim. Biophys. Acta* **116,** 362 (1966).
58. I. Björkhem and H. Danielsson, *Europ. J. Biochem.* **2,** 403 (1967).
59. I. Björkhem and H. Danielsson, *Acta Chem. Scand.* **19,** 2298 (1965).
60. I. Björkhem, *Acta Chem. Scand.* **21,** 2561 (1967).
61. I. Björkhem, H. Danielsson, and K. Einarsson, *Europ. J. Biochem.* **2,** 294 (1967).
62. I. Björkhem, H. Danielsson, K. Einarsson, and G. Johansson, *J. Clin. Invest.* **47,** 1573 (1968).
63. P. P. Nair, M. Gordon, S. A. Tepper, and D. Kritchevsky, *J. Biol. Chem.* **243,** 4034 (1968).
64. N. Wachtel, S. Emerman, and N. B. Javitt, *J. Biol. Chem.* **243,** 5207 (1968).
65. H. Danielsson, *Ark. Kemi* **17,** 373 (1961).
66. D. S. Fredrickson, *J. Biol. Chem.* **222,** 109 (1956).
67. I. Björkhem, H. Danielsson, and K. Einarsson, *Europ. J. Biochem.* **4,** 458 (1968).
68. H. Danielsson and T. T. Tchen, *in* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. II, p. 117, Academic Press, New York (1968).
69. D. Mendelsohn, L. Mendelsohn, and E. Staple, *Biochemistry* **5,** 3194 (1966).
70. D. Mendelsohn and L. Mendelsohn, *Biochemistry* **7,** 4167 (1968).
71. H. Danielsson, *Acta Chem. Scand.* **14,** 348 (1960).
72. H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.* **237,** 338 (1962).
73. O. Berséus, *Acta Chem. Scand.* **19,** 325 (1965).
74. K. A. Mitropoulos and N. B. Myant, *Biochem. J.* **97,** 26c (1965).
75. D. Mendelsohn and L. Mendelsohn, *Biochem. J.* **114,** 1 (1969).
76. G. A. D. Haslewood, "Bile Salts," Methuen, London (1967).
77. P. P. Shah, E. Staple, and J. L. Rabinowitz, *Arch. Biochem. Biophys.* **123,** 427 (1968).
78. K. Okuda and N. Hoshita, *Biochim. Biophys. Acta* **164,** 381 (1968).
79. T. Cronholm and G. Johansson, *Europ. J. Biochem.* **16,** 373 (1970).
80. K. Okuda and H. Danielsson, *Acta Chem. Scand.* **19,** 2160 (1965).
81. T. Masui, R. Herman, and E. Staple, *Biochim. Biophys. Acta* **117,** 266 (1966).
82. K. Okuda and N. Takigawa, *Biochim. Biophys. Acta* **176,** 873 (1969).
83. E. Staple, *in* "Methods in Enzymology" (R. B. Clayton, S. P. Colowick, and N. O. Kaplan, eds.), Vol. XV, p. 562, Academic Press, New York (1969).
84. K. Okuda and N. Takigawa, *Biochem. Biophys. Res. Commun.* **33,** 788 (1968).
85. K. Okuda, N. Takigawa, R. Fukuba, and T. Kuwaki, *Biochim. Biophys. Acta* **185,** 1 (1969).
86. G. Waller, H. Theorell, and J. Sjövall, *Arch. Biochem. Biophys.* **111,** 671 (1965).
87. P. D. G. Dean and M. W. Whitehouse, *Biochem. J.* **98,** 410 (1966).
88. T. Masui and E. Staple, *Biochim. Biophys. Acta* **104,** 305 (1965).
89. T. Masui and E. Staple, *J. Biol. Chem.* **241,** 3889 (1966).
90. T. Masui and E. Staple, *Steroids* **9,** 443 (1967).
91. P. D. G. Dean and M. W. Whitehouse, *Biochem. J.* **101,** 632 (1966).

92. M. W. Whitehouse, J. L. Rabinowitz, E. Staple, and S. Gurin, *Biochim. Biophys. Acta* **37,** 382 (1960).
93. M. W. Whitehouse, E. Staple, and S. Gurin, *J. Biol. Chem.* **236,** 68 (1961).
94. K. Shimizu, T. Fujioka, M. Otagaki, and K. Yamasaki, *Yonago Acta Med.* **3,** 158 (1959).
95. T. Yamada, *Hiroshima J. Med. Sci.* **15,** 375 (1966).
96. E. Staple, *in* "Bile Salt Metabolism" (L. Schiff, J. B. Carey, Jr., and J. M. Dietschy, eds.), p. 127, C. C Thomas, Springfield, Ill. (1969).
97. K. A. Mitropoulos and N. B. Myant, *Biochem. J.* **103,** 472 (1967).
98. K. A. Mitropoulos and N. B. Myant, *Biochim. Biophys. Acta* **144,** 430 (1967).
99. J. B. Carey, Jr., and G. Williams, *J. Clin. Invest.* **42,** 450 (1963).
100. A. Norman and R. H. Palmer, *J. Lab. Clin. Med.* **63,** 986 (1964).
101. G. Johansson, *Acta Chem. Scand.* **20,** 240 (1966).
102. H. Danielsson and K. Einarsson, *in* "The Biological Basis of Medicine" (E. E. Bittar and N. Bittar, eds.), Vol. V, p. 279, Academic Press, London (1969).
103. W. Voigt, P. J. Thomas, and S. L. Hsia, *J. Biol. Chem.* **243,** 3493 (1968).
104. W. Voigt, S. L. Hsia, D. Y. Cooper, and O. Rosenthal, *FEBS Letters* **2,** 124 (1968).
105. W. Voigt, E. P. Fernandez, and S. L. Hsia, *Proc. Soc. Exptl. Biol. Med.* **133,** 1158 (1970).
106. S. Bergström, H. Danielsson, and T. Kazuno, *J. Biol. Chem.* **235,** 983 (1960).
107. T. Masui, F. Ueyama, H. Yashima, and T. Kazuno, *J. Biochem. (Tokyo)* **62,** 650 (1967).
108. A. Kallner, *Acta Chem. Scand.* **22,** 2361 (1968).
109. R. S. Rosenfeld and L. Hellman, *Biochemistry* **7,** 3085 (1968).
110. I. Björkhem, K. Einarsson, and G. Johansson, *Acta Chem. Scand.* **22,** 1595 (1968).
111. H. J. Karavolas, W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy, *J. Biol. Chem.* **240,** 1568 (1965).
112. S. A. Ziller, Jr., E. A. Doisy, Jr., and W. H. Elliott, *J. Biol. Chem.* **243,** 5280 (1968).
113. A. F. Hofmann, V. Bokkenheuser, R. L. Hirsch, and E. H. Mosbach, *J. Lipid Res.* **9,** 244 (1968).
114. A. F. Hofmann and E. H. Mosbach, *J. Biol. Chem.* **239,** 2813 (1964).
115. H. Danielsson, A. Kallner, and J. Sjövall, *J. Biol. Chem.* **238,** 3846 (1963).
116. A. Kallner, *Acta. Chem. Scand.* **21,** 87 (1967).
117. I. Björkhem and J. Gustafsson, *Europ. J. Biochem.* **18,** 207 (1971).
118. I. Björkhem and K. Einarsson, *Europ. J. Biochem.* **13,** 174 (1970).
119. T. Hoshita, *J. Biochem. (Tokyo)* **61,** 440 (1967).
120. T. Hoshita, *J. Biochem. (Tokyo)* **61,** 633 (1967).
121. T. Hoshita, *J. Biochem. (Tokyo)* **66,** 313 (1969).
122. T. Hoshita, S. Shefer, and E. H. Mosbach, *J. Lipid Res.* **9,** 237 (1968).
123. I. Björkhem, *Europ. J. Biochem.* **8,** 345 (1969).
124. J. Bremer, *Acta Chem. Scand.* **9,** 268 (1955).
125. J. Bremer, *Acta Chem. Scand.* **9,** 683 (1955).
126. J. Bremer and U. Gloor, *Acta Chem. Scand.* **9,** 689 (1955).
127. M. D. Siperstein, *Fed. Proc.* **14,** 282 (1955).
128. M. D. Siperstein and A. E. Murray, *Science* **123,** 377 (1956).
129. W. H. Elliott, *Biochem. J.* **62,** 427 (1956).
130. W. H. Elliott, *Biochem. J.* **62,** 433 (1956).
131. T. Scherstén, *Biochim. Biophys. Acta* **141,** 144 (1967).
132. T. Scherstén, P. Björntorp, P. H. Ekdahl, and S. Björkerud, *Biochim. Biophys. Acta* **141,** 155 (1967).

133. R. H. Palmer, *Proc. Natl. Acad. Sci.* **58,** 1047 (1967).
134. L. Peric-Golia and R. S. Jones, *Proc. Soc. Exptl. Biol. Med.* **110,** 327 (1962).
135. L. Peric-Golia and R. S. Jones, *Science* **142,** 245 (1963).
136. B. A. Gordon, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem. Physiol.* **41,** 77 (1963).
137. D. M. Tennent, H. Siegel, M. E. Zanetti, G. W. Kuron, W. H. Ott, and F. J. Wolf, *J. Lipid Res.* **1,** 469 (1960).
138. J. W. Huff, J. L. Gilfillan, and V. M. Hunt, *Proc. Soc. Exptl. Biol. Med.* **114,** 352 (1963).
139. D. G. Gallo, R. W. Harkins, A. L. Sheffner, H. P. Sarett, and W. M. Cox, Jr., *Proc. Soc. Exptl. Biol. Med.* **122,** 328 (1966).
140. W. T. Beher, M. E. Beher, and B. Rao, *Proc. Soc. Exptl. Biol. Med.* **122,** 881 (1966).
141. S. Bergström and H. Danielsson, *Acta Physiol. Scand.* **43.** 1 (1958).
142. W. T. Beher, G. D. Baker, and W. L. Anthony, *Am. J. Physiol.* **199,** 736 (1960).
143. W. T. Beher, G. D. Baker, W. L. Anthony, and M. E. Beher, *Henry Ford Hosp. Med. Bull.* **9,** 201 (1961).
144. W. T. Beher, G. D. Baker, and W. L. Anthony, *Proc. Soc. Exptl. Biol. Med.* **109,** 863 (1962).
145. S. M. Grundy, A. F. Hofmann, J. Davignon, and E. H. Ahrens, Jr., *J. Clin. Invest.* **45,** 1018 (1966).
146. M. J. Lee, D. V. Parke, and M. W. Whitehouse, *Proc. Soc. Exptl. Biol. Med.* **120,** 317 (1965).
147. P. D. G. Dean and M. W. Whitehouse, *Biochim. Biophys. Acta* **137,** 328 (1967).
148. M. J. Lee and M. W. Whitehouse, *Biochim. Biophys. Acta* **100,** 317 (1965).
149. M. T. Subbiah, A. Kuksis, and S. Mookerjea, *Can. J. Biochem.* **47,** 847 (1969).
150. T. Cronholm and J. Sjövall, *Europ. J. Biochem.* **2,** 375 (1967).
151. J. D. Wilson, W. H. Bentley, and G. T. Crowley, *in* "Bile Salt Metabolism" (L. Schiff, J. B. Carey, Jr., and J. M. Dietschy, eds.), p. 140, C. C Thomas, Springfield, Ill. (1969).
152. S. Shefer, S. Hauser, I. Bekersky, and E. H. Mosbach, *J. Lipid Res.* **10,** 646 (1969).
153. K. Einarsson and G. Johansson, *FEBS Letters* **1,** 219 (1968).
154. J. Renson, J. Van Cantfort, B. Robaye, and J. Gielen, *Arch. Int. Physiol.* **77,** 972 (1969).
155. S. Shefer, S. Hauser, J. Bekersky, and E. H. Mosbach, *J. Lipid Res.* **11,** 404 (1970).
156. N. B. Myant and H. A. Eder. *J. Lipid Res.* **2,** 363 (1961).
157. J. M. Dietschy and J. D. Wilson, *J. Clin. Invest.* **47,** 166 (1968).
158. M. D. Siperstein and M. J. Guest, *J. Clin. Invest.* **39,** 642 (1960).
159. N. L. R. Bucher, P. Overath, and F. Lynen, *Biochim. Biophys. Acta* **40,** 491 (1960).
160. M. D. Siperstein and V. M. Fagan, *J. Biol. Chem.* **241,** 602 (1966).
161. M. D. Siperstein, *Am. J. Clin. Nutr.* **8,** 645 (1960).
162. H. J. Weis and J. M. Dietschy, *J. Clin. Invest.* **48,** 2398 (1969).
163. W. Seitz and V. von Brand, *Klin. Wchschr.* **39.** 891 (1961).
164. G. M. Fimognari and V. W. Rodwell, *Science* **147,** 1038 (1965).
165. W. L. Miller and J. L. Gaylor, *Biochim. Biophys. Acta* **137,** 400 (1967).
166. J. M. Dietschy, *Fed. Proc.* **26,** 1589 (1967).
167. P. Back, B. Hamprecht, and F. Lynen, *Arch. Biochem. Biophys.* **133,** 11 (1969).
168. J. M. Dietschy, *J. Clin. Invest.* **47,** 286 (1968).
169. D. S. Fredrickson, A. V. Loud, B. T. Hinkelman, H. S. Schneider, and I. D. Frantz, Jr., *J. Exptl. Med.* **99,** 43 (1954).
170. S. Eriksson, *Proc. Soc. Exptl. Biol. Med.* **94,** 582 (1957).
171. O. Strand, *Proc. Soc. Exptl. Biol. Med.* **109,** 668 (1962).
172. T. H. Lin, R. Rubinstein, and W. L. Holmes, *J. Lipid Res.* **4,** 63 (1963).

173. O. Strand, *J. Lipid Res.* **4,** 305 (1963).
174. K. A. Mitropoulos and N. B. Myant, *Biochem. J.* **94,** 594 (1965).
175. O. Berséus, *Acta Chem. Scand.* **19,** 2131 (1965).
176. G. A. D. Haslewood, *J. Lipid Res.* **8,** 535 (1967).
177. T. Hoshita and T. Kazuno, *Advan. Lipid Res.* **6,** 207 (1968).
178. T. Masui, Y. Hijikata, and T. Kazuno, *J. Biochem. (Tokyo)* **62,** 279 (1967).
179. S. Enomoto, *J. Biochem. (Tokyo)* **51,** 393 (1962).
180. S. Enomoto, *J. Biochem. (Tokyo)* **52,** 1 (1962).
181. S. Betsuki, *J. Biochem. (Tokyo)* **60,** 411 (1966).
182. S. Betsuki, *Hiroshima J. Med. Sci.* **15,** 25 (1966).

Chapter 2

BILE SALT TRANSPORT SYSTEMS*

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I. INTRODUCTION

There are three sites where the active transport of bile salts has been described. Two of these, the liver and the ileum, are integral parts of the enterohepatic circulation. The third site is the proximal renal tubule, which can reabsorb bile salts from the glomerular filtrate in those situations where appreciable concentrations of bile salts exist in the peripheral (i.e., non-portal) plasma. In addition to these presumed active transport sites, passive transepithelial movement of bile salts has been described in various regions of the gastrointestinal tract. These passive processes play a recognizable role in the enterohepatic circulation of the total bile salt pool, but the quantitative contribution of these passive processes is not precisely known. A number

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of fairly extensive reviews on the transport of bile salts and related substances have appeared (1–3).

The enterohepatic circulation of bile salts involves the cycling of fairly large quantities of material. It has been estimated that the human liver secretes some 30 g of bile salts per day. Of these 30 g, approximately 0.8 g per day is newly synthesized material (4). This emphasizes the efficiency of the intestinal reabsorptive processes. The liver also is remarkably efficient in extracting bile salts from portal blood, as evidenced by the fact that the concentration of bile salts in peripheral plasma is a small fraction of that of portal plasma (5–7). Direct determination of taurocholate and glycocholate extraction by the liver in the dog has been measured by O'Maille *et al.* (8) and found to be 92%.

As used in this chapter, the term "active transport" will of necessity be loosely defined. A rigorous definition would include the requirement that transport occur against an electrochemical gradient. Some authors (9) would also require that this movement be directly coupled to metabolism. Although it would be desirable to be completely rigorous, there is insufficient information concerning the intimate mechanism of transport of bile salts to allow this. For example, it is generally agreed that the hepatic secretion of bile salts represents an active transport process. However, it has not yet been feasible to measure directly the chemical activity of bile salts within the bile canaliculi, nor have appropriate electrical potential measurements been made. In this treatment, we will emphasize those lines of evidence which are consistent with active transport.

II. ACTIVE TRANSPORT IN THE INTESTINE

The existence of a specialized transport system for bile salts might have been predicted from Tappeiner's results at the end of the last century. At that time, it was already known that enteral administration of bile was necessary for the maintenance of normal rates of bile flow in bile fistula animals. Tappeiner identified bile salts as the components of bile responsible for this phenomenon. Furthermore, he found that absorption of bile salts from the intestine occurred only in the ileum (10). The latter finding has in general been amply confirmed (see later). Although subsequent work with more sensitive techniques has demonstrated that bile salts are absorbed from other regions of the gastrointestinal tract, the predominant role of the ileum in absorption is widely accepted. This predominance is attributable to the fact that active transport of bile salts is not found elsewhere in the intestine. This was first recognized in 1961 (11) as the result of experiments utilizing the everted gut sac technique of Wilson and Wiseman (12). The technique

consists of incubating sacs of intestine, previously turned inside out, in a physiological salt solution containing the compound of interest, in this case a bile salt. The sac is filled with an identical solution. In the case of an actively absorbed substance, one observes the depletion of that substance from the mucosal bathing solution and an increase in its concentration in the serosal solution (inside the sac) This results in raising the ratio of concentrations, serosal/mucosal, to values exceeding the initial value of 1. The results of such experiments utilizing rat small bowel are given in Fig. 1. Taurocholate was the bile salt used. Final serosal/mucosal concentration ratios exceeding unity were not found in the first two segments representing the proximal half of the small intestine.

There is some small transport activity in the third quarter of the gut, but the major activity resides in distal fourth. Using smaller segments of rat tissue, Dietschy (3) showed a progressive increase in transport activity within the distal 40% of the intestine. Similar localization of activity has been demonstrated with tissue from pigeons, chickens, mice, hamsters, guinea pigs, and monkeys (13) (see Fig. 2). The absence of this activity in the large intestine was demonstrated by Holt (14).

The high serosal/mucosal concentration ratios demonstrated by the transporting segments do not by themselves constitute sufficient evidence for an active process. However, two alternative interpretations have been excluded. First, the results cannot be attributed to the ion-trapping mechanism whereby a nonpenetrating anion of a diffusible acid will be concentrated in the compartment of higher *p*H (15). In fact, the final *p*H differences between the serosal and mucosal fluids operate against such a mechanism (see Table I).

A second possibility would have the ion distribute itself between the two compartments in accordance with the transmucosal electrical potential.

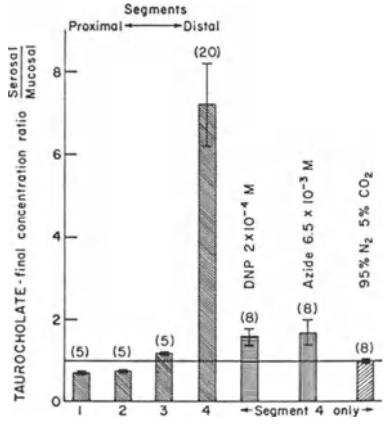


Fig. 1. Transport of taurocholate by everted sacs of rat intestine. Each segment was one-quarter the length of the small intestine. Numbers in parentheses represent the number of animals in each group. I bars give ± SE. DNP, 2,4-dinitrophenol. From Lack and Weiner (11).

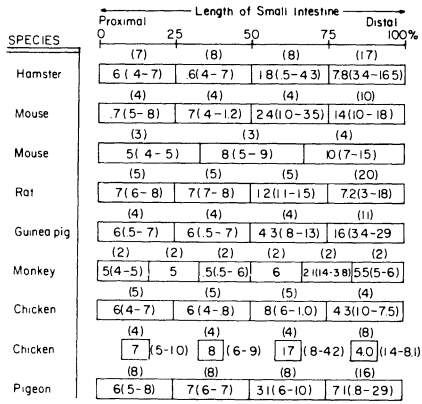


Fig. 2. Final serosal/mucosal concentration ratios of taurocholate established by everted sacs of small intestine from seven species. Segments are drawn to indicate positions and relative sizes of the intestinal segments. With either of the two species mouse and chicken, two different methods of dividing the intestine were employed. These are indicated on separate lines. Values given are means, with ranges indicated in parentheses within or alongside segments. Numbers above segments indicate number of animals. From Glasser *et al.* (13).

TABLE I. Final *p*H Gradients of Serosal and Mucosal Media after 90 min Incubation of Everted Gut Sacs with Taurocholate[a]

*p*H		Taurocholate concentration
Mucosal	Serosal	ratio, serosal/mucosal
	Rat	
7.50	6.70	7.7
7.48	6.72	11.3
7.55	6.65	19.0
	Guinea pig	
7.45	6.68	33.0
7.50	6.75	34.0
7.55	6.85	25.0

From Lack and Weiner (11).
[a] Initially, the *p*H was 7.3 in both compartments.

However, these potentials are too small to account for the observed concentration ratios (16). Moreover, gut preparations in which the transmucosal potential was put at zero by a short-circuiting technique could still develop such concentration differences (17).

Consistent with the notion that this is an active process was the observation that metabolic inhibitors compromised transport (Fig. 1). The mechanism of coupling taurocholate translocation to metabolic energy is poorly understood. Some evidence is consistent with recent hypotheses developed for the transport of other organic compounds. An example of these hypotheses was presented by Curran *et al.* (18) and involves the interaction of sodium ions and amino acid transport. It is postulated that the uphill transport of amino acids into the mucosal cell depends on the maintenance of high extracellular and low intracellular sodium ion concentrations. The high extracellular sodium concentration, by enhancing sodium binding to a

postulated membrane carrier, simultaneously increases the affinity of the same carrier for the amino acid. This complex dissociates more readily on the intracellular face of the membrane, where Na ions are more dilute. This produces a higher concentration of the amino acid within the cell and provides a favorable concentration gradient for its egress from the basal side of the cell. This process is totally dependent on the maintenance of a concentration difference of sodium across the cell membrane; thus only the outflow of sodium need be coupled to metabolic energy. In light of this and other related hypotheses, it is of interest that the ileal transport of taurocholate is dependent on the presence of extracellular sodium ions (14,19).

Ouabain inhibits sodium extrusion from the cell and thereby obliterates its critical concentration differences. Ouabain acts on the energy utilization step for the active extrusion of sodium ions, as evidenced by its inhibition of "transport adenosine triphosphatase" (20). The inhibition of bile salt transport by ouabain (21,22) resembles similar actions against other transport systems (e.g., those for amino acids and sugars). The omission of cations other than sodium from the incubation media does not greatly impair taurocholate transport (22).

A characteristic of all thoroughly studied active transport systems is structural specificity. In general, however, the structural requirements for transport are considerably less restrictive than those for many enzymes. A large variety of cholanic acid derivatives are susceptible to active ileal transport. Thus all naturally occurring bile salts and a number of synthetic analogues have been shown to be transported. In all cases, active movement was restricted to the distal small bowel (Fig. 3). This discussion of structural requirements will include the following: (1) hydroxylation of the steroid nucleus, (2) steric configuration of the cholanic acid moiety, (3) the nature of the amino acid grouping in the conjugated bile acid, and (4) the ionic state of the bile salt.

No single hydroxyl group appears to be essential for transport. Thus compounds 9, 11, and 15 of Fig. 3, which are derivatives of taurocholate each lacking one of the three hydroxyl groups, are transported. Compound 17, glycodehydrocholic acid, a triketo compound possessing no hydroxyl groups, is also transported. This again suggests that ring hydroxylation is not required.

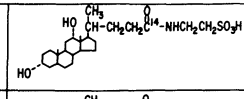
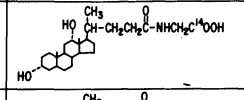
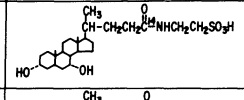
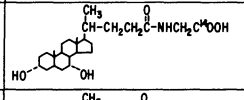
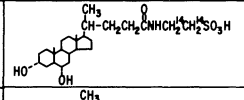
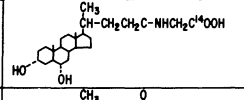
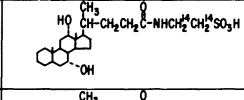
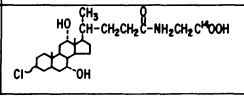
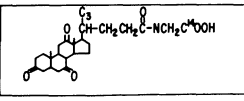
Bile salts in general belong to the coprostane group of cholesterol derivatives, which entails the β-configuration for hydrogen at position 5. Alteration of the molecule to the 5α-configuration does not preclude transport (Alan Hofmann, personal communication).

Bile acids conjugated with either taurine or glycine may be transported: indeed, the ileal system can transport cholic acid itself. When an analogue of taurocholate containing a positively charged group (compound 8, Fig. 3)

COMPOUND	FORMULA	FINAL SEROSAL/MUCOSAL CONCENTRATION RATIOS			
		1st Quarter	2nd Quarter	3rd Quarter	4th Quarter
TRIHYDROXYCHOLANIC ACID DERIVATIVES					
1 Cholic Acid		.63 (.52-.72)	.59 (.56-.64)	2.9 (2.8-3.1)	3.7 (2.5-5.1)
2 Taurocholic Acid		.59 (.53-.65)	.62 (.48-.68)	4.3 (.80-13.0)	13.0 (3-26)
3 Glycocholic Acid		.85 (.82-.87)	.87 (.82-.90)	2.6 (1.2-3.3)	11.2 (10.0-13.3)
4 N-Cholyl n Saminovaleric Acid		.52 (.50-.56)	.52 (.50-.56)	1.4 (1.1-1.8)	5.2 (2.7-7.6)
5 N-Cholylaspartic Acid		.68 (.64-.77)	.62 (.55-.70)	.79 (.70-.98)	1.2 (1.1-1.3)
6 N-Cholyl-O-phosphorylethanalamine		.63 (.52-.80)	.56 (.51-.60)	.51 (.40-.55)	.85 (.79-.88)
7 N-Cholylaminoethylphosphonic Acid		.63 (.60-.80)	.78 (.70-.80)	1.3 (.86-1.7)	2.0 (2.0-2.1)
8 N-Cholyl-N^2 Trimethylethylenediamine		.69 (.68-.70)	.72 (.68-.75)	.77 (.70-.78)	.71 (.67-.75)

Fig. 3. Transport of various bile acids by everted gut sacs of guinea pig small bowel. Values are means of three to six sacs. The range of values is given in parentheses. From Lack and Weiner (22).

was tested, no transport was observed. This material at the concentrations tested did not interfere with the transport of its naturally occurring analogue. The apparent requirement for a negative charge on the side chain prompted studies of bile salts containing two negative charges. In these instances, transport was minimal. This suggests that a single negative charge on the side chain is essential for activity. This hypothesis was supported by transport studies in media of different *p*H. As illustrated in Figs. 4a and 4b, the transport of dibasic bile acids is enhanced relative to the transport of monobasic bile acids at lower *p*H. Since lower *p*H increases the concentration of singly charged species of the dibasic acids, this result is consistent with the idea that a single negative charge on the side chain is a requirement for transport (23). Further support for this notion is provided by the demon-

COMPOUND	FORMULA	FINAL SEROSAL/MUCOSAL CONCENTRATION RATIOS			
		1st Quarter	2nd Quarter	3rd Quarter	4th Quarter
DIHYDROXYCHOLIC ACID DERIVATIVES					
9 Taurodeoxycholic Acid		.63 (.54-.68)	.70 (.63-.83)	4.0 (2.1-5.3)	5.5 (5.3-6.5)
10 Glycodeoxycholic Acid		.55 (.52-.60)	.58 (.44-.65)	1.5 (1.0-2.0)	1.8 (1.3-2.2)
11 Taurochenodeoxycholic Acid		.67 (.63-.70)	.66 (.62-.69)	3.7 (2.1-5.3)	6.5 5.3-6.5
12 Glychochenodeoxycholic Acid		.63 (.55-.70)	.65 (.60-.73)	1.2 (.80-2.0)	2.1 (1.6-2.3)
13 Taurohyodeoxycholic Acid		.69 (.67-.75)	.65 (.66-.78)	4.2 (3.0-5.5)	5.7 (5.0-6.8)
14 Glycohyodeoxycholic Acid		.78 (.70-.80)	.70 (.65-.76)	2.4 (1.5-3.7)	6.9 (4.6-8.3)
15 7,12 Dihydroxycholanyltaurine		.66 (.67-.71)	.62 (.60-.63)	2.9 1.2-3.9	4.5 (4.1-5.5)
16 N-3-chloro-7,12 dihydroxycholanylglycine		.67 (.56-.72)	.65 (.57-.69)	1.5 (.99-2.0)	2.0 (1.3-2.7)
TRIKETOCHOLANIC ACID DERIVATIVE					
17 Glycodehydrocholic Acid		.81 (.78-.85)	.83 (.79-.89)	1.1 (.95-1.3)	3.4 (3.2-3.6)

stration that dibasic acids are more effective competitors for ileal bile salt transport when *p*H of the incubation medium is decreased (Table II).

Apparently, the requirement for a single negative charge applies primarily to the region of the side chain. The introduction of an additional negative charge at the 3-position of ring A does not preclude transport. This was demonstrated with the 3α-sulfate ester of taurolithocholic acid (24). This is one of a series of recently described natural bile salts (25).

A characteristic feature of active transport mechanisms is the existence of a maximal rate, which is ascribed to saturation kinetics analogous to the kinetics for enzymic reactions (Michaelis–Menton). A maximal rate for the transport of taurocholate can be demonstrated *in vitro* and *in vivo* (11,14,17, 19). Unfortunately, the interpretation that the observed maximal transport

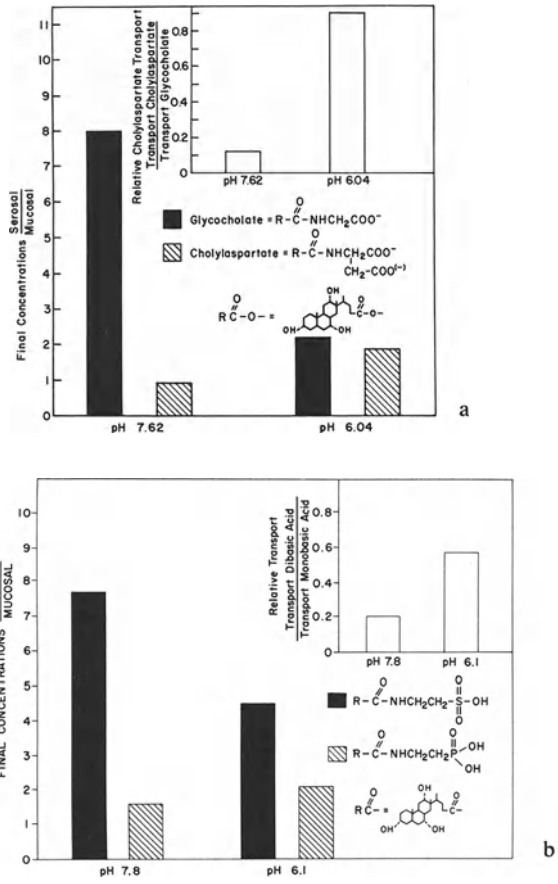


Fig. 4. (a) Effects of *p*H of incubation medium on the relative transport of glycocholate and *N*-cholylaspartate by everted sacs. (b) Effect of *p*H of incubation medium on the relative transport of taurocholate and *N*-cholylaminoethylphosphonic acid. The dibasic compound is a relatively better substrate for transport at the lower *p*H, presumably because a greater proportion of the molecules exist as the singly charged species. The pK_a of taurocholate (1.54) is such that the quantity of anion present is not significantly different at the two *p*H values. From Lack and Weiner (23).

rates are characteristic of the transport system exclusively may not be entirely valid. This uncertainty stems from the observation that bile salts can be rather nonspecific inhibitors of a variety of intestinal functions. In addition to some metabolic alterations (26), bile salt preparations had been shown to

TABLE II. Effect of *p*H on the Inhibition of Bile Salt Transport by *N*-Cholylaspartate[a]

Expt. No.	Final *p*H	Substrate	Initial concn. (mM)	Inhibitor	Concn. (mM)	Substrate[b] transported (μmoles)	Control activity (%)
A	7.7	Glycocholate	0.24	None	—	1.55	100
	7.7	Glycocholate	0.24	*N*-cholylaspartate	0.56	1.42	92
	6.1	Glycocholate	0.24	None	—	0.98	100
	6.1	Glycocholate	0.24	*N*-cholylaspartate	0.56	0.58	59
B	7.8	Taurocholate	0.20	None	—	1.36	100
	7.8	Taurocholate	0.20	*N*-cholylaspartate	0.28	1.38	102
	6.2	Taurocholate	0.20	None	—	1.06	100
	6.2	Taurocholate	0.20	*N*-cholylaspartate	0.28	0.80	75
C	7.8	Taurocholate	0.20	None	—	1.51	100
	7.8	Taurocholate	0.20	*N*-cholylaspartate	0.56	1.34	88
	6.1	Taurocholate	0.20	None	—	1.20	100
	6.1	Taurocholate	0.20	*N*-cholylaspartate	0.56	0.77	64

From Lack and Weiner (23).

[a] Incubation was at 37°C for 60 min. Krebs–Ringer phosphate solution was used without Ca^{2+} but with three times the conventional amount of phosphate buffer. All values are the mean of four gut sacs. Uninhibited transport is assigned 100%.

[b] Removed from the mucosal compartment.

inhibit the transmucosal transport of sugars, amino acids, and electrolytes *in vitro* (27–29). Originally, these effects were even observed in the jejunum, a region where the bile salts themselves are not transported. Many of these pharmacological actions in the jejunum were clearly shown by Pope *et al.* (30) to be attributable to unconjugated bile salts present as contaminants. Indeed, Lack and Weiner (22), working with highly purified conjugated bile salts, had previously observed that inhibition of jejunal transport of glucose or tyrosine did not occur. However, they found that ileal transport of these substances was seriously compromised by the presence of these purified bile salts. This difference in sensitivity of the two regions of the intestine is probably attributable to the fact that bile salts are concentrated within the cells that transport them. Thus the maximal transport rate for bile salts seen at elevated concentrations may be a reflection of general depression of cell function in addition to the saturation of the transport system. These results were obtained from experiments involving guinea pig tissue. Whether or not they apply to kinetic data with tissue from other species (see below) is not known.

The fact that the active transport of all bile salts occurs in the same anatomical site allows the consideration that they are transported by a single system. This conjecture is supported by demonstrations that the mutual inhibition of transport between pairs of bile salts in hamster and rat ileum appears to follow the kinetic patterns consistent with competition (19,31). Lack and Weiner (22), who made a fairly extensive study of mutual inhibition between pairs of bile salts with guinea pig ileum *in vitro,* did not attempt a similar kinetic treatment because of the above-mentioned observations concerning the general transport depression caused by the bile salts. In this tissue, the fractional depression of the transport of one bile salt by another was the same as the fractional depression of glucose or tyrosine transport. In *in vivo* experiments involving the perfusion of ileal segments, Heaton and Lack (32) were able to demonstrate mutual inhibition of transport with different pairs of bile salts without this complication (see Fig. 5a and 5b). This is consistent with competitive interaction. As might be expected, inhibition of one bile salt by another was found to be less marked in the *in vivo* studies.

In all the aforementioned findings, dihydroxy bile salts were more potent inhibitors than the trihydroxy compounds. This is demonstrated for the *in vivo* studies in Table III. This would imply that the affinity of K_m for bile salts containing two hydroxyl groups was greater than that for the trihydroxylated substances. However, Schiff and Dietschy (33) in a preliminary report state that the affinity constant of the conjugated bile salts was independent of the number of hydroxyl groups. At the time of writing, it is impossible to resolve this apparent contradiction.

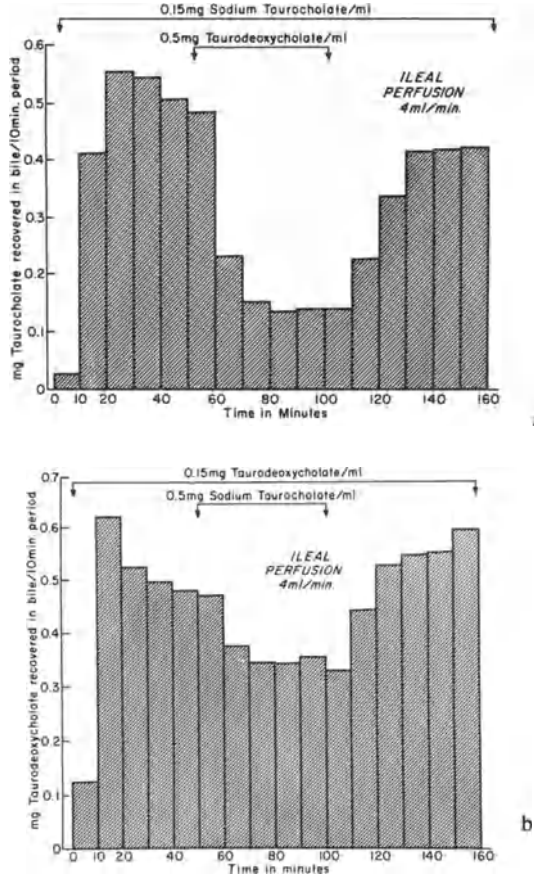


Fig. 5. (a) Effect *in vivo* of taurodeoxycholate on the ileal transport of sodium taurocholate. Guinea pigs maintained under light ether anesthesia were used. In each experiment, the cystic bile duct was tied off and the common bile duct cannulated for bile collections. Ileal segments, 20 cm, were perfused in the following order: (1) a saline solution containing 0.15 mg sodium taurocholate, (2) a solution containing 0.15 mg sodium taurocholate plus 0.5 mg sodium deoxycholate, and (3) the original solution with only sodium taurocholate. The arrows indicate the order of addition. Transmucosal movement, i.e., intestinal transport, was quantitated by measuring the rate of appearance of the perfused taurocholate in the bile. Each bar represents the total recovery for a 10-min period. (b) Effect of sodium taurocholate on the *in vivo* ileal transport of taurodeoxycholate. From Heaton and Lack (32).

TABLE III. Mutual Inhibition *in Vivo* Between Different Pairs of Bile Salts in the Guinea Pig[a]

Expt. No.	Substrate	Concn. (μmole/ml)	Inhibitor	Concn. (μmole/ml)	Substrate transported (μmole/10 min) Control periods[b] (−inhibitor)	Substrate transported (μmole/10 min) Experimental[c] (+inhibitor)	Percent inhibition
1	Taurocholate	0.28	Taurodeoxycholate	0.96	0.54	0.16	70
2	Taurocholate	0.28	Taurodeoxycholate	0.96	0.89	0.24	73
2A	Taurocholate	0.28	Taurodeoxycholate	0.96	0.90	0.25	72
3	Taurocholate	0.28	Taurodeoxycholate	0.96	0.72	0.29	60
4	Taurocholate	0.28	Taurodeoxycholate	0.96	0.54	0.17	68
5	Taurocholate	0.28	Glycodeoxycholate	1.06	0.60	0.14	81
6	Taurocholate	0.28	Taurochenodeoxycholate	0.96	0.47	0.18	62
7	Taurocholate	0.28	Glycochenodeoxycholate	1.06	0.43	0.14	67
8	Glycocholate	0.32	Glycodeoxycholate	1.06	0.35	0.14	60
9	Taurodeoxycholate	0.29	Taurocholate	0.94	1.06	0.68	36
10	Taurodeoxycholate	0.29	Taurocholate	0.94	0.60	0.41	32
11	Glycochenodeoxy-cholate	0.32	Taurocholate	0.94	1.07	0.66	39
12	Taurocholate	0.28	Glycodehydrocholate	1.10	0.97	0.91	5
13	Glycodehydro-cholate	0.33	Taurocholate	0.94	0.39	0.16	59
14	Glycodehydrocholate	0.33	Taurocholate	0.94	0.38	0.15	61

From Heaton and Lack (32).

[a] The experimental details are explained in the caption of Fig. 5A.

[b] The control values were obtained from the mean of the two immediate preinhibition periods averaged with the highest recovery in the post-inhibition period.

[c] The experimental (inhibitor) values are obtained from the biliary recoveries of the last three periods of inhibition.

III. PASSIVE PROXIMAL INTESTINAL ABSORPTION OF BILE SALTS

Dietschy *et al.* (17) have defined in terms of current concepts the factors involved in the passive absorption of bile salts across the mucosa of the jejunum. Three processes have been considered. One is movement of bile salts with the osmotic flow of water. The important parameter in this regard is the reflection coefficient, which can be visualized as the sieving effect of the membrane to retard the movement of bile salts when water moves across the membrane. The values of this coefficient may vary between 0 (no sieving) and 1 (complete retardation). The values for taurocholate and cholate average 0.96, indicating that this path for absorption is virtually inoperative.

A second mode of movement is passive ionic diffusion. In this process, the bile salt anion moves through the membrane barrier driven by electrochemical difference. Anions of both conjugated and unconjugated bile salts are involved in this process.

The final passive mechanism is nonionic diffusion. In this case, the solute diffuses as the undissociated acid through the cell membranes, which act as lipid barriers. Concentration difference of the undissociated acid is the driving force. The extent of this process is determined by three factors. The first two, pH of the medium and pK_a of the bile acid, define the proportion of molecules in the undissociated state. The third factor, solubility in the lipid membrane, determines the ease of penetration. Taurocholic acid, by virtue of its poor solubility in lipid-like phases and its very low pK_a, does not participate significantly in this process. On the other hand, unconjugated cholic acid and other unconjugated acids which have appropriate physiochemical properties can be extensively absorbed by this mechanism. Under normal

TABLE IV. Absorption of Taurocholate from Proximal and Distal Small Intestine in Anesthetized Animals[a]

Species	Load (mg)	Absorption in 90 min[b] (%)		
		Proximal	Distal	Ratio
Rabbit	183	0.9	51.4	57.0
Rat	15	13.2	83.4	6.3
Guinea pig	30	1.6	40.3	25.0
Dog	183	13.2	84.8	6.4
Spider monkey	86	0.2	19.9	100.0
	172	2.3	32.0	13.9
	344	4.0	8.4	2.1

From Glasser *et al.* (13).

[a] Separate animals were used for proximal and distal studies. Figures for monkeys represent values from individual animals; for all other, the mean of three or four animals.

[b] Cumulative radioactivity collected in common bile duct expressed as percent of amount injected into the intestinal segment.

physiological conditions, this is not a significant process in the jejunum since the bile salts in this region are of the conjugated variety.

Studies of proximal passive diffusion have demonstrated that consideration of the species of animal as well as the experimental technique is critical. As shown in Table IV, the rate of taurocholate egress from the proximal small bowel is negligible in the rabbit and guinea pig but is significantly higher in other species. In the case of those species having gallbladders, one would expect bile to be present in the intestine for the most part only during the digestion of a meal. The mixture of bile salts, other bile constituents, digesting foodstuffs, and other secretions represents a millieu much different from the experimental conditions of Table IV, where simple buffered solutions of bile salts were placed in tied-off segments of intestine in anesthetized animals. In these experiments, the dog returned a significant amount of taurocholate to the liver from tied-off segments, suggesting that in this species a degree of proximal (i.e., passive) absorption of taurocholate is possible. However, studies of the enterohepatic circulation in dogs with resections of various segments of the intestine strongly suggest that in the functioning state minimal absorption takes place proximally. Playoust *et al.* (34) studied the turnover of radioactive taurocholate in five normal animals and five with resection of the proximal small bowel. The results with both groups were identical. However, with ileal resection the digestion of one fatty meal resulted in almost complete loss of the radioactivity. Representative experiments comparing results from dogs with ileal and jejunal re-

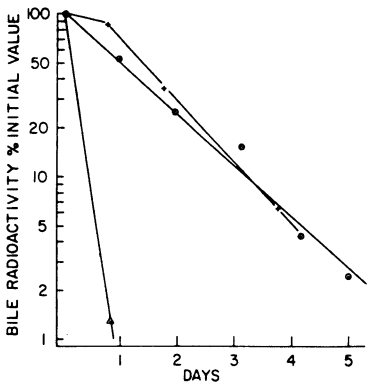


Fig. 6. Decline of radioactivity in the bile of dogs given sodium taurocholate-^{14}C. The upper curves are from two experiments in the same dog with jejunal resection. The lower curve is from an experiment using a dog with resection of the ileum. Sampling of bile was achieved through a plastic tube placed in the gallbladder and exteriorized through a stab wound. The gallbladders functioned normally. Experiments were initiated after a 24-hr fast by the intravenous injection of sodium taurocholate-^{14}C. Five hours later, the gallbladder was emptied, the volume determined, and a small aliquot used for analysis. The bulk of the bile was returned to the gallbladder. The dog was then allowed to eat its daily meal. This procedure was repeated at 24-hr intervals. The peak of radioactivity in bile is assigned a value of 100 and its occurrence is assigned at zero time. These are representative experiments of a study involving five dogs with ileal resection, five dogs with jejunal resection, and five sham-operated animals. The experiments were performed several weeks after surgery. From Playoust *et al.* (34).

sections are presented in Fig. 6. The apparent discrepancy between the experiments with tied-off segments of intestine *in situ* and these results can probably be resolved by the observation of Small (cited in reference 3). He found that taurocholate absorption by diffusion was considerably less in the presence than in the absence of phospholipid. Presumably, this phenomenon is attributable to the fact that taurocholate is incorporated into bulkier mixed micellar particles with phospholipid.

In man, the enterohepatic circulation of taurocholate and glycocholate has been studied in subjects lacking varying lengths of the ileum. These investigations employed in principle essentially the same experimental design that was used with the resected dogs, and they are discussed in greater detail and extended context by Tyor in Chapter 4 of this volume. In the absence of the ileum, labeled bile salts virtually disappeared from the hepatic biliary space within 24 hr, suggesting that the duodenojejunum participates to a minimal extent in the enterohepatic circulation. However, during the initial 24 hr these patients consumed three meals. It has been estimated that under normal physiological conditions bile salts circulate at least twice per meal (35). It is possible that in these situations removal of the ileum resulted in only a fractional loss in intestinal bile salt absorption. Such a loss compounded several times could cause a complete loss of labeled bile salts within 24 hr. Recently, Van Deest *et al.* (36) measured the disappearance of injected radioactive taurocholate in ileectomized patients over shorter time periods. They reported a half-life based on the decline of specific activity of taurocholate to be as low as 1.3 hr, a value consistent with the idea that taurocholate absorption from the proximal regions is negligible and that the ileum is the major site for transmucosal movement of this primary bile acid. Low-Beer *et al.* (37), employing a different approach involving double isotopes, studied the enterohepatic circulation of another bile salt, glycocholate, following the digestion of one meal in ileectomized and normal subjects. A measured amount of ^{14}C-labeled glycocholate of known specific activity was injected intravenously 2 hr prior to the evening meal. The next morning before breakfast, a known dose of tritium-labeled glycocholate was injected, the patient was intubated with a catheter to permit sampling of the duodenal contents, and the gallbladder was emptied by the administration of cholecystokinin. From the bile recovered via the duodenum, a sample of pure glycocholic acid was isolated and assayed simultaneously for its specific activity based on tritium and carbon-14. The tritium dilution allowed estimation of total glycocholate present in the hepatic and biliary space prior to the administration of cholecystokinin. From this and the specific activity of the carbon-14 in glycocholic acid, one calculates the total glycocholic acid returning to the liver and gallbladder after the digestion of one meal. The results of these studies in four patients with ileectomy indicate that little glycocholate leaves the alimentary tract during the digestion of one meal. The

average retention in these patients was 3.6%, compared with 68% for normal controls. Thus it would seem that under physiological conditions passive diffusion of both glyco- and taurocholate is of minor quantitative significance.

In contrast to the results with trihydroxy acids, Hislop *et al.* (38) reported in a preliminary communication that glycochenodeoxycholic acid and glycodeoxycholic acid can be absorbed from the jejunum. The authors employed a triple lumen tube to perfuse 50-cm jejunal segments. Twenty-five percent of these glycine conjugates were taken up, compared with 3.3% of the taurine conjugates. The authors considered the value for absorption of the conjugates of taurine negligible. Their results with the glycine conjugates, however, suggest that a considerable fraction of the total enterohepatic circulation of bile salts is via the jejunal region, since glycochenodeoxycholic acid and glycodeoxycholic acid comprise as much as 45% of the bile salt pool of man. It is not known whether these findings are applicable in conditions with normal intestinal contents. A consequence of proximal absorption would be conservation of the glycine-conjugated dihydroxy bile salts in subjects lacking an ileum. In a single patient, Hardison and Rosenberg (39) report that ileectomy resulted in the depletion of the deoxycholate pool, a condition which would be unexpected if circulating glycodeoxycholate could return in considerable amounts to the liver via absorption from the proximal segments.

IV. PASSIVE ABSORPTION OF BILE SALTS IN THE LOWER GASTROINTESTINAL TRACT

Direct measurement of passive transport in the ileum has not been made because of the presence of the active mechanism. It has been assumed that these processes are quantitatively similar to those in the jejunum. However, there are known differences in permeability characteristics of various parts of the small bowel (40).

Unlike the normal proximal small intestine, there have been reports of appreciable quantities of unconjugated bile acids in the ileum (41). This is not a uniform finding (42) and may reflect differences in diet or other experimental variables. Insofar as there are appreciable quantities of unconjugated acids, which undoubtedly arise from bacterial hydrolysis, nonionic diffusion plays an important role.

It is known from the work of Norman and Sjövall (42) that approximately 50% of bile salts injected into the cecum of a rat is absorbed and re-enters the enterohepatic circulation. In all cases, hydrolysis of conjugated bile salts precedes this absorption. A large fraction of the bile salts are additionally

modified to less polar derivatives before absorption by bacterial action. Both of these phenomena would favor a nonionic diffusion mechanism in this region of the gastrointestinal tract, which lacks an active transport system (14). Dietschy has recently shown that nonionic diffusion from the large bowel can indeed occur (cited in reference 36).

V. THE TRANSPORT OF BILE SALTS IN THE LIVER

At the present time, the most widely accepted hypothesis concerning the formation of bile attaches primary importance to the secretion of organic anions, of which the bile salts are quantitatively most important. According to this hypothesis, it is the osmotic effect exerted by these substances which provides the driving force for the ingress of water, electrolytes, and other substances (9,43–45) into the canaliculi. Subsequently, the bile so formed is modified in the hepatic portions of the biliary tree. In animals equipped with gallbladders, considerable concentration occurs during storage. This latter process is effected by the active transport of sodium chloride out of the gallbladder followed by the osmotic extraction of water (46). Bile salts and other organic compounds do not traverse the gallbladder epithelium to a large extent and are thereby concentrated (47,48). The organic anion secretion hypothesis regarding the formation of canalicular bile requires that secretion of these anions be an active transport process. There are three lines of evidence which have been used to support this: (1) the high hepatic bile/plasma concentration ratios of bile salts, (2) the apparent saturation kinetics of the secretory process, and (3) the evidence for competitive interactions in the transport of different anions. As indicated in the *Introduction,* all of this evidence is strongly suggestive but does not constitute rigorous proof of active transport.

Concentration ratios of taurocholate between hepatic bile and plasma have been reported to be as high as 1000. However, since bile salts tend to aggregate into micelles (see Small, Chapter 8 of Volume 1), the actual activity ratios are not as high.

The maximal capacity for secretion of taurocholate in chickens is 10 μmoles/min/kg (45). The corresponding figures for rat and dog are 15 and 6–8.6, respectively (8,45). The maximal rate for glycocholate in the dog is approximately the same as that for taurocholate (8). There is a vast literature on the extraction and excretion of anionic dyes by the liver. This has been amply reviewed by Sperber (43), who also marshalled the evidence supporting the notion that these substances are secreted by the same mechanism that applies to the bile salts. The major evidence is depression of dye excretion following the administration of taurocholate, glycocholate, dehydrocholate,

and cholate. The converse result, depression of taurocholate by bromcresol green, has also been demonstrated. Not all workers have observed inhibition of dye excretion by taurocholate administration. In fact, some have observed enhancement (49). These results are not necessarily inconsistent with the foregoing hypothesis. A variety of other physiological factors such as changes in bile flow rate (45) or hepatic blood flow (50) might account for these apparently divergent results. There are, however, two recent observations which may require partial re-evaluation of the notion that bile salts and dyes are handled by identical mechanisms. Humans exhibiting the Dubin–Johnson syndrome and a mutant strain of sheep have apparently normal excretory function for taurocholate but almost completely impaired excretion of bromsulfophthalein (BSP) and other organic anions (51,52). This might be interpreted as evidence for separate excretory pathways for bile salts and BSP.

If it is of interest that biliary storage of BSP in patients with the Dubin–Johnson syndrome fell within the normal range (52,53), allowing the possibility that the mechanism for the extraction of the dye from plasma by the liver is unimpaired. Thus one may speculate that the site of competition between bile salts and anionic dyes is at the hepatic uptake (or storage) stage and that subsequent secretion from the cell into the canaliculus of the two substances may proceed by separate mechanisms.

VI. RENAL TRANSPORT OF BILE SALTS

In the absence of disease, bile salt concentrations in peripheral plasma are quite low. Human serum values from normal subjects vary from 0.03 to 0.23 mg/100 ml (54). Higher levels are seen after feeding, apparently caused by a small fractional escape of bile salts from the enterohepatic circulation, over which traffic is greatly enhanced by feeding in species with gallbladders. Peripheral blood values averaging 0.08 mg/100 ml have been reported for rats (6) and peripheral serum values of 0.3 mg/100 ml for cebus monkeys (7). Of course, higher values are encountered in hepatic disease, with correspondingly greater loads presented to the kidneys (55).

Because of results (see below) suggesting an interaction of bile salts with the renal tubular mechanism for organic anion secretion, it is interesting to consider the consequences of efficient renal secretion of these substances, were it to occur. At a mean plasma level of 1 μg/ml and renal plasma flow of 700 ml/min, urinary excretion would be 1 g/day. This exceeds the daily turnover of bile salts in man and is many times greater than the highest excretory rates observed.

Rudman and Kendall (55) were the first to appreciate that the observed

excretory rates of bile salts in urine required the participation of a reabsorptive mechanism in man. First, they demonstrated that circulating bile salts were extensively bound to plasma proteins, mainly albumin (56). They calculated the renal clearances of bile salts based on estimations of unbound plasma concentrations and derived values much lower than reasonable values of filtration rate.

This was confirmed in dogs (57) in a study which also demonstrated (for cholic, taurocholic, and glycocholic acids) that reabsorption occurs in the proximal tubule (Fig. 7) and that the reabsorptive process involves active transport. The latter conclusion was based on estimates of the tubular fluid/ultrafiltrable plasma concentration ratio for taurocholate and an estimate of transtubular electrical potential taken from the literature. In addition, it was shown that the reabsorptive process for bile salts had a maximal rate (saturation kinetics) (Fig. 8).

Although entirely consistent with the earlier clinical evidence, this work did not provide an explanation for observations which suggested (but did not require) an interaction of bile salts with a secretory process. The latter was inferred from the observation of Woo and Hong (58) that bile salts inhibit

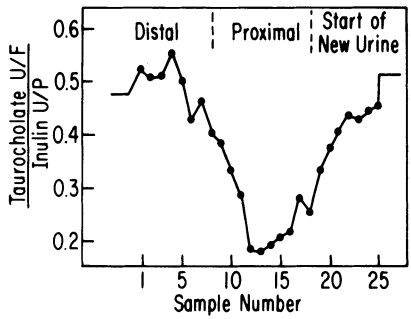


Fig. 7. Stop-flow analysis of taurocholate reabsorption. This experiment was performed during the intravenous infusion of taurocholate and inulin into a dog undergoing massive osmotic diuresis. After an equilibration period, urinary outflow from the kidney was stopped by occluding the ureter, leaving the urine in contact with the various tubular segments for several minutes. This allowed additional time for the operation of transport processes, thus exaggerating their effect on the composition of the urine. After release of the occlusion, small serial samples of urine were collected for analyses. Samples of urine exposed to the more distal nephron segments emerged first. More precise anatomical localization was provided by appropriate marker substances (not shown). The results are expressed as the concentration of taurocholate in urine divided by the concentration in plasma ultrafiltrate (U/F) factored by the corresponding ratio for inulin (U/P). The latter corrects for concentration differences caused by the movement of water, since inulin cannot cross the tubular epithelium. The lowest ratios occur in the samples exposed to the proximal tubular epithelium, indicating that this is the site of active transport. A thorough discussion of this technique is given by Malvin (61). From Weiner *et al.* (57).

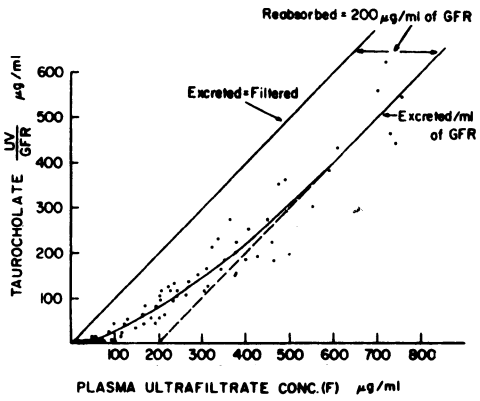


Fig. 8. Relationship of excretion to filtered load. Values for excretion (UV) have been divided by glomerular filtration rate (GFR) to correct for differences among dogs. The dashed line depicts the idealized situation in which maximal rate of reabsorption is 20 mg/min, GFR is 100 ml/min, and reabsorption is complete until the maximal rate is attained. Obviously, the actual approach to the maximal rate is more gradual. From Weiner *et al.* (57).

renal secretion of other organic anions as well as the structural similarity of conjugated bile salts and known substrates for tubular secretion.

At least partial clarification is provided by recent work fo Zins and Weiner (59) which demonstrated a secretory flux for taurocholate in the proximal tubule which can be inhibited by the administration of *p*-aminohippurate, a secreted substance. This flux, which presumably traverses the well-known mechanism for organic anion secretion, is normally overshadowed by the larger active reabsorptive process which occurs in the same tubular segment. This is not a unique example of bidirectional transport in a single tubular segment; uric acid is similarly handled (60).

REFERENCES

1. J. R. Senior, *J. Lipid Res.* **5,** 495 (1964).
2. I. M. Weiner and L. Lack, *in* "Handbook of Physiology, Alimentary Canal," p. 1439, Academic Press, New York (1968).
3. J. M. Dietschy, *J. Lipid Res.* **9,** 297 (1968).
4. S. Bergström, *Fed. Proc.* **20,** 121 (1961).
5. T. Olivercrona and J. Sjövall, *Acta Physiol. Scand.* **46,** 284 (1959).
6. S. M. Grundy and J. Sjövall, *Proc. Soc. Exptl. Biol. Med.* **107,** 306 (1961).
7. O. W. Portman and S. Shah, *Arch. Biochem. Biophys.* **96,** 516 (1962).

8. E. R. L. O'Maille, T. G. Richards, and A. H. Short, *J. Physiol.* **189,** 337 (1967).
9. H. O. Wheeler, *in* "Progress in Liver Diseases" (H. Popper and F. Schoffner, eds.), Vol. 2, p. 15, Grune & Stratton, New York (1965).
10. F. Verzar, and E. J. McDougal, "Absorption from the Intestine," p. 218, Longmans, London (1936).
11. L. Lack and I. M. Weiner, *Am. J. Physiol.* **200,** 313 (1961).
12. T. H. Wilson and G. Wiseman, *J. Physiol. (London)* **123,** 116 (1954).
13. J. Glasser, I. M. Weiner, and L. Lack, *Am. J. Physiol.* **208,** 359 (1965).
14. P. R. Holt, *Am. J. Physiol.* **207,** 1 (1964).
15. B. B. Brodie and C. A. Hogben, *J. Pharm. Pharmacol.* **9,** 345 (1957).
16. P. F. Curran and A. K. Solomon, *J. Gen. Physiol.* **41,** 143 (1957).
17. J. M. Dietschy, H. S. Solomon, and M. D. Siperstein, *J. Clin. Invest.* **45,** 832 (1966).
18. P. F. Curran, S. G. Schultz, R. A. Chez, and R. E. Fuisz, *J. Gen. Physiol.* **50,** 1261 (1967)
19. M. R. Playoust and K. J. Isselbacher, *J. Clin. Invest.* **43,** 467 (1964).
20. J. C. Skou, *Physiol. Rev.* **45,** 596 (1965).
21. T. M. Parkinson, *Biochim. Biophys. Acta* **86,** 425 (1964).
22. L. Lack and I. M. Weiner, *Am. J. Physiol.* **210,** 1142 (1966).
23. L. Lack and I. M. Weiner, *Biochim. Biophys. Acta* **135,** 1065 (1967).
24. T. Low-Beer, M. P. Tyor, and L. Lack, unpublished work.
25. R. H. Palmer, *Proc. Natl. Acad. Sci.* **58,** 1047 (1968).
26. A. M. Dawson and K. J. Isselbacher, *J. Clin. Invest.* **39,** 730 (1960).
27. R. G. Faust, *J. Cell. Comp. Physiol.* **63,** 55 (1964).
28. A. S. Nunn, R. D. Baker, and G. W. Searle, *Life Sci.* **9,** 646 (1963).
29. T. M. Parkinson and J. A. Olson, *Life Sci.* **2,** 393 (1963).
30. J. L. Pope, T. M. Parkinson, and J. A. Olson, *Biochim. Biophys. Acta* **130,** 218 (1966).
31. P. R. Holt, *Am. J. Physiol.* **210,** 635 (1966).
32. K. W. Heaton and L. Lack, *Am. J. Physiol.* **214,** 585 (1968).
33. E. R. Schiff and J. M. Dietschy, *J. Clin. Invest.* **47,** 87 (1968).
34. M. R. Playoust, L. Lack, and I. M. Weiner, *Am. J. Physiol.* **208,** 363 (1965).
35. B. Borgström, A. Dahlquist, G. Lundh, and J. Sjövall, *J. Clin. Invest.* **36,** 1521 (1957).
36. B. W. Van Deest, J. S. Fordtran, S. G. Morawski, and J. D. Wilson, *J. Clin. Invest.* **47,** 1314 (1968).
37. T. Low-Beer, L. Lack, and M. P. Tyor, unpublished work.
38. I. G. Hislop, A. F. Hofmann, and L. S. Schoenfield, *J. Clin. Invest.* **46,** 1070 (1967).
39. W. G. M. Hardison and I. H. Rosenberg, *New Engl. J. Med.* **277,** 337 (1967).
40. J. S. Fordtran, F. C. Rector, Jr., F. E. Maynard, and J. Kinney, *J. Clin. Invest.* **44,** 1935 (1965)
41. J. M. Dietschy, *Proc.* **26,** 1589 (1967).
42. A. Norman and J. Sjövall, *J. Biol. Chem.* **233,** 872 (1958).
43. I. Sperber, *Pharmacol. Rev.* **11,** 109 (1959).
44. I. Sperber, *Proc. First Internat. Pharmacol. Meet.* **4,** 137 (1961).
45. I. Sperber, *in* "The Biliary System" (W. Taylor, ed.), p. 457, Blackwell, Oxford (1965).
46. J. M. Diamond, *in* "The Biliary System" (W. Taylor, ed.), p. 495, Blackwell, Oxford (1965).
47. J. D. Ostrow, *Clin. Res.* **13,** 258 (1965).
48. J. D. Ostrow, *J. Clin. Invest.* **46,** 2035 (1967).
49. D. Ritt, J. S. Schenker, and B. Combes, *Gastroenterology* **52,** 322 (1967).
50. R. W. Brauer, *in* "Liver Function" (R. W. Brauer, ed.), p. 113, American Institute of Biological Science, Washington, D.C. (1958).
51. I. M. Arias, *Medicine* **45,** 513 (1966).

52. S. Gustein, S. Alpert, and I. M. Arias, *Israeli J. Med. Sci.* **4,** 36 (1968).
53. H. O. Wheeler, J. I. Meltzer, and S. E. Bradley, *J. Clin. Invest.* **39,** 1131 (1960).
54. D. H. Sandberg, J. Sjövall, and D. A. Turner, *J. Lipid Res.* **6,** 182 (1965).
55. D. Rudman and F. E. Kendall, *J. Clin. Invest.* **36,** 530 (1957).
56. D. Rudman and F. E. Kendall, *J. Clin. Invest.* **36,** 538 (1957).
57. I. M. Weiner, J. E. Glasser, and L. Lack, *Am. J. Physiol.* **207,** 964 (1964).
58. T. H. Woo and S. K. Hong, *Am. J. Physiol.* **204,** 776 (1963).
59. G. R. Zins and I. M. Weiner, *Am. J. Physiol.* **215,** 840 (1968).
60. G. R. Zins and I. M. Weiner, *Am. J. Physiol.* **215,** 411 (1968).
61. R. L. Malvin, *Progr. Cardiovasc. Dis.* **3,** 432 (1961).

Chapter 3

BILE SALT METABOLISM IN MAN

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I. INTRODUCTION

This chapter deals with the metabolism of bile salts in man, especially as it relates to disease, particularly liver disease. The important role of bile salts in lipid absorption and gallstone formation is discussed in other chapters and will therefore not be considered here. The alterations in bile salt metabolism will be discussed in relation to disease categories in an effort to present a clearer overview of the general subject. A more detailed presentation of alterations in specific diseases has recently been prepared (1).

II. PRIMARY BILE SALTS IN MAN

There are two 24-carbon primary bile acids in man, cholic and chenodeoxycholic acids, shown as compounds I and II, respectively, in Fig. 1. There are two 27-carbon bile acids in human bile, trihydroxycoprostanic acid, III, and dihydroxycoprostanic acid, IV. These may also be regarded as primary bile acids, but they occur in very small quantities compared to the amounts of 24-carbon bile acids and consequently the term "primary bile acids" usually refers to the two 24-carbon bile acids in man, cholic and chenodeoxycholic.

*Deceased March 1970.

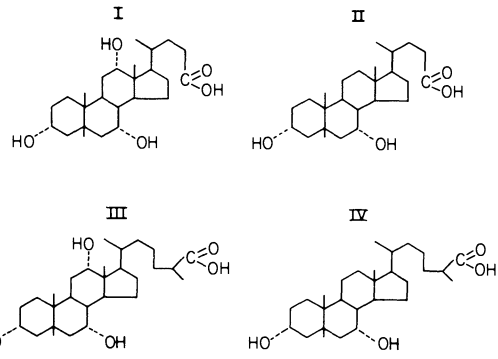

Fig. 1. The two 24-carbon primary bile acids in man are I, 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid), and II, 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid). Their immediate precursors are, respectively, III, 3α,7α,12α-trihydroxy-5β-cholestanoic acid, and IV, 3α,7α-dihydroxy-5β-cholestanoic acid.

Cholate and chenodeoxycholate are the only two 24-carbon bile salts which can be isolated from the bile of patients with total external bile fistula (2), an observation which supports the validity of the primary and secondary bile salt concept.

III. PATHWAYS FOR PRIMARY BILE SALT FORMATION IN MAN

The pathways for primary bile salt synthesis shown in Fig. 2 are derived from studies in man (3–7) and are believed to represent the major synthetic routes. Several of the intermediates shown in Fig. 2 have been isolated from human bile. Trihydroxycoprostanic acid, XV, has been crystallized from human fistula bile (8) and shown to be derived from cholesterol (4,5). The major metabolite of trihydroxycoprostanic acid in man is cholic acid (5). 5β-Cholestane-3α,7α-diol, X, has been identified as a product of cholesterol oxidation (6) and 3α,7α-dihydroxycoprostanic acid, XI, has been isolated from human fistula bile (7).

In addition to these naturally occurring compounds in human bile, a number of other intermediates have been identified in fractions of human liver homogenates (3) and are shown in Fig. 2 as compounds VI, VII, VIII, IX, X, XIII, XIV.

The observations upon which the pathways in Fig. 2 are based are consistent with the view (14) that oxidation of the cholesterol side chain inhibits 12α-hydroxylation. The 12α-hydroxylase enzyme (or enzymes) ap-

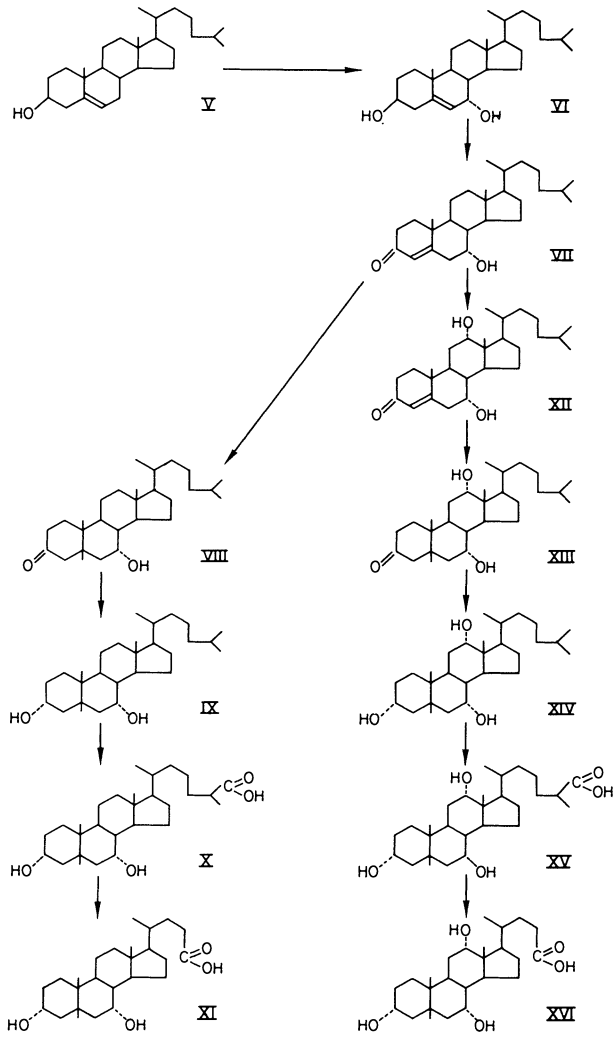

Fig. 2. Possible pathways for primary bile salt synthesis in man compiled from various sources (see text). V, Cholesterol; VI, cholest-5-ene-3β,7α-diol; VII, 7α-hydroxycholest-4-en-3-one; VIII, 7α-hydroxy-5β-cholestan-3-one; IX, 5β-cholestane-3α,7α-diol; X, 3α,7α-dihydroxy-5β-cholestanoic acid; XI, 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid); XII, 7α,12α-dihydroxy-cholest-4-en-3-one; XIII, 7α,12α-dihydroxy-5β-cholestan-3-one; XIV, 5β-cholestane-3α,7α,12α-triol; XV, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; XV, 3α,7α,12α-trihydroxy-5β-cholanoic acid; XVI, 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid).

pears to have a high substrate specificity for 7α-hydroxycholest-4-en-3-one (3,109), and thus the route for cholic acid synthesis may be restricted, perhaps, to a single pathway. The implications of this with respect to liver injury will be discussed in a later section.

Chenodeoxycholate synthesis may possibly proceed along several pathways. As shown in Fig. 2, one route is similar to that for cholate, in which ring alterations are completed before side-chain oxidation. A second suggested route begins with the oxidation of a terminal methyl group of cholesterol, yielding 26-hydroxycholesterol (9,10), which is readily converted to chenodeoxycholate but not cholate in the rat. Such a route involving 26-hydroxycholesterol remains speculative in man, however, as this compound has not yet been identified as a metabolite of cholesterol in human bile and there is some recent evidence that 26-hydroxycholesterol is not an important intermediate in bile salt formation (11).

The reaction sequence in the conversion of cholesterol into 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol has been shown to be the same in human and rat liver homogenates (3). All of these reactions occur with the 20,000*g* supernatant fluid of homogenates of human liver. The 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one proceeds efficiently in the presence of a microsomal fraction fortified with NADPH (3).

IV. SYNTHESIS RATES

The rate of cholate synthesis in man is about 200–300 mg/day, as measured by isotope dilution studies. The chenodeoxycholate synthesis rate is similar, so that the total primary bile salt synthesis is about 400–600 mg daily for a healthy adult, and when in the steady state this amount is also the daily fecal excretion rate (12).

Values for turnover time, pool size, and half-life of cholate and chenodeoxycholate and the excretion rates for both are given in Table I. When turnover time or synthesis rates are calculated from isotope dilution studies, the values are usually in the same range as those derived from isotope balance studies provided that the latter are done by properly validated and reproducible methods.

V. MAXIMUM SYNTHESIS RATES

Maximum synthesis rates in man can be induced by complete interruption of the enterohepatic circulation so that no bile salts are returned to the liver to suppress synthesis. Surgical construction of a total external bile

TABLE I. Values for Bile Salt Turnover, Half-life, and Pool Size in Man

Subjects and conditions (No. subjects)	Total formation or excretion (mg/day)	Cholate			Chenodeoxycholate			References
		Turnover (mg/day)	Half-life (days)	Pool size (g)	Turnover (mg/day)	Half-life (days)	Pool size (g)	
Normal subjects (2)	490	190–200	2–4	0.54–1.19	290–390	4.3–6.0	2.42–2.52	Danielsson (12)
Normal subjects (8)		360	2.8	1.38			1.45	Lindstedt (22)
Hypercholesterolemia (5)	272	137	1.1–3.7	0.32–0.65	134	1.4–5	0.30–0.78	Kottke (20)
Mixed hyperlipidemia (3)	1172	766			397			Kottke (20)

fistula for biliary decompression as a palliative procedure in a group of nine patients with abdominal malignancy provided an opportunity to study maximum bile salt synthesis rates in man (13). Observed values for maximum synthesis rates are shown in Table II.

This is approximately a four- to sixfold increase over normal synthesis rates. The rat can increase its synthesis rate for cholic acid ten- to twentyfold (14). The pool size under these circumstances (bile fistula) is probably small and confined largely to the liver and biliary tree.

When the ileum is removed, bypassed, or severely diseased, the enterohepatic circulation is again interrupted, which results in a diminished pool size (15) and increased daily fractional excretion rates from 0.12 to values of 0.5–1.5 (16).

In four patients with ileectomy (17) when in the fasting state (overnight), total bile salt pool size was slightly decreased (from 2.9 to 2.2 g) and contained more deoxycholate (0.55 g). With each succeeding meal, however, excessive loss occurs in the feces and the pool becomes greatly depleted.

Cholestyramine resin forms a nonabsorbable complex with bile salts in the gut lumen and increases fecal excretion about three times and may thus be expected to have an effect on bile salt pool size and synthesis rates similar to ileectomy (18).

Fasting in obese but otherwise normal subjects results in cessation of cholate turnover (and presumably synthesis) and disappearance of bile salts from the stools (19).

Patients with hypercholesterolemia do not appear to have significant alterations in bile salt synthesis rates, but patients with combined hypercholesterolemia and hypertriglyceridemia have increased synthesis rates for both cholate and chenodeoxycholate (20). Bile salt synthesis rates are not appreciably changed when nicotinic acid feeding lowers plasma cholesterol concentrations (20). Synthesis rates may also be affected by thyroid hormones. Cholic acid synthesis is decreased and half-life prolonged in hypothyroid subjects. These alterations may be corrected with thyroid hormone (21). Bile acid synthesis is increased in thyrotoxicosis (21).

Manipulation of dietary fats to alter serum cholesterol concentrations has relatively little effect on bile salt metabolism in man when measured by

TABLE II. Maximum Synthesis Rates in Grams per Day in Man

	Range	Average[a]
Cholate	0.5–3.2	2.1 (0.25)
Chenodeoxycholate	0.25–2.1	1.2 (0.25)
Total	0.83–5.1	3.3 (0.50)
Cholate/chenodeoxycholate	1.2–3.2	2.1 (1)

[a] Normal values in parentheses.

isotope dilution studies (22,23). When isotope balance studies are used, however, significant increase in fecal bile acid excretion occurs when feeding of unsaturated fats is accompanied by a lowering in plasma cholesterol concentrations (24).

In cirrhosis of the liver, cholic acid pool size and turnover rates are increased but half-life (in plasma) remains about the same as in normal subjects (25). In viral hepatitis, the pool size is apparently decreased, as is the daily production (26).

VI. RELATIVE SYNTHESIS RATES IN LIVER DISEASE

As will be discussed in a later section, patients with certain types of liver injury have chenodeoxycholate as the predominant primary bile salt in their serum. Primary bile salt concentration ratios in serum are a fairly accurate reflection of the primary bile salt concentration ratio in bile. The evidence for this is given in Fig. 3, in which the primary bile salt concentration ratio in serum is plotted against that in bile in 14 patients. The correlation coefficient for these two variables is 0.86 ($p<0.01$). When chenodeoxycholate predominates in bile its metabolites (lithocholate and others) predominate in feces, and when cholate predominates in bile its metabolites (deoxycholate and others) predominate in feces (27). This relationship is shown in Fig. 4. It thus appears that primary bile salt concentrations in blood and bile are related to their relative synthesis rates and that the predominant bile salt in blood and bile has the greater synthesis rate, since its metabolites predominate in feces. This assumes of course that there is a steady state and

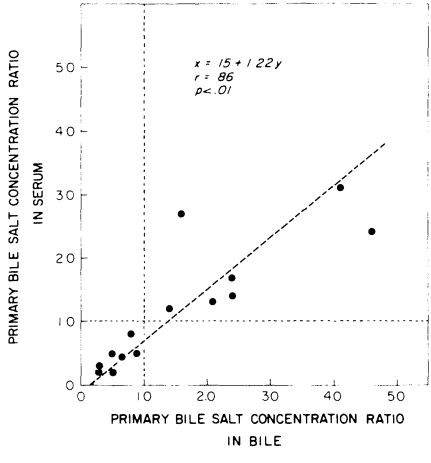

Fig. 3. The primary bile salt (cholate/chenodeoxycholate) concentration ratio in serum is plotted against the same concentration ratio in bile. The correlation coefficient is 0.86, showing that the primary bile salt concentration ratio in serum is a fairly accurate reflection of that in bile.

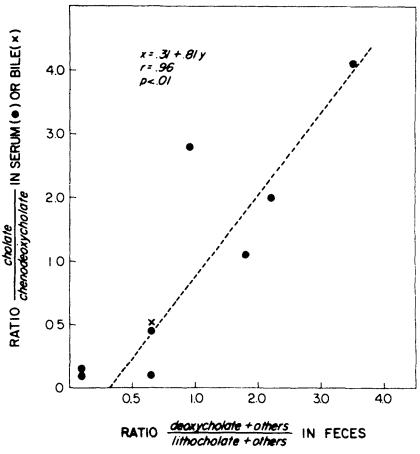

Fig. 4. The primary bile salt concentration ratio in serum or bile is plotted against the concentration ratio of their metabolites in feces. The correlation coefficient is 0.96, showing that the primary bile salt with the highest concentration in serum or bile has the highest synthesis rate (see text).

that synthesis equals excretion. These observations may be interpreted as showing that patients with portal cirrhosis synthesize mainly chenodeoxycholate when it predominates in their serum and those with cholestasis synthesize mainly cholate when it predominates in serum.

VII. FORMATION OF SECONDARY BILE ACIDS IN MAN

The predominant bile salts in feces are not the same as those in bile. When ^{14}C-labeled cholesterol is given intravenously to an adult human, the two major metabolites excreted in approximately equal quantities in the feces are deoxycholate and lithocholate, with much smaller amounts of other secondary bile salts. Deoxycholate and lithocholate, which account for slightly more than half of the excreted activity (28), are derived from 7α-dehydroxylation of cholate and chenodeoxycholate, respectively, by colon bacteria. The process has been shown to proceed by direct removal of the 7α-OH group with the adjacent 6β-hydrogen, yielding a Δ^6-cholenoic acid; this unsaturated acid is then hydrogenated to give deoxycholate (29).

With isotopically labeled primary bile salts, cholate and chenodeoxycholate, it is possible to show that in addition to deoxycholate a wide variety of secondary bile salts are derived from cholate, the chief ones being 12α-hydroxy-3-keto-5β-cholanoic acid, 3β,12α-dihydroxy-5β-cholanoic acid, 3α-hydroxy-12 keto-5β-cholanoic acid, and 3β-hydroxy-12-keto-5β-cholanoic acid (30). A smaller number are derived from chenodeoxycholate, mainly lithocholate, 3β-hydroxy-5β-cholanoic acid, and 3-keto-5β-cholanoic acid (31). The secondary bile acids which have been identified in man are listed in Table III.

TABLE III. Bile Acids Identified in Human Feces[a]

Systematic name	Common name
3α,7α-Dihydroxy-12-keto-5β-cholanoic acid	
3α,12α-Dihydroxy-7-keto-5β-cholanoic acid	
3α,7β,12α-Trihydroxy-5β-cholanoic acid	
3β,7β,12α-Trihydroxy-5β-cholanoic acid	
3β,7α,12α-Trihydroxy-5β-cholanoic acid	
3α,7α,12α-Trihydroxy-5β-cholanoic acid	Cholic
3α,12α-Dihydroxy-5β-cholanoic acid	Deoxycholic
3,12-Diketo-5β-cholanoic acid	Dehydrodeoxycholic
3α-Hydroxy-12-keto-5β-cholanoic acid	
3β-Hydroxy-12-keto-5β-cholanoic acid	
12α-Hydroxy-3-keto-5β-cholanoic acid	
3α,12β-Dihydroxy-5β-cholanoic acid	
3β,12α-Dihydroxy-5β-cholanoic acid	
3β,12β-Dihydroxy-5β-cholanoic acid	
3α,7α-Dihydroxy-5β-cholanoic acid	Chenodeoxycholic
7α-Hydroxy-3-keto-5β-cholanoic acid	
3β,7α-Dihydroxy-5β-cholanoic acid	
3α-Hydroxy-7-keto-5β-cholanoic acid	7-Ketolithocholic
3α,7β-Dihydroxy-5β-cholanoic acid	Ursodeoxycholic
7β-Hydroxy-5β-cholanoic acid	
3α-Hydroxy-5β-cholanoic acid	Lithocholic
3β-Hydroxy-5β-cholanoic acid	Isolithocholic
3-Keto-5β-cholanoic acid	
5β-Cholanoic acid	Cholanic
3α,7α,12α-Trihydroxy-5α-cholanoic acid	Allocholic

[a] Hill and Drasar (110) have isolated bacterial strains that possess 12α-dehydroxylase, and it is thus possible that some lithocholic acid in feces is derived from cholic acid via 12α-dehydroxylation of deoxycholic acid. See text for references.

Secondary bile acids produced in the colon are absorbed by the colon into the enterohepatic circulation to a limited extent. Deoxycholate is usually the chief secondary bile salt found in human bile, with smaller amounts of lithocholate (1). The predominance of deoxycholate in human bile suggests that it is more readily absorbed by the colon than lithocholate.

VIII. AMINO ACID CONJUGATES

The most active subcellular fraction for conjugation of bile salt CoA intermediates with taurine or glycine is microsomal (32). The system is strongly stimulated by the addition of a lysosome fraction, which may contain some peroxisome particles (33). Taurine and glycine are the only amino acids known to conjugate with bile acids in man, and the conjugation appears to be limited to the liver. In healthy adults, the usual glycine/taurine ratios are

2.0–3.2, but values up to 4 or 6 sometimes occur (34,35). During the first few days of life and in the fetus, however, virtually all bile salts are taurine conjugates (36–38). Glycine/taurine ratios and conjugating capacity are altered in hepatobiliary disease; this will be discussed in Section X. The process of conjugation is fairly complete, as only trace amounts of unconjugated bile salts are found in the bile of healthy subjects or in that from patients with various types of liver disease (35). When the enterohepatic circulation is partially or completely interrupted, however, up to 3% of intravenously injected ^{14}C-labeled bile salts may occur in bile (2) or duodenal content (17) in the nonconjugated form, so that conjugation is not required for excretion.

Conjugation lowers the ionization constant of unconjugated bile salts (pK_a about 6) to a pK_a of 1.8–3.7, and they become largely ionized at the pH of luminal content, which ranges from 6 to 8. Ionized bile salts are absorbed in jejunal and proximal ileal segments by ionic diffusion at rates proportional to their intraluminal concentrations and activities (39). This process is relatively slow, and thus conjugation serves to prevent premature absorption of bile salts by jejunal and proximal ileal segments and maintains these detergent compounds at intraluminal concentrations sufficient to assist with the micellar phase of fat digestion and absorption.

When bile salts are deconjugated by abnormal bacterial proliferation in the small bowel (40), yielding free or unconjugated bile salts with a pK_a of 4–6, they are quickly absorbed by nonionic diffusion in the jejunum so that intraluminal bile salt concentration may be insufficient for efficient fat absorption and steatorrhea may result (41).

Under normal circumstances, bacterial deconjugation is limited largely to the colon (numerous species of bacteria have been found capable of deconjugation (42–44), and an enzyme from one strain has been isolated (45). Under normal circumstances, bacterial deconjugation is limited largely to the colon so that bile salts excreted in the feces are almost entirely unconjugated. When antibiotics are administered, conjugated bile salts are excreted in the feces (46). Free bile salts may be absorbed from the colon (47), which may account for relatively high concentration of free bile salts in the serum of patients with cirrhosis (25), a condition in which removal from the blood by the liver is impaired (48) as is conjugation (32).

IX. SULFATE ESTERS

The occurrence of sulfate esters (taurolithocholic sulfate and glycolithocholic sulfate) has been described in human bile (49). Palmer has suggested that sulfation of lithocholate may modify its toxic properties and may have important effects on other aspects of its metabolism.

X. ALTERED BILE SALT METABOLISM WITH HEPATOBILIARY DISORDERS

The two primary bile salts in man, cholate and chenodeoxycholate, are synthesized exclusively in the liver, and consequently measurement of their concentrations in blood or bile often yields useful information about the liver and its response to injury. Bile salt metabolism is altered in two important ways with liver injury. The concentrations of bile salts in the blood increase, and the concentration ratio in both blood and bile changes.

Normally (healthy state), the liver is very efficient in removing the bile salts from portal blood to bile, thus confining them largely to the enterohepatic circulation so that bile salt concentrations in healthy subjects rarely exceed 5μg/ml in peripheral blood. Values for primary and secondary bile salt concentrations in the serum of healthy subjects are given in Table IV. The concentrations of the two secondary bile salts (deoxycholate and lithocholate) in serum are very low and seldom exceed 2–3 μg/ml even when primary bile salt concentrations are increased over 20 times normal values. Consequently, when serum bile salt concentrations are increased with hepatobiliary disease, almost all of the increase is accounted for by elevation in the primary bile salt concentrations, and the primary bile salt concentration ratio will include nearly all of the bile salts quantitated.

The concentrations of the primary bile salts in serum are very nearly equal but sufficiently small so that they approach in magnitude the error of the method. For this reason, a primary bile salt concentration ratio has little or no significance when the concentrations are not increased above the normal range. If the concentration of at least one of the bile salts is increased above the normal range, however, the error remains the same, the concentration is not significantly altered by error, and thus the concentration ratio becomes valid. The general observation has been made that when serum bile salt concentrations are elevated in the presence of severe liver injury, chenodeoxycholate predominates and the cholate/chenodeoxycholate ratio is usually less than 1 (50). A primary bile salt ratio of greater than 1 is most commonly found in jaundiced patients who have bile duct disease, either intra- or extrahepatic (50).

When the concentrations of bile salts are measured simultaneously in blood and bile and expressed as a concentration ratio, it is found that the concentration ratios of blood and bile are very similar and related as shown in Fig. 3, in which the correlation coefficient is 0.86.

Thus primary bile salt concentration ratios in serum are an accurate reflection of primary bile salt concentrations in bile. Alterations in bile salt concentrations in various disease states have been more extensively studied in blood than in bile. Bile salts are more easily quantitated in bile than in blood

TABLE IV. Normal Bile Salt Concentrations and Concentration Ratios in Blood and Bile

Source and method	Cholate	Chenode-oxycholate	Deoxy-cholate	Litho-cholate	Total	Tri-hydroxy	Di-hydroxy	References
Concentrations								
Serum GLC, μg/ml (Sandberg)	0.03–0.65	0.05–1.30	0.05–0.45	—	0.3–2.3			(111)
Serum GLC μg/ml (Carey)	0–0.95	0.22–0.95	0.01–0.27	0–0.11	0.33–2.11			(1)
Serum UV spectrum, μg/ml					0–5.3	0–3.4	0–1.9	(50)
Duodenal content GLC, mg/ml	0.98	0.59	0.50	0.01	2.08	0.98	1.1	A.L.[a]
Fistula bile UV spectrum, mg/ml					4.4	3.1	1.3	A.L.
Hepatic bile, mg/ml					18.0			(34)
Gallbladder bile GLC, mg/ml	18.3	11.5	6.7	1.5	7–64			(113)
Ratio of average values								
Serum	0.45	1	0.38	0.09		1.9	1	
Duodenal content	1.6	1	0.8	0.01		0.9	1	
Fistula bile						2.4	1	
Gallbladder bile	1.6	1	0.6	0.1				

[a] A. L. values obtained in author's laboratory

but bile is much more difficult to obtain and bile salt concentrations in bile, if collected from the duodenum, are much more variable.

XI. DISEASE GROUPS

Table V shows average serum bile salt concentrations and concentration ratios for groups of patients with various hepatobiliary disorders.

XII. PORTAL CIRRHOSIS

In 123 patients with portal cirrhosis, the trihydroxy fraction ranged up to 77.6 μg/ml and the highest dihydroxy value was 55.0 μg/ml. The concentration ratio ranged from 0.001 to 5. The scatter diagram (Fig. 5) shows the distribution of the concentration ratio for the portal cirrhosis group compared with the bile duct disease group. In the portal cirrhosis group, 78% have a concentration ratio of less than 1, whereas 82% of the bile duct disease group have a ratio of greater than 1. High bile salt concentrations occur in both groups, but the concentration ratio does tend to partially separate obstructive and cholestatic disorders from the type of liver injury associated with portal cirrhosis.

The serum bile salt concentration ratio in patients with portal cirrhosis or other types of hepatic cell injury has some usefulness, therefore, in the differential diagnosis of jaundice. Jaundiced patients with a bile salt concentration ratio of less than 1 will more likely be jaundiced because of hepatic cell injury, whereas those with a ratio greater than 1 will more likely be jaun-

TABLE V. Comparison of Average Serum Concentrations of Bile Salts, Bilirubin, and Alkaline Phosphatase for Groups of Patients with Various Hepatobiliary Disorders

Disease	No. patients	Serum bile salt concentrations (μg/ml) Trihydroxy[a]	Dihydroxy[a]	Total	Ratio tri/di	Serum bilirubin (mg %)	Alkaline phosphatase (KA units)
Portal cirrhosis	123	4.39	8.50	12.89	0.6	7.40	26.69
Bile duct disease	115	21.57	10.12	31.69	2.9	12.53	59.69
Biliary cirrhosis	29	13.03	8.43	21.46	1.8	6.15	81.13
Viral hepatitis	56	24.68	18.25	42.93	1.9	8.77	25.65

[a] Trihydroxy bile salts in serum are mainly cholate and dihydroxy bile salts mainly chenodeoxycholate, especially when the serum concentrations are elevated above normal values.

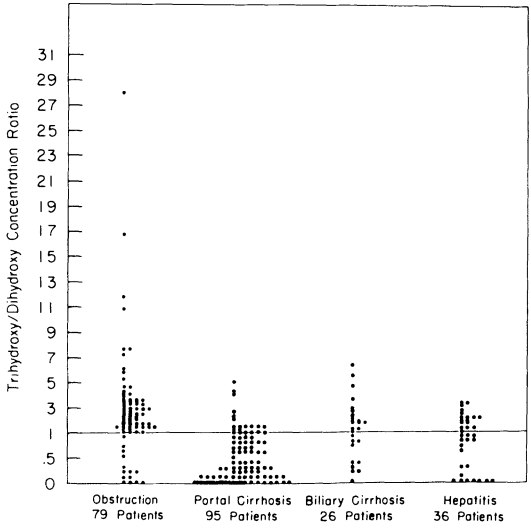

Fig. 5. Serum bile salt concentration ratios are plotted by disease group. The group with bile duct obstruction, whether intra- or extrahepatic, tends to have a higher ratio (greater than 1) than the portal cirrhosis group, in which the ratio is usually greater than 1 (see text for details).

diced because of cholestasis or bile duct obstruction either within the liver or in extrahepatic bile ducts. As with all other liver "function" tests, however, the concentration ratio cannot be taken as an absolute or infallible guide but must be interpreted in conjunction with other studies.

Although useful in diagnosis, the concentration ratio is perhaps more helpful in revealing the extent of liver injury. Jaundiced patients with a concentration ratio of less than 1 are more likely to have other evidence of extensive liver injury such as hypoalbuminemia, 3+ to 4+ cephalin flocculation, and increased serum immunoglobulin concentrations. Patients with impending or actual hepatic coma often have only chenodeoxycholate detectable in their serum, and all have had ratios of less than 1. A concentration ratio of less than 1 is by no means an irreversible event. Acute alcoholic liver injury is the most common example of its reversibility. When such patients are admitted to the hospital, their concentration ratio is frequently less than 1 but reverts to higher values with abstinence from alcohol, rest, good diet, and time (50).

The primary bile salt concentration ratio also has prognostic significance in patients with chronic liver disease, since it is related to the extent of liver injury. Patients with a ratio of less than 1 usually have other evidence of

extensive liver injury and therefore are more likely to die sooner of hepatic failure than those who have a ratio greater than 1.

XIII. BILE DUCT OBSTRUCTION

As mentioned in Section XII, patients with obstruction of the bile ducts, either intrahepatic or extrahepatic, usually have a bile salt concentration ratio of greater than 1 (82% of those in Table V and Fig. 5). Serum bile salt concentrations in this group range up to 110.7 μg/ml for trihydroxy bile salts and 60.3 μg/ml for dihydroxy bile salts. These concentrations are composed almost exclusively of primary bile salts. Analysis of serum bile salts by gas–liquid chromatography in patients with bile duct obstruction often fails to reveal the presence of any significant quantities of secondary bile salts (1). This circumstance has also been observed in duodenal contents of patients with bile duct obstruction (35). With bile duct obstruction or cholestasis of long standing, the concentration ratio may decline and reach a value of less than 1 as liver damage becomes more extensive, as it often does with prolonged bile duct obstruction.

Serum bile salt concentrations or concentration ratios are of no value in distinguishing between intrahepatic and extrahepatic bile duct obstruction or cholestasis. Patients with metastatic or primary hepatic tumors may have values identical to those with cancer or stone occluding the common bile duct. Drug-induced cholestasis or the cholestatic phase of viral hepatitis may give identical results.

XIV. HEPATITIS

Serum bile salt concentrations may reach high values in patients with viral hepatitis. In the group of patients studied, trihydroxy values ranged up to 90.4 μg/ml and dihydroxy values to 73.6 μg/ml. The highest concentrations usually occur in the first week or 10 days of the disease, and the concentration ratio usually reflects the obstructive or cholestatic phase of the disease; i.e., it is greater than 1. As shown in Fig. 6, the serum bile salt concentrations may fall rapidly after the initial rise and reach nearly normal values before the serum bilirubin or alkaline phosphatase values become normal.

These observations differ from those of Frosh, who has reported that serum bile salt concentrations remain elevated after bilirubin concentrations and enzyme activities return to normal and who thus regards serum bile salt measurements as superior to other tests in revealing abnormalities in hepatitis (51).

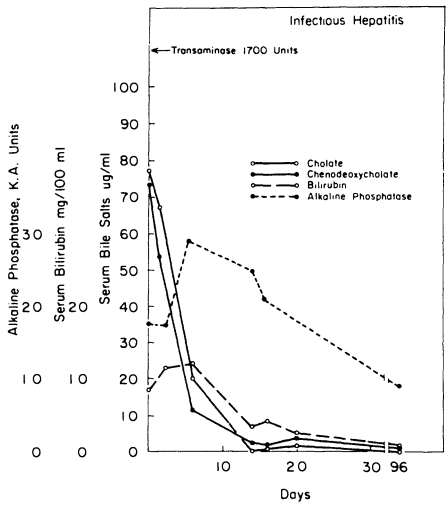

Fig. 6. Bile salt, bilirubin, and alkaline phosphatase values in the serum of a patient with hepatitis plotted against time, showing that serum bile salt concentrations can reach normal values before serum bilirubin concentration becomes normal.

The average concentration ratio for the 56 patients in the hepatitis group was 1.9. The ratio may frequently become less than 1 during some stage of the illness, usually when the concentrations are falling rapidly. Under such circumstances, a reversed ratio does not signify severe liver damage or a poor prognosis, especially when other tests show improvement. Such low ratios are usually transient, but if the ratio remains low for any extended period it may represent more serious liver injury and be accompanied by a rising bilirubin concentration.

Serum bile salt concentrations and concentration ratios in patients with post-transfusion hepatitis appear to have a pattern similar to that seen in infectious hepatitis except that the rapid changes seen in infectious hepatitis are less commonly encountered in the post-transfusion group.

A more detailed account of serum bile salt alterations in a variety of specific diseases categories is given elsewhere (1).

XV. ALCOHOLIC LIVER INJURY

Examples of bile salt alterations in patients with acute alcoholic liver injury are given in Table VI. The changes are quite pronounced, especially during stages of acute alcoholism. In the group shown in Table VI, serum bile salt concentrations declined and the ratio (except for the first patient) increased 10 days following admission. These changes may be interpreted as additional evidence that ethanol itself may have a direct toxic action on

TABLE VI. Serum Bile Salt Concentrations and Concentration Ratios in Acute Alcoholic Liver Injury

	Admission			10 days after admission		
Patient	Trihydroxy (μg/ml)	Dihydroxy (μg/ml)	Ratio	Trihydroxy (μg/ml)	Dihydroxy (μg/ml)	Ratio
1	10.8	23.4	0.5	1.1	11.0	0.1
2	2.5	15.1	0.2	1.1	3.8	0.3
3	4.1	53.6	0.1	1.0	1.4	0.7
4	0.8	32.4	0.02	5.4	3.7	1.5
5	0.4	19.2	0.02	1.6	5.9	0.3

hepatic cells. Ethanol decreases cholate synthesis in rats (52), and it is reasonable to suppose that it would have a similar effect on the human hepatic cell.

XVI. DRUG-INDUCED JAUNDICE

Jaundice induced by certain medications is usually associated with alterations in serum bile salt concentrations. Increased serum bile salt concentrations have been observed with chlorpromazine, 5-fluorouracil, nilevar, Carbarsone, tolbutamide, and Dilantin (1,53). With all of these drugs, cholate predominated in the serum, and thus the cholate/chenodeoxycholate ratio was greater than 1. This is consistent with the elevation in alkaline phosphatase and cholestasis that occurred in all of these patients. The estrogenic component of Enovid, mestranol, has also been shown to produce similar changes in cholate/chenodeoxycholate concentration ratio and cholestasis (1,54). In jaundice associated with isoniazid ingestion, chenodeoxycholate predominates and the concentration is less than 1, which is compatible with the more severe type of hepatocellular injury induced by this group of compounds (1).

XVII. BILE SALTS AND PRURITUS

Despite some conflicting observations (55), the bulk of recent evidence supports the hypothesis that increased concentrations of bile salts in the skin, resulting from sustained elevations in serum concentrations, are the responsible circumstance for pruritic jaundice. Higher concentrations of bile salts occur in the skin of patients who itch than in those who do not (56). A reduction in high skin bile salt concentrations in a jaundiced patient with pancreatic carcinoma followed cholecystojejunostomy, and this was accompanied

by a lowering of serum bile salt concentrations and relief of pruritus (56). Bile salts are the only compounds which have been regularly shown to occur in increased concentrations in blood and skin of patients with the type of pruritus associated with jaundice. High concentrations of bilirubin, cholesterol, phospholipids, neutral lipids, or free fatty acids are not associated with pruritus when serum bile salt concentrations are not elevated.

A nonabsorbable strongly basic anion exchange resin which binds bile salts in the intestinal lumen, thereby removing them from the enterohepatic circulation for excretion in the feces, will relieve pruritus in patients who do not have complete biliary obstruction. When serum bile salt concentrations are measured serially at frequent intervals during cholestyramine feeding, it can be shown that concentrations reach near normal levels before pruritus stops, and when cholestyramine is withheld, high serum concentrations are reached before pruritus starts. This lag period, which may be a day or more, may represent the period of time required for concentrations of bile salts sufficient to induce pruritus to accumulate in or leave the skin (57).

XVIII. PRURITUS AND PREGNANCY

Healthy pregnant women have normal serum bile salt concentrations, but when pruritus occurs serum bile salt concentrations increase and the concentration ratio is usually greater than 1 (1,58). In one patient with eclampsia, however, the ratio was 0.9. Estrogens with phenolic A-rings appear to play a role in the idiopathic pruritus of pregnancy and pruritus gravidarum. They have been shown to impair hepatic bromsulfophthalein (BSP) disposal (59), and ethinyl estradiol produced pruritus and jaundice in patients who had such symptoms during pregnancy (60). Increased serum bile salt concentrations and other evidence of liver injury were induced by mestranol, the estrogen component of Enovid, in a patient who was not so affected when given the progestational component norethynodrel (1,54).

The type of pruritus occurring during pregnancy which is caused by increased concentrations of bile salts in the blood and skin is readily relieved by cholestyramine; this is discussed in Section XXVIII.

XIX. LITHOCHOLIC ACID AND LIVER INJURY

The toxicity of bile has long been recognized, and early observations have been reviewed by Horrall (61). Cirrhosis of the liver following lithocholate feeding was first observed by Holsti (62,63), who also demonstrated that its precursor, chenodeoxycholate, had the same effect (64). The cirrhotogenic

properties of lithocholate were also demonstrated in a variety of mammals (65), birds (66,67), and a primitive lizard, the iguana (68). In acute experiments, intravenously administered sodium taurolithocholate produced cholestasis in rats (69) and hamsters (70), but there is no cholestasis when lithocholate is *fed* to rats (71). Lithocholic acid occurs naturally in human bile and feces. It is derived from chenodeoxycholate by 7α-dehydroxylation of chenodeoxycholate by intestinal bacteria (72). It therefore becomes of importance to know if the concentrations of lithocholate to which the liver is exposed are ever sufficiently high or sustained to play a role in the perpetuation of liver injury, especially in patients who already have mainly chenodeoxycholate in their blood and bile.

The quantities of lithocholate required to produce cirrhosis when fed to rats (about 625 mg/kg) exceed by approximately 100 times the amounts found in man. The concentration of lithocholate in human gallbladder bile ranges from 0.2 to 5 mg/ml (mean 1.5 mg/ml), while that in human blood serum rarely exceeds 1 μg/ml (1). Rats, however, readily convert lithocholate to other less toxic bile salts, mainly 3α, 6β-dihydroxy-5β-cholanoic acid, in feeding experiments (71). The human liver does not readily convert lithocholate to other compounds to any significant extent (2). It thus may be possible that on a mg/kg basis lithocholate may be more toxic to man than to rats. Rabbits, like man, are unable to convert lithocholate to other compounds (73), and cirrhosis develops in these animals when lithocholate is fed at the 0.25% dietary level (about 0.17 mg/kg/day). An estimate of lithocholate synthesis in a healthy adult would be about 3–4 mg/kg/day.

XX. DISSOCIATED JAUNDICE

"Dissociated jaundice" (*ictere dissociée*) refers to a dissociation of bilirubin and bile salt excretion in which bilirubin is retained, leading to jaundice, but bile salts are excreted in a normal manner so that such patients are icteric but do not have pruritus and no bile salts are detected in blood or urine. The concept was held that only the hepatic cell could selectively retain bilirubin but excrete bile salts and that bile ducts or gallbladder cells could not. The observation of dissociated jaundice was therefore regarded as useful in distinguishing jaundice due to hepatitis or cirrhosis from that caused by bile duct obstruction (74–76).

This concept has now been largely abandoned in favor of more suitable methods for making this distinction, but it is of interest to re-examine it with more specific analytical methods than were available when the concept was popular. Table VII gives the results of comparing serum bile salt concentrations with total serum bilirubin concentrations and shows that jaundice with

TABLE VII. Incidence of Dissociated Jaundice in Various Disease Groups

Disease	Number of patients	Percent of patients with jaundice but normal serum bile salt concentrations
Portal cirrhosis	123	35
Viral hepatitis	56	21
Bile duct disease	115	12

normal serum bile salt concentrations (dissociated jaundice) occurred in 35% of the cirrhosis group, 21% of the hepatitis group, and 12% of the bile duct disease group. When the cirrhosis and hepatitis groups are combined, dissociation occurred in 31%. The reverse situation, in which serum bile salt concentrations are increased in the absence of jaundice, is less commonly seen, occurring in only 3% of all groups combined.

Thus when the concept of dissociated jaundice is examined by modern analytical techniques it is found to be true only to a very limited extent (i.e., more patients with cirrhosis or hepatitis will have dissociated jaundice than will those with bile duct disease), and the incidence of dissociated jaundice is so low in all groups as to be of little diagnostic usefulness—for the fact remains that more jaundiced patients (65% or more) will have increased serum bile salt concentrations regardless of the type of disease. Dissociated jaundice is of limited diagnostic value only when it is present.

XXI. CONJUGATION IN HEPATOBILIARY DISORDERS

Homogenates prepared from the livers of patients with stone or cancer occluding the common bile duct have a reduced capacity to conjugate bile salts (32), and a relative decrease in glycine conjugation has been found in patients with toxic or infectious hepatitis, cirrhosis, and obstructive jaundice (77). This observation is supported by the finding of a lowered glycine/taurine ratio in four of seven patients with obstructive jaundice (35). Glycine/taurine ratios are variable in patients with cirrhosis and hepatitis, but the trend is usually in the direction of a reduced ratio. A notable exception is myxedema, in which glycine/taurine ratios exceed those in any other condition thus far studied.

XXII. STAGNANT LOOP SYNDROME

The serum bile salt pattern is altered in patients with bacterial proliferation in the small intestinal content (stagnant loop or blind loop syndrome). Bacterial deconjugation of bile salts in the lumen of the small intestine per-

mits rapid absorption, and the concentrations of free bile salts in the blood become greatly increased (78), exceeding the concentration of conjugated bile salts. Primary bile salts predominate, but the relative proportion of deoxycholate becomes increased. Taurine conjugation is diminished under these circumstances. The pattern can be restored to near normal by antibiotic administration. A similar alteration in the serum bile salt pattern occurs in patients with extensive intestinal resection (78). Bacterial proliferation sometimes occurs in the lumen of small bowel in such patients, and if the ileum has been resected more bile salt reaches the colon, so that greater absorption of deconjugated bile salts is possible from the colon.

In circumstances where there is a stagnant loop or shortened bowel, bile salt patterns in serum reflect those found in the intestinal content (78).

XXIII. BILE SALTS AND TARGET CELLS

The shape and fragility of red cell membranes are greatly influenced by their free cholesterol content, and bile salts have been shown to affect the free cholesterol content of red cell membranes in at least two ways (79). Serum cholesterol transesterase activity is inhibited by bile salts, and thus removal of free cholesterol from the membrane, which is facilitated by esterification, is delayed. Bile salts induce a shift in the cell/serum free cholesterol partition ratio in favor of the cell, so that supranormal concentrations of cholesterol occur in the cells. Such cells are flat and osmotically resistant and appear as target cells on stained smears. Patients with jaundice, especially obstructive jaundice, have numerous target cells, and it has been postulated that increased concentrations of bile salts in the serum of such patients may account for the target cell formation (79).

XXIV. EXCRETION IN URINE AND FECES

Urinary excretion of bile salts by healthy subjects is apparently very limited. The urine contained 2% of the radioactivity administered orally as ^{14}C-cholic acid to a healthy subject in whom 100% of the radioactivity was recovered (80) and 0.12% of radioactivity administered to four normal subjects when ^{14}C-cholate was given intravenously (25). Conventional methods do not detect bile salts in the urine of healthy subjects (81,82). In jaundice patients, however, bile salts are excreted in the urine regularly (83). The highest 24-hr excretion rates reported by Gregg occurred in patients with common bile duct obstruction (58 mg/24 hr) and drug-induced cholestasis (40 mg/24 hr). The cholate/chenodeoxycholate ratio was greater than 0.59

in the obstruction–cholestasis group and less than 0.50 in the cirrhosis–hepatitis group. Thus the primary bile salt ratio in urine reflects the changes observed in blood in that the ratio is higher in the bile duct disease group and lower in the cirrhosis–hepatitis group. In four jaundiced patients with cirrhosis, 8.6–67.4% of the radioactivity from intravenously injected ^{14}C-cholic acid was recovered in the urine in 7 days; 60–90% was conjugated, 40–60% as a dihydroxy compound, presumably deoxycholate (25). In four patients with hepatitis, 3.1–35.1% of intravenously injected ^{14}C-cholate was recovered in the urine over a period of 96 hr (26).

XXV. FECES

Most bile salts excreted in the feces are of the secondary type. Their formation is discussed in Section VII. The daily fecal excretion of bile salts in healthy subjects is highly variable and easily influenced by dietary alterations. Values from several studies are given in Table VIII. Bile salts virtually disappear from the stools during prolonged fasting, and turnover nearly ceases (19). Primary bile salts appear in the stools of patients with diarrhea (1). Patients taking cholestyramine excrete the usual pattern of secondary bile salts (57), so that apparently bacterial dehydroxylation of bile salts can occur in the presence of this resin. Patients with total external bile fistulas have no bile salts in the feces (2); this does not exclude transintestinal excretion of bile salts but makes it unlikely. As mentioned earlier, the predominance of chenodeoxycholic acid in blood and bile is often reflected in a predominance of lithocholate over deoxycholate in the feces (27).

TABLE VIII. Daily Fecal Excretion of Bile Acids in Man

Subjects and conditions	Fecal bile acid excretion (mg/day)	References
Normal—high cholesterol feeding	280–1070	(108)
Normal—low cholesterol feeding	230–830	(108)
Hypercholesterolemia	330	(18)
Hypercholesterolemia with cholestyramine feeding	1076	(18)
Hypercholesterolemia—coconut oil feeding	790	(23)
Hypercholesterolemia—safflower oil feeding	540	(23)
Normal subjects—fat free	130–650	(28)
Cholesterol-"free" diets	189–431	(107)
Moderate cholesterol diets	93–799	(107)
Normal—butter diet	473	(24)
Normal—safflower oil diet	564	(24)

XXVI. ENTEROLITHS

Enteroliths are concretions of precipitated bile acids which form in the intestinal tract and thus differ from gallstones, which consist mainly of cholesterol, bilirubin, or both. All but two enteroliths of which the composition has been determined have been found to be composed of choleic acid, a molecular coordinate complex of deoxycholic acid, and usually a fatty acid in a proportion of about 8:1. Of the remaining two, one enterolith was found in an afferent gastroenterostomy loop (84) and the other in a diverticulum of the second portion of the duodenum (85). Both of these enteroliths consisted of a mixture of unconjugated primary bile acids, cholic and chenodeoxycholic acids.

It is quite probable that enterolith formation in man is a consequence of stasis and bacterial proliferation in the intestine. Microbial enzymes deconjugate bile salts, which raise their pK_a to a value of about 6, so that much of the free bile acid is un-ionized and precipitates at the pH of intestinal content. When microbial enzymes also convert cholic acid to deoxycholic acid, the latter compound forms choleic acids, which have an even higher pK_a and thus precipitate more readily.

XXVII. THERAPEUTIC AGENTS IN BILE SALTS

Bile salts have been used for centuries as digestive aids and cathartics. Their use for these purposes has become more limited recently, and this is probably appropriate until better preparations become available and indications for their administration become more clearly defined. Most commercial bile salt preparations are desiccated or crude extracts of cattle bile containing mainly unconjugated bile salts with small amounts of pigment and lipids. They are generally supplied as 0.2- or 0.3-g tablets. The customary dose is 0.4–0.6 g three times daily with meals. This is probably an inadequate dose, as will be explained later. Dehydrocholic acid, 3,7,12–triketo-5β-cholanoic acid, is oxidized cholic acid and is supplied in pure form but does not form micelles and hence probably does not assist fat absorption significantly and would be of little value for replacement therapy. Sodium dehydrocholate is supplied as a 20% solution in ampules and is widely used to measure blood circulation times. It does not appear to be conjugated by the liver (86).

The most common situations in which bile salts are deficient in the small intestine are (1) T-tube drainage of the common bile duct and (2) partial or complete interruption of ileal absorption, as in regional enteritis or ileal resection or bypass. In the former group, bile drainage is usually incomplete and temporary, so that most patients tolerate this period well without

bile salt administration. If near total biliary drainage is maintained for more than a few weeks, however, oral bile salts are indicated, since they aid fat absorption and are required for efficient absorption of vitamins A, D, and K. The customary dose of bile salts is probably too small for patients in whom all bile is being drained. The total bile salt pool (about 3.5 g) is largely in the gallbladder in normal fasting subjects and is estimated to circulate at least twice during a meal (7 g), so that theoretically a dose approximating this quantity should be given with each meal. Such large amounts, however, often cause diarrhea, and thus a smaller dose of 3.4 g with meals is usually preferable, increasing to a larger dose if the patient can tolerate it. Animals with total bile fistulas may survive for years without apparent ill-effect if vitamins A, D, and K are supplied parenterally (87,88).

Administration of 3.5 g bile salts with meals is therapeutic in postileectomy steatorrhea (89). Bile salts in this condition may aggravate diarrhea, however, and the use of medium-chain triglycerides as a fat source and cholestyramine to "bind" bile salts has been recommended as a means of dealing with steatorrhea in the presence of cholerheic diarrhea (90,91).

Administration of bile salts in jaundice caused by hepatobiliary disease is contraindicated, especially if pruritus is present, as bile salts will only aggravate this.

XXVIII. ANION EXCHANGE RESINS

Cholestyramine is very useful in certain disorders of bile salt metabolism. It is a polystyrene polymer with a 2% divinyl benzene cross-linkage and quaternary ammonium groups in the chloride form. The resin is not absorbed by the intestine and exchanges a chloride ion for an ionized bile salt molecule, which is carried out in the feces. Despite the close affinity of the resin for the bile salt, bacteria deconjugate and convert primary to secondary bile salts so that the feces of patients fed cholestyramine contain unconjugated secondary bile salts as do normal feces but the fecal excretion of bile salts is increased about three times (18). Cholestyramine has been used chiefly for relief of pruritus in jaundice, especially in chronic forms such as biliary cirrhosis (57,92–94). The usual starting dose is 10 g/day in three divided doses with meals. Children with absent or a diminished number of intrahepatic bile ducts ("ductopenia"), in whom growth is retarded, sometimes return toward a normal growth curve when serum bile salt concentrations are lowered to near normal levels by effective cholestyramine treatment. This sometimes requires larger doses of up to 20–30 g/day (95). Cholestyramine usually requires 5–10 days to achieve relief so that it may be of little use for transient pruritus such as that sometimes observed during the first few days of infectious hepatitis.

Calcification of the biliary tree with cholestyramine feeding has been reported (93,96). Gallstone formation has been induced (97) and prevented (98) with cholestyramine in animals. The effects of cholestyramine on bile salt synthesis are discussed in a preceding section.

XXIX. ANTIBIOTICS

Antibiotics in sufficient amounts suppress bacterial deconjugation and formation of secondary bile salts (46). The stagnant loop syndrome provides a circumstance in which antibiotics are useful in treating altered states of bile salt metabolism. In the stagnant loop syndrome, bacterial proliferation in the small bowel content deconjugates bile salts and leads to the formation of secondary bile salts. Unconjugated bile salts are rapidly absorbed from the small bowel, and thus their intraluminal concentration is diminished and fat absorption impaired. Tetracycline (40) or erythromycin (41) suppresses bacterial growth and restores conjugated bile salts to the small bowel lumen, with disappearance of steatorrhea.

Neomycin is a polybasic, poorly absorbed antibiotic which forms insoluble precipitates with bile salts (99). It lowers serum cholesterol concentrations in man (100–102) and chickens (99) and increases fecal bile acid excretion. It inhibits the hepatotoxic effects of lithocholic acid ingestion in chickens (99) and prevents bacterial conversion of cholate to deoxycholate (103). Neomycin, 6–12 g/day, induces a malabsorption syndrome, with mucosal changes similar to those of sprue (104). Bile salt metabolism is thus affected in at least three ways by neomycin: (1) a binding effect similar to that of cholestyramine, (2) suppression of deconjugation and secondary bile formation caused by antimicrobial properties, and (3) possible impairment of absorption of bile salts by intestinal mucosa. The first probably accounts for most of the increased fecal excretion of bile salts.

Serum lithocholate concentrations can be lowered with either neomycin or cholestyramine, as can the serum concentrations of the other bile salts if they are elevated. This is often associated with a similar decline in serum bilirubin concentrations, alkaline phosphatase activity, and BSP retention (7,95,105,106). This response has been interpreted as at least one piece of evidence that high serum bile salt concentrations, especially of lithocholate, may be injurious to the liver. In three jaundiced children with a paucity of intrahepatic bile ducts and pruritus, lowering of the serum bile salt concentrations to normal values with cholestyramine was accompanied by a return to normal of all previously abnormal liver chemistries except alkaline phosphatase, and the patients' growth curves returned to normal (95).

This is in contrast to studies in adults (92,93) with biliary cirrhosis in

which cholestyramine did not alter liver function studies significantly, but it was not determined in these studies if normal serum bile salt concentrations were ever established. It appears that sufficiently large amounts of cholestyramine must be given to produce normal or near normal serum bile salt concentrations if beneficial effects on the liver are to be achieved.

REFERENCES

1. J. B. Carey, Jr., *in* "Liver Disease" (L. Schiff, ed.), J. B. Lippincott, Philadelphia, p. 103 (1969).
2. J. B. Carey, Jr., and G. Williams, *J. Clin. Invest.* **42,** 450 (1963).
3. I. Björkhem, H. Danielsson, K. Einarsson, and G. Johansson, *J. Clin. Invest.* **47,** 1573 (1968).
4. E. Staple and J. L. Rabinowitz, *Biochim. Biophys. Acta* **59,** 735 (1962).
5. J. B. Carey, Jr., *J. Clin. Invest.* **43,** 1443 (1964).
6. J. L. Rabinowitz, R. H. Herman, D. Weinstein, and E. Staple, *Arch. Biochem. Biophys.* **114,** 233 (1966)
7. J. B. Carey, Jr., I. D. Wilson, F. G. Zaki, and R. F. Hanson, *Medicine* **45,** 461 (1966).
8. J. B. Carey, Jr., and G. A. D. Haslewood, *J. Biol. Chem.* **238,** 855 (1963).
9. K. A. Mitropoulos and N. B. Myant, *Biochem. J.* **103,** 472 (1967).
10. K. A. Mitropoulos and N. B. Myant, *Biochim. Biophys. Acta* **144,** 430 (1967).
11. D. Mendelsohn and L. Mendelsohn, *Biochemistry* **7,** 4167 (1968).
12. H. Danielsson, P. Eneroth, K. Hellström, S. Lindstedt, and J. Sjövall, *J. Biol. Chem.* **238,** 2299 (1963).
13. J. B. Carey, Jr., and G. Williams, unpublished observations.
14. S. Bergström, H. Danielsson, and B. Samuelsson, *in* "Lipid Metabolism" (K. Bloch, ed.), Wiley, New York (1960).
15. K. W. Heaton, W. I. Austad, L. Lack, and M. P. Tyor, *Gastroenterology* **55,** 5 (1968).
16. M. M. Stanley and B. Nemchausky, *J. Lab. Clin. Med.* **70,** 627 (1967).
17. R. Abaurre, S. G. Gordon, J. G. Mann, and F. Kern, Jr., *J. Lab. Clin. Med.* **72,** 849 (1968).
18. R. B. Moore, C. A. Crane, and I. D. Frantz, Jr., *J. Clin. Invest.* **47,** 1664 (1968).
19. M. M. Stanley, *J. Lab. Clin. Med.* **72,** 1020 (1968).
20. B. A. Kottke, *J. Lab. Clin. Med.* **70,** 990 (1967).
21. K. Hellström and S. Lindstedt, *J. Lab. Clin. Med.* **63,** 666 (1964).
22. S. Lindstedt, J. Avigan, D. S. Goodman, J. Sjövall, and D. Steinberg, *J. Clin. Invest.* **44,** 1754 (1965).
23. J. Avigan and D. Steinberg, *J. Clin. Invest.* **44,** 1845 (1965).
24. R. B. Moore, J. T. Anderson, H. L. Taylor, A. Keys, and I. D. Frantz, Jr., *J. Clin. Invest.* **47,** 1517 (1968).
25. M. Blum and N. Spritz, *J. Clin. Invest.* **45,** 187 (1966).
26. E. Theodore, N. Spritz, and M. H. Sleisenger, *Gastroenterology* **55,** 183 (1968).
27. R. F. Hanson and J. B. Carey, Jr., *J. Clin. Invest.* **49,** 39a (1970) (Abstr.).
28. S. S. Ali, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem.* **44,** 957 (1966).
29. B. Samuelsson, *J. Biol. Chem.* **235,** 361 (1960).
30. P. Eneroth, B. Gordon, and J. Sjövall, *J. Lipid Res.* **7,** 524 (1966).
31. P. Eneroth, B. Gordon, R. Ryhage, and J. Sjövall, *J. Lipid Res.* **7,** 511 (1966).

32. T. Schersten, *Biochim. Biophys. Acta* **141,** 144 (1967).
33. T. Schersten, P. Björntorp, P. H. Ekdahl, and S. Björkerud, *Biochim. Biophys. Acta* **141,** 155 (1967).
34. W. Burnett, *in* "The Biliary System" (W. Taylor, ed.), Blackwell Scientific Publs. (Davis), Philadelphia, p. 601 (1965).
35. J. Sjövall, *Clin. Chim. Acta* **5,** 30 (1960).
36. A. M. Bongiovanni, *J. Clin. Endocrinol. Metab.* **25,** 678 (1965).
37. J. C. Encrantz and J. Sjövall, *Clin. Chim. Acta* **4,** 793 (1959).
38. J. R. Poley, J. C. Dower, C. A. Owen, Jr., and G. B. Stickler, *J. Lab. Clin. Med.* **63,** 838 (1964).
39. J. M. Dietschy, H. S. Solomon, and M. D. Siperstein, *J. Clin. Invest.* **45,** 832 (1966).
40. R. M. Donaldson, Jr., *J. Clin. Invest.* **44,** 1815 (1965).
41. I. H. Rosenberg, W. G. Hardison, and D. M. Bull, *New Engl. J. Med.* **276,** 1391 (1967).
42. A. Norman and R. Grubb, *Acta Pathol. Microbiol. Scand.* **36,** 1 (1955).
43. M. Frankel, *Biochem. J.* **30,** 2111 (1936).
44. S. Tabaqchali and C. C. Booth, *Lancet* **2,** 12 (1966).
45. P. P. Nair, M. Gordon, and J. Reback, *J. Biol. Chem.* **242,** 7 (1967).
46. A. Norman, *Acta Physiol. Scand.* **33,** 100 (1955).
47. P. Samuel, G. M. Saypol, E. Meilman, E. H. Mosbach, and M. Chafizadeh, *J. Clin. Invest.* **47,** 2070 (1968).
48. B. Josephson, *J. Clin. Invest.* **18,** 343 (1939).
49. R. H. Palmer, *Proc. Natl. Acad. Sci.* **58,** 1047 (1967).
50. J. B. Carey, Jr., *J. Clin. Invest.* **37,** 1494 (1958).
51. B. Frosch and H. Wagener, *Nature* **213,** 404 (1967).
52. A. Lefevre and C. S. Lieber, *Clin. Res.* **16,** 531 (1968).
53. D. H. Gregory, G. F. Zaki, G. A. Sarosi, and J. B. Carey, Jr., *Arch. Pathol.* **84,** 194 (1967).
54. E. Urban, B. W. Frank, and F. Kern, Jr., *Ann. Int. Med.* **68,** 598 (1968).
55. D. V. Datta and S. Sherlock, *Brit. Med. J.* **1,** 216 (1963).
56. L. J. Schoenfield, J. Sjövall, and E. Perman, *Nature* **213,** 93 (1967).
57. J. B. Carey, Jr., and G. Williams, *J. A. M. A.* **176,** 432 (1961).
58. K. Sjövall and J. Sjövall, *Clin. Chim. Acta* **13,** 207 (1966).
59. T. F. Gallagher, Jr., M. N. Meller, and A. Kappas, *Medicine* **45,** 471 (1966).
60. M. J. Kreek, E. Weser, M. H. Sleisenger, and G. H. Jeffries, *New Engl. J. Med.* **277,** 1391 (1967).
61. O. H. Horrall, "Bile: Its Toxicity and Relation to Disease," University of Chicago Press, Chicago (1938).
62. P. Holsti, *Acta Pathol. Microbiol. Scand. Suppl.* **113,** 1 (1956).
63. P. Holsti, *Nature* **186,** 250 (1960).
64. P. Holsti, *Acta Pathol. Microbiol. Scand.* **54,** 479 (1962).
65. R. D. Hunt, G. A. Leveille, and H. E. Sauberlich, *Proc. Soc. Exptl. Biol. Med.* **115,** 277 (1964)
66. R. D. Hunt, G. A. Leveille, and H. E. Sauberlich, *Proc. Soc. Exptl. Biol. Med.* **113,** 139 (1963).
67. H. M. Edwards and F. M. Boyd, *Proc. Soc. Exptl. Biol. Med.* **113,** 294 (1963).
68. A. Stolk, *Experientia* **16,** 507 (1960).
69. N. B. Javitt, *Gastroenterology* **50,** 394 (1966).
70. F. Schaffner and N. B. Javitt, *Lab. Invest.* **15,** 1783 (1966).
71. F. G. Zaki, J. B. Carey, Jr., F. W. Hoffbauer, and C. Nwokolo, *J. Lab. Clin. Med.* **69,** 737 (1967).

72. A. Norman and M. S. Shorb, *Proc. Soc. Exptl. Biol. Med.* **110,** 552 (1962).
73. G. Johansson, *Acta Chem. Scand.* **20,** 240 (1966).
74. L. Lyon-Caen, *J. Physiol. Pathol. Gen.* **12,** 758 (1910).
75. A. Lemierre, M. Brule, and H. Garban, *Semaine Med.* **34,** 301 (1914).
76. M. Brule, "Recherches sur les icteres," 3rd ed., Masson, Paris (1922).
77. P. H. Ekdahl and T. Bennike, *Acta Chem. Scand.* **115,** 203 (1958).
78. B. Lewis, S. Tabaqchali, D. Panveliwalla, and I. D P. Wootton, *Lancet* **1,** 219 (1969).
79. R. A. Cooper and J. H. Jandl, *J. Clin. Invest.* **47,** 809 (1968).
80. S. M. Grundy, E. H. Ahrens, and T. A. Miettinen, *J. Lipid Res.* **6,** 397 (1965).
81. D. Rudman and F. E. Kendall, *J. Clin. Invest.* **36,** 530 (1957).
82. T. Usui, S. Nakasone, and M. Kawamoto, *Yonago Acta Med.* **10,** 250 (1966).
83. J. A. Gregg, *Nature* **214,** 29 (1967).
84. J. C. Fisher, E. F. Bernstein, and J. B. Carey, Jr., *Gastroenterology* **49,** 272 (1965).
85. P. Fantl, A. J. Rollo, and H. Strosberg, *Gut* **6,** 384 (1965).
86. R. I. Ellin, A. I. Mendeloff, and D. A. Turner, *Anal. Biochem.* **4,** 198 (1962).
87. A. J. Quick, C. Hussey, and G. E. Collentine, *J. Lab. Clin. Med.* **44,** 909 (1954).
88. A. J. Quick, C. V. Hussey, and G. E. Collentine, *Am. J. Physiol.* **176,** 239 (1954).
89. W. G. M. Hardison and I. H. Rosenberg, *New Engl. J. Med.* **277,** 337 (1967).
90. A. F. Hofmann, *Gastroenterology* **52,** 752 (1967).
91. R. B. Zurier, S. A. Hashim, and T. B. VanItallie, *Gastroenterology* **49,** 490 (1965).
92. D. V. Datta and S. Sherlock, *Gastroenterology* **50,** 323 (1966).
93. F. Schaffner, F. M. Klion, and A. J. Latuff, *Gastroenterology* **48,** 293 (1965).
94. T. B. VanItallie, S. A. Hashim, R. S. Crampton, and D. M. Tennent, *New Engl. Med.* **265,** 469 (1961).
95. H. L. Sharp, J. B. Carey, Jr., J. G. White, and W. Krivit, *J. Pediat.* **71,** 723 (1967).
96. R. F. Wells, J. H. Knepshield, and C. Davis, *Am. J. Digest. Dis.* **13,** 86 (1968).
97. L. J. Schoenfield and J. Sjövall, *Am. J. Physiol.* **211,** 1069 (1966).
98. F. Bergman and W. Vanderlinden, *Gastroenterology* **53,** 418 (1967).
99. H. Eyssen, E. Evrard, and H. Vanderhaeghe, *J. Lab. Clin. Med.* **68,** 753 (1966).
100. P. Samuel and A. Steiner, *Proc. Soc. Exptl. Biol. Med.* **100,** 193 (1959).
101. G. A. Goldsmith, J. G. Hamilton, and O. N. Miller, *Trans. Ass. Am. Physicians* **72,** 207 (1959).
102. G. A. Goldsmith, J. G. Hamilton, and O. N. Miller, *Arch. Int. Med.* **105,** 512 (1960).
103. J. G. Hamilton, *Arch. Biochem. Biophys.* **101,** 7 (1963).
104. E. D. Jacobson, R. B. Chodos, and W. W. Faloon, *Am. J. Med.* **38,** 524 (1960).
105. J. B. Carey, Jr., I. D. Wilson, F. G. Zaki, and G. Williams, *J. Lab. Clin. Med.* **68,** 862, 1966 (Abst.).
106. J. B. Carey, Jr., I. D. Wilson, G. Onstad, and F. G. Zaki, *J. Clin. Invest.* **46,** 1042 (1967).
107. S. M. Grundy and E. H. Ahrens, Jr., *J. Lipid Res.* **10,** 91 (1969).
108. J. D. Wilson and C. A. Lindsey, Jr., *J. Clin. Invest.* **44,** 1805 (1965).
109. K. Einarsson, *Europ. J. Biochem.* **5,** 101 (1968).
110. M. J. Hill and B. S. Drasar, *Biochem. J.* **104,** 55 (1967).
111. D. H. Sandberg, J. Sjövall, K. Sjövall, and D. A. Turner, *J. Lipid Res.* **6,** 182 (1965).
112. G. A. D. Haslewood, "Bile Salts," Methuen & Co., London, p. 63 (1967).
113. F. Nakayama, *J. Lab. Clin. Med.* **69,** 594 (1967).

Chapter 4

NEWER ASPECTS OF BILE SALT METABOLISM IN INTESTINAL DISEASE*

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I. GENERAL CONSIDERATIONS OF EXPERIMENTAL METHODS USED IN THESE CLINICAL INVESTIGATIONS

In the absence of a disordered hepatobiliary system, the bile salt pool is virtually confined to the enterohepatic circuit. The intravenous administration of a ^{14}C-labeled bile salt results in its rapid hepatic clearance and prompt entry into bile (1). Recovery of the injected labeled bile salt from the duodenum has averaged 90% 3 hr after intravenous injection when cholecystokinin is administered intravenously over a 1-hr period (2). In this situation, a balloon is used to obstruct the lumen of the small intestine at the level of the ligament of Treitz. The total duodenal (bile) collection will average 230 ml in normal man (2). Measurements of the specific activity of the previously injected bile salt recovered from the pooled duodenal contents provide a measure of the available bile salt pool present in the hepatobiliary system when such studies are performed after an overnight fast (3). Obviously, this method of estimation of the bile salt pool does not provide a measure of that portion of endogenous bile salt which may be sequestered in other regions of the enterohepatic space during the 3-hr period in which the labeled material

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is injected and collected. Additionally, incomplete evacuation of the gallbladder could lead to inadequate mixing with the contents of the biliary tree, and seepage of bile around the obstructed site would result in incomplete recovery. These several potential errors may be eliminated through use of the isotope dilution principle, in which a steady-state situation prevails and the injected labeled bile salt distributes throughout the endogenous pool. Thus measurements of specific activity obtained from small samples of duodenal fluid obtained serially after the injection of the labeled bile salt do not require either complete gallbladder evacuation or complete luminal sampling. In such studies, single aliquots are representative when obtained after complete mixing with the endogenous bile salt pool. Such mixing is hastened by normal physiological processes which follow the ingestion of a meal, inasmuch as current evidence suggests that the entire bile salt pool circulates enterohepatically two to three times with each meal (4). The methods of calculation of bile salt pool and of the rate of loss of the labeled bile salt from the enterohepatic circulation using the daily exponential decline of specific activity are standard procedures and have been reviewed recently in detail (5). Lindstedt (6) in 1957 used cholic acid-^{14}C to study the kinetics of the enterohepatic circulation in normal man. More recently, two groups (7,8) have obtained such measurements following the simultaneous administration of cholic acid and chenodeoxycholic acid, one labeled with ^{3}H and the other with ^{14}C. Observations (7) in two normal subjects indicated a larger pool for chenodeoxycholic acid than for cholic acid, as well as a more prolonged half-life ($t_{1/2}$) and turnover rate. In another study (8), six hypercholesteremic subjects exhibited a longer $t_{1/2}$ for chenodeoxycholate, but the pool size and turnover rate for cholic acid appeared relatively larger. It is apparent from such studies that simultaneous measurements of the kinetics of the two primary bile salts are required in additional normal individuals and that such measurements utilizing a single bile salt need not be representative of the total picture. Observations in our laboratory have demonstrated that hydrolysis of conjugated bile salts occurs regularly during enterohepatic circulation in normal man (9). Such metabolic alterations of sodium taurocholate-24-^{14}C and sodium glycocholate-24-^{14}C were apparent (Fig. 1) following their intravenous administration to normal medical students. In these studies, aliquots of duodenal fluid were obtained at the designated time intervals, individual bile salts were fractionated* by thin-layer chromatography, and the ^{14}C radioactivity of each fraction was compared with the total ^{14}C radioactivity recovered from the chromatogram (3,9). Following taurocho-

*Taurodeoxycholate and taurochenodeoxycholate were not separable nor were their glycine-conjugated counterparts.

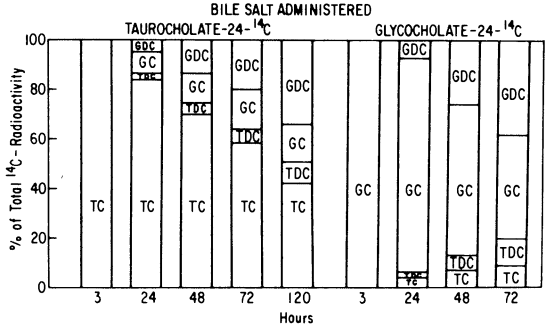

Fig. 1. Composition of residual ^{14}C radioactivity in duodenal fluid obtained at the designated time periods after the intravenous injection of either taurocholate-^{14}C (R. A.) or glycocholate-^{14}C (S. L.) into two student volunteers. Data are expressed in terms of percentage of ^{14}C radioactivity contributed by each bile salt fraction to the total recoverable ^{14}C radioactivity. GDC, glycodeoxycholate; GC, glycocholate; TDC, taurodeoxycholate; TC, taurocholate.

late-^{14}C administration, the percentage of glycocholate-^{14}C recovered increased progressively with time, whereas the administration of glycocholate-^{14}C was associated with a progressive increase in the percentage of taurocholate-^{14}C recovered (Fig. 1). This sequence could only arise as a result of bacterial hydrolysis followed by reabsorption of the cholate-^{14}C and subsequent hepatic conjugation with glycine or taurine. Similar measurements have not been performed with glycine or taurine conjugates of chenodeoxycholic acid. However, it would seem likely that such bacterial metabolism and altered patterns of distribution of the label would pertain. Injection of unconjugated bile salts cannot detect these phenomena. In such experiments (6–8), the injected cholate-^{14}C would be partitioned by the liver between the taurocholate and glycocholate pools, and interconversions of taurocholate and glycocholate (or other primary bile salts) dependent in part on enteric bacteria could not be ascertained. Similarly, measurements of ^{14}C-radioactivity in fecal collections obtained over successive 24-hr periods following intravenous administration of ^{14}C-labeled bile salts do not detect such bacterial hydrolysis and hepatic reconjugation. More importantly, the excretion method (10,11), when applied to estimates of turnover rate, measures indiscriminately the radioactivity present in metabolites of the injected primary bile salt which recirculate enterohepatically at rates which may differ significantly from that of the parent compound. This aspect of the problem is

illustrated graphically in Fig. 1. It is apparent that ^{14}C radioactivity accumulated progressively in glycine and taurine conjugates of deoxycholate following intravenous administration of either taurocholate-24-^{14}C or glycocholate-24-^{14}C to a normal medical student. Indeed, by 120 hr in the former instance and 72 hr in the latter, 49% of the residual total ^{14}C-radioactivity in duodenal fluid was present in conjugates of this secondary bile salt. At these time periods, residual total ^{14}C-radioactivity represented a considerable portion of the initial 3-hr value, i.e., 42 and 48%, respectively. These observations are consistent with well-recognized metabolic pathways for trihydroxy bile salt metabolism (12,13). The presence of organisms in the intestinal flora of normal man which have been shown capable of both hydrolysis and 7α-dehydroxylation adds further physiological significance to such data (14). Here, deoxycholate-^{14}C formed either in the more distal regions of the small intestine or colon may be absorbed by passive diffusion, returning to the liver and there conjugating with glycine or taurine in ratios which approximate 3:1 (9), prior to excretion into bile. The ^{14}C-conjugated deoxycholate then recirculates enterohepatically at rates which have not thus far been measured but probably are similar to those of primary bile salts. Such observations may also be pertinent to the metabolism of conjugates of chenodeoxycholic acid, although its metabolic by-product, lithocholate (13), may circulate at significantly different rates than conjugated deoxycholate. Indeed, recent studies suggest a biphasic rate of recirculation which may be related to the formation of conjugated lithocholate sulfate (R. H. Palmer, personal communication) and to differences in intestinal transport of sulfated and unsulfated compounds. (15)

The concentration of bile salts in the intestinal lumen of normal man during the digestion of a test meal was reported by Borgström *et al.* in 1957 (4) and by Sjövall in 1959 (16). From these measurements, obtained during 3–4 hr of digestion and absorption, and the isotope dilution studies of Lindstedt (6), it appeared that bile salts were excreted into the intestine and absorbed at least two times during the digestion of a test meal. More recently, several groups (17–20) have obtained such measurements of luminal bile salt concentration in normals and hospitalized controls. The methods used, including considerations of dilutional factors, preparation of duodenal fluid, and chemical procedures, have been reviewed recently in detail (5). It has been suggested that such measurements may provide a measure of the effectiveness of synthetic and absorptive mechanisms to maintain the bile salt pool and enable luminal concentration of bile salts capable of physiological function. There have been no combined studies of luminal bile salt concentration and isotope dilution measurements following injection of labeled bile salts in normals. Such observations would serve as a basis for comparative observation in pathophysiological states.

II. STUDIES OF ENTEROHEPATIC CIRCULATION OF BILE SALTS IN PATIENTS WITH DISEASES PRIMARILY INVOLVING THE DISTAL SMALL INTESTINE

A. Quantitative Changes in Recirculating Bile Salts

It has been established that the ileum is the sole site of active transport of bile salts in a variety of species of animals (21,22). The physiological significance of this active transport system in the maintenance of bile salt enterohepatic circulation has been suggested by the studies of Playoust *et al.* (23), in which ileectomy and jejunectomy were compared in dogs. Although studies (24) in normal humans have suggested that taurocholate absorption occurs at a more distal location than fat absorption, information on the role of the ileum in the maintenance of enterohepatic bile salt circulation in man has been obtained chiefly from studies in patients with a variety of disorders of this intestinal region. The vast majority of reported observations have been performed in patients with resection or bypass of varying lengths of the distal small intestine, usually with concomitant resection of portions of the proximal colon or distal area of the proximal small intestine (3,9–11,17–20,25). Regional enteritis was the basic illness in approximately 60%, and obstruction due to volvulus was the reason for resection in approximately 20%. The methods of study have included sequential measurements of specific activity of duodenal contents following intravenous or oral administration of ^{14}C-labeled trihydroxy bile salts [cholate-24-^{14}C (9,17), taurocholate-24-^{14}C (3,9, 18,25), and glycocholate-24-^{14}C (3)], measurements of fecal excretion following oral or intravenous administration of cholate-24-^{14}C (10,11), and assay of the concentration of bile salts (17–20) in luminal fluid obtained during digestion and absorption of a test meal. The problems of precise localization of the presence and extent of residual disease, as in the case of resected regional enteritis, as well as the extent of surgical excision of the small intestinal length, are well recognized (26). Nevertheless, these observations have demonstrated clearly that resection of the distal one-half to one-third of the small intestine results in marked reductions in enterohepatic circulation of trihydroxy conjugated bile salts and an increased rate of fecal excretion of ^{14}C-radioactivity. For example, the specific activity of sodium taurocholate-^{14}C and sodium glycocholate-^{14}C in duodenal fluid 24 hr after their intravenous injection was virtually zero in two patients with resection of the distal one-third to one-half of the small intestine for obstruction due to volvulus and in three patients who had a jejunocolic fistula (three-fourths of the distal small intestine bypassed) for the management of obesity (Fig. 2) (3). Additionally, the specific activity obtained at 24 hr in five patients with resection of the distal one-half to one-third of the small intestine for regional enteritis

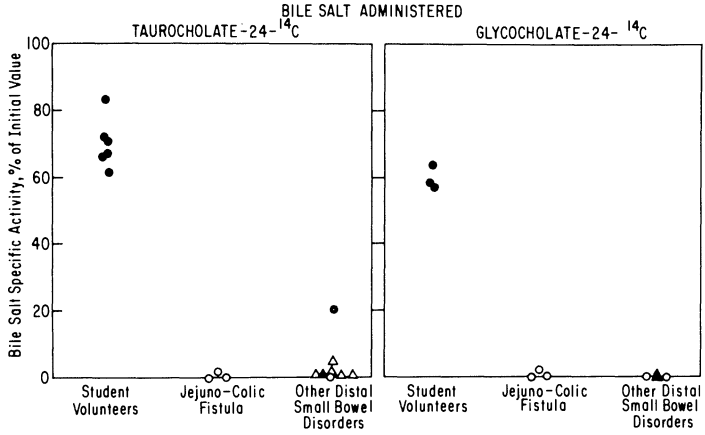

Fig. 2. Rate of disappearance of ^{14}C-labeled conjugated trihydroxy bile salts from the enterohepatic circulation 24 hr after intravenous injection into student volunteers, patients with jejunocolic fistula, and patients with other distal small bowel disorders. O, Resection, volvulus; △, resection, regional enteritis; ▲, no resection, radiation; ⊘no resection, active regional enteritis (see Table I). In part from Heaton *et al.* (3).

was reduced similarly (Fig. 2) (9). In these studies, the available bile salt accumulated in the hepatobiliary system after an overnight fast was estimated by the isotope dilution formula using the specific activity of the initial 2–3-hr duodenal sample and the specific activity of the known amount of injected ^{14}C-labeled conjugated trihydroxy bile salt (3). These results (Table I) are subject to the methodological errors discussed previously. Nevertheless, the results following taurocholate injection are comparable to those reported by Van Deest *et al.* (18) (Table I), in which the specific activity of duodenal samples was measured on four occasions (each during the digestion of a standard meal) over the 26-hr period which followed intravenous administration of sodium taurocholate-24-^{14}C to five patients with distal small intestinal resection for similar illnesses. In these studies (18), the half-life ($t_{1/2}$) of injected taurocholate-^{14}C and pool size were estimated from the exponential decline in specific activity (Fig. 3). Concomitant measurements of total bile salt concentration in small bowel contents revealed values after an overnight fast (during breakfast) which were within the normal range in three patients with resection but were approximately half normal in two other patients. However, in each of these patients the concentration fell over the second and third meal to values which were clearly below normal and then tended to rise somewhat by the next morning (fourth meal). These combined observations (3,18) indicate considerably increased rates of bile salt synthesis in ileectomized patients which are most readily demonstrated after an over-

TABLE I. Measurements of Taurocholate-^{14}C and Glycocholate-^{14}C Recirculation and Metabolism

Subject	Reason for resection or illness	Approximate amount resected or bypassed		Taurocholate-^{14}C Half-life (hr)	Taurocholate-^{14}C Estimated pool size (mg)	Glycocholate-^{14}C Half-life (hr)	Glycocholate-^{14}C Estimated pool size (mg)
E. D.[a]	Obesity	2/3			64		836
J. H.[a]	Obesity	2/3			41		825
E. R.[a]	Volvulus	1/2					322
E. B.[a]	Volvulus	1/3			59		535
M. V.[b]	Volvulus	4/5		5.5	50		
L. C.[b]	Radiation	1/3		3.8	104		
F. R.[b]	Regional enteritis	1/2		1.3	43		
H. A.[b]	Regional enteritis	1/2		2.5	147		
P. G.[b]	Regional enteritis	1/3		2.5	191		
J. B.[a]	Regional enteritis	1/3			50		
D. H.[a]	Regional enteritis	1/3			94		
P. L.[a]	Regional enteritis	1/3			80		
N. M.[a]	Regional enteritis	1/3			168		
S. R.[a]	Regional enteritis	1/3			84		
E. C.[c]	Regional enteritis	0		10.8	71		
N. S.[a]	Radiation	0			40		625
Student volunteers[c]			Range	35–54	272–633	30–37	735–1400
			Mean ± 1 S.D.	45 ± 8	377 ± 130	33 ± 4	1165 ± 403

[a] Pool size estimated by isotope dilution formula, using the specific activity of the duodenal sample obtained 3 hr after injection of the labeled bile salt (3).

[b] Pool size estimated from the exponential decline in specific activity of duodenal samples obtained serially after injection of the labeled bile salt (18).

[c] Pool size estimated from the exponential decline in specific activity of duodenal samples obtained serially after injection of the labeled bile salt (3).

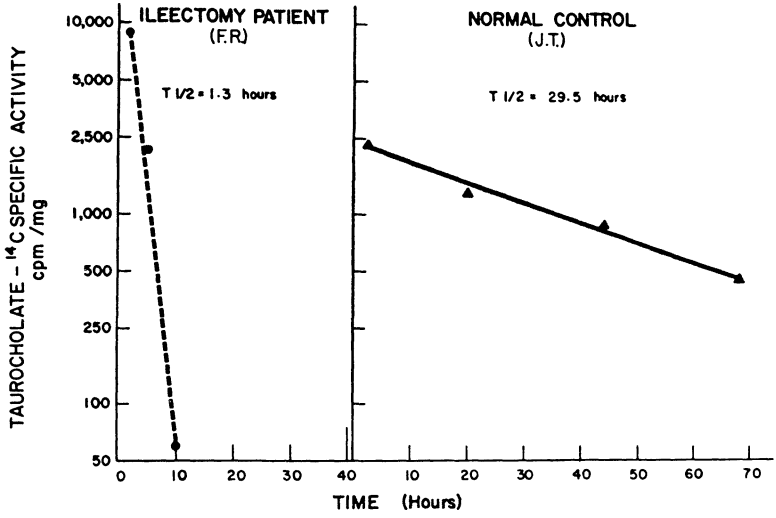

Fig. 3. The rate of disappearance of sodium taurocholate-24-^{14}C from the duodenal contents of a normal subject (J. T.) and an ileectomy patient (F. R.). After the intravenous administration of taurocholate-24-^{14}C (5.6×10 cpm), duodenal samples were aspirated at 2, 6, 10, and 26 hr in F. R. and at 2, 20, 44, and 68 hr in J. T. The specific activity of the 26-hr sample in F. R. was too low for assay. From Van Deest *et al.* (18).

night fast. This enhanced synthesis is incapable of maintaining levels exhibited by normals throughout a day of feeding (18). The studies of Van Deest *et al.* (18) showed that the deficits in luminal concentration of total bile salts were not as great as would be expected from their estimates of taurocholate pool size measured simultaneously. This lack of correlation may be explained partially by the estimates of available glycocholate-^{14}C in the hepatobiliary system of ileectomized patients (3) (Table I). Although values for taurocholate-^{14}C were approximately 13% of normal in such patients, those for glycocholate-^{14}C were approximately 60% of normal. Thus in these patients who have had a two-thirds to one-third loss of small intestine there is a relative increase in the ratio of the glycocholate pool to the taurocholate pool: range 9.5–20.1 as compared with 2.5 and 4.7 in two normal students in whom the bile salt pool was measured with both cholate conjugates (3). An increased relative conjugation of bile salts with glycine has been reported by McLeod and Wiggins (20) in five patients with ileectomy and also by Garbutt *et al.* (9) in 11 patients with similar disorders (Table II). In the latter study (9), the relative conjugation of cholate with glycine and taurine (G:T ratio) was measured colorimetrically in extracts of duodenal fluid fractionated by thin-layer chromatography, whereas the G:T ratio of the

TABLE II. Relative Conjugation of Cholate and Deoxycholate-^{14}C with Glycine and Taurine in Duodenal Fluid

	Cholate conjugates			Deoxycholate-^{14}C conjugates[b]		
	Number of determinations	G:T		Number of determinations	G:T	
		Mean ± 1 S.D.	Range		Mean ± 1 S.D.	Range
Ileal disorders	13	15.3 ± 6.0	7.5–27.5	5[a]	16.8 ± 7.5	12.1–18.0
Duodenal–jejunal disorders	4	3.4	1.4–6.4[c]	4[c]	4.9	2.3–7.5
Nontropical sprue	2	1.6	1.4–1.8	2	2.8	2.3–3.8
Whipple's disease	2	5.4	4.4–6.4	2	7.1	6.7–7.5
Student volunteers	5	2.8 ± 0.8	1.8–3.5[c]	5[c]	3.4 ± 1.4	2.3–5.7

From Garbutt *et al.* (9).

[a] Each determination is the average of two values in one patient and one value in four patients obtained during a single study period.

[b] Obtained following intravenous administration of sodium taurocholate-24-^{14}C. See text (9).

[c] Average of two to four values obtained during a single study period.

dihydroxy bile salt was assayed from the radioactivity present in glycine and taurine conjugates of deoxycholate-^{14}C of duodenal fluid obtained following the intravenous administration of taurocholate-^{14}C (Figs. 1 and 4). In additional studies (9), the G:T ratio of cholic acid-^{14}C and deoxycholic acid-^{14}C was determined in duodenal fluid obtained over a 3-hr period following their intravenous injection into ileectomized patients. Values obtained, which were similar to those shown in Table II, established the hepatic origin of these altered G:T ratios for both the primary trihydroxy and secondary dihydroxy bile salts. Although the precise mechanism involved in the production of these changes has not been determined, these results were related to the enhanced synthesis of primary bile salts resulting from the loss of enterohepatic circulation and a consequent increased demand for conjugation with glycine and taurine. It was apparent from these studies (9) that ileectomized patients had not lost the capacity for conjugation with taurine, since a marked decrease in G:T ratio followed oral taurine administration. These results suggest a decrease in the relative availability of taurine. In their entirety (3,9,18, 20), these observations indicate that taurine-conjugated bile salts comprise an extremely small increment of the bile salt pool in patients with loss of ileal bile salt transport and that a closer correlation between luminal bile salt concentration and estimates of bile salt pool size may be obtained with

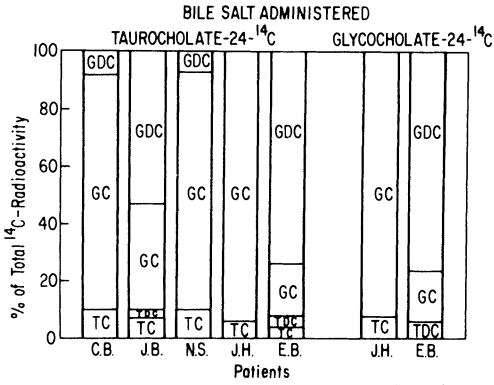

Fig. 4. Composition of residual ^{14}C radioactivity in duodenal fluid obtained 24 hr after intravenous injection of either taurocholate-^{14}C or glycocholate-^{14}C to patients with disorders of the distal small bowel (see Table I). Data are expressed in terms of percentage of ^{14}C radioactivity contributed by each bile salt fraction to the total recoverable ^{14}C radioactivity. GDC, glycodeoxycholate; GC, glycocholate; TDC, taurodeoxycholate; TC, taurocholate. In part from Heaton *et al.* (3).

measurements of glycine-conjugated bile salts. In addition, such correlative information suggests that measurements of luminal bile salt concentration during the entire day (18) may provide a useful estimate of bile salt reabsorption and synthetic capcaity. It should be noted that neither isotopic dilution methods nor measurements of fecal radioactivity have been applied in such patients using conjugates of chenodeoxycholate-^{14}C. The absence of deoxycholate conjugates in duodenal fluid obtained from four of six patients with ileal disorders thus far studied (17,20) and the observation that ^{14}C radioactivity, following intravenous injection of deoxycholate-^{14}C was virtually zero in duodenal fluid obtained at 24 hr in one patient and 72 hr in another, both with ileal resection (9), suggest a similar loss of dihydroxy bile salts from enterohepatic recirculation. Nevertheless, differences in synthetic rates of primary trihydroxy and dihydroxy bile salts may exist, as suggested by the previously cited observations in normal humans.

Similar studies to those described have been performed in only a limited number of patients with ileal disease in the absence of resection or bypass (3,9–11): five with active regional ileitis and two with radiation damage. The results of isotope dilution studies (Table I, Fig. 2), fecal excretion measurements, and measurements of luminal bile salt concentration have thus far been similar to those reported in ileectomized patients, suggesting that inflammatory disease of the ileum *per se* results in a loss of enterohepatic circulation of bile salts. It is not known whether this absorptive defect is repaired in remission of regional enteritis. The course of this disease is characterized by variations in activity; whether or not these defects in bile salt metabolism vary in accordance with this natural history of the disease is one of several questions requiring study.

B. Qualitative Changes in Recirculating Bile Salts

Bacterial modifications of primary bile salts enable such material to traverse the intestinal mucosa via nonionic diffusion processes (27). A small intestinal bacterial flora may develop in all patients with ileal resection and removal of the ileocecal valve but might be a more likely concomitant in patients with regional enteritis with recurrent disease and partial obstruction of the new terminal small bowel. Moreover, this latter sequence may be a feature of regional enteritis not complicated by previous resection or bypass. Such findings have been reported by Krone *et al.* (19) in two patients, one with resection and one without, who exhibited multiple strictures. Bile salts in the unconjugated form comprised 50–100% of the bile salts in luminal contents obtained from a site 20–30 cm distal to the ligament of Treitz. In studies of similar patients reported by Van Deest *et al.* (18), Heaton *et al.* (3), and Garbutt *et al.* (9), the most distal site of sampling was at the ligament of

Treitz, and bile salts were present only as conjugates. Nevertheless, the latter two reports include observations which clearly demonstrate a tendency for microbial metabolites of taurocholate and glycocholate to persist in the enterohepatic circulation longer than the parent compound. In these studies, total ^{14}C radioactivity, normalized for volume change, was measured in duodenal fluid 24 and 48 hr after the intravenous injection of sterol-^{14}C labeled conjugates of cholic acid. The residual ^{14}C radioactivity represented the sum of the remaining unaltered bile salt and its microbial metabolites. Sufficient total ^{14}C radioactivity was present at 24 hr to measure the relative chemical composition in four of 11 patients with ileal resection or bypass studied (3) (Fig. 4); patient N. S. had ileal disease due to extensive irradiation (25). In one of these (J. B.), a patient with recurrent regional enteritis, such measurements were obtained from a 48-hr sample (9). It is apparent (Fig. 4) that the residual radioactivity after taurocholate administration is present chiefly as reconjugated microbial metabolites. The residual radioactivity after glycocholate administration to patient E. B. was also present chiefly as such metabolites. Indeed, the similarity in distribution of radioactivity following administration of either bile salt to patients E. B. and J. H. is striking and is in accord with the concept that the radioactivity present in the 24-hr sample was derived from a common precursor; in this case, cholate-^{14}C was formed from bacterial hydrolysis. It is also apparent that bacterial hydrolysis and 7α-dehydroxylation may predominate, with subsequent reconjugation of deoxycholate-^{14}C. The entire study with sodium taurocholate-^{14}C was repeated in E. B. and J. B., following the administration of tetracycline, 0.25 g every 6 hr, which was continued for the duration of the study. During this period, ^{14}C radioactivity of the untreated duodenal sample was zero at 24 hr, whereas these values were 17 and 12% prior to tetracycline. Although the intestinal flora was not studied and the luminal site of these metabolic transformations was unknown, these observations with tetracycline lend additional support to the role of passive diffusion as a mechanism of absorption of unconjugated bile acids in some patients with ileectomy. These considerations of bacterial metabolism may not be quantitatively important to the enterohepatic circulation of many patients with ileectomy. However, they may contribute materially in some instances. One patient with active regional enteritis without surgery, which appeared to involve the distal one-third of the small intestine, has been studied in this manner after intravenous sodium taurocholate-24-^{14}C (9). The specific activity was 22 and 4.5% of the 3-hr value at 24 and 48 hr, respectively ($t_{1/2}$10. 8 hr) (Fig. 5). However, the total ^{14}C-radioactivity in luminal fluid was 55 and 36% of the 3-hr value, indicating a persistence in enterohepatic circulation of altered bile salts. The composition of this residual ^{14}C radioactivity is shown in Fig. 5. At 24 and 48 hr, conjugates of deoxycholate-^{14}C comprised 48 and 93% of the residual

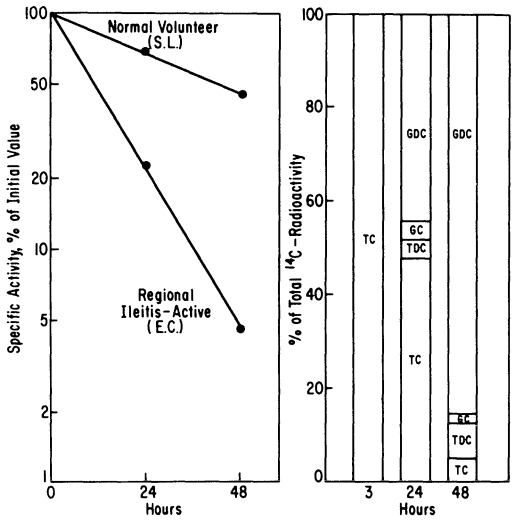

Fig. 5. Rate of disappearance of sodium taurocholate-24-^{14}C from the enterohepatic circulation 24 and 48 hr after intravenous injection into a patient with active regional enteritis without resection (E. C.) (left panel). Composition of residual ^{14}C radioactivity in duodenal fluid obtained 3, 24, and 48 hr after the intravenous injection of sodium taurocholate-24-^{14}C into patient E. C. (right panel). Data are expressed in terms of percentage of ^{14}C radioactivity contributed by each bile salt fraction to the total recoverable ^{14}C radioactivity. GDC, glycodeoxycholate; GC, glycocholate; TDC, taurodeoxycholate; TC, taurocholate. In part from Grabutt *et al.* (9).

radioactivity, respectively. Thus it would appear that bacterial modification with reabsorption of bile salts by both passive and active processes may combine to maintain enterohepatic bile salt recirculation in this disorder.

III. STUDIES OF ENTEROHEPATIC CIRCULATION OF BILE SALTS IN PATIENTS WITH DISEASES PRIMARILY INVOLVING THE PROXIMAL SMALL INTESTINE

A. Nontropical Sprue and Whipple's Disease

Since nontropical sprue and Whipples disease involve the proximal small intestine primarily, quantitative alterations of bile salt enterohepatic cir-

culation would not be expected except in the relatively uncommon instance of concomitant ileal disease. Indeed, measurements of enterohepatic circulation of taurocholate-^{14}C have been reported as normal in three patients with nontropical sprue (9,18) and one with Whipple's disease (9). Kinetic data obtained from the exponential decline in specific activity, including the $t_{1/2}$ of the disappearance rate and pool size, were also similar to normal values. However, the average bile salt concentration of duodenal fluid sampled during the digestion and absorption of a test meal was reduced in one of the patients with nontropical sprue (18), suggesting that factors other than loss of enterohepatic circulation may be operative in such patients. Similar studies of luminal bile salt concentration in two other patients with sprue (19,20) and one with Whipple's disease (20) have been reported as normal. Garbutt *et al.* (9) have reported a decreased rate of enterohepatic circulation of taurocholate-^{14}C in one patient with Whipple's disease (Fig. 6) in whom the total

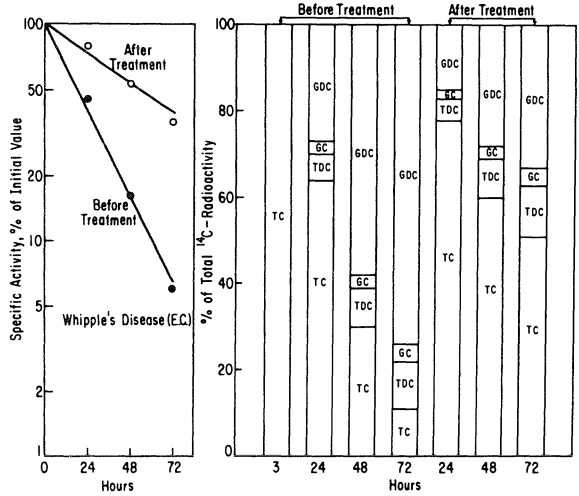

Fig. 6. Rate of disappearance of sodium taurocholate-24-^{14}C from the enterohepatic circulation 24, 48, and 72 hr after intravenous injection into a patient with Whipple's disease before and 3 months after treatment with antibiotics (left panel). Composition of residual ^{14}C radioactivity in duodenal fluid obtained at 3, 24, 48, and 72 hr after the intravenous injection of sodium taurocholate-24-^{14}C into patient E. C. with Whipple's disease before and 3 months after antibiotic treatment (right panel). Data are expressed in terms of percentage of ^{14}C radioactivity contributed by each bile salt fraction to the total recoverable ^{14}C radioactivity. GDC, glycodeoxycholate; GC, glycocholate; TDC, taurodeoxycholate; TC, taurocholate. In part from Garbutt *et al.* (9).

residual ^{14}C radioactivity was similar to that obtained from normal volunteers at 24, 48, and 72 hr after injection of the labeled bile salt. The chemical composition of this residual radioactivity (Fig. 6) indicates prominent bacterial deconjugation and 7α-dehydroxylation, with glycodeoxycholate-^{14}C predominating in all samples obtained after the initial 3 hr. The morphological alteration in this disease invariably involves the duodenum (28,29). However, luminal bile salt samples from this site were consistently present as conjugates, suggesting the bacterial metabolism of bile salts is not operative at this level of the small intestine. It seems unlikely, therefore, that these observations are related to the basic pathology of Whipple's disease (28,29). More likely, a resident bacterial flora capable of significant qualitative alterations of trihydroxy bile salt may develop at some more distal intestinal site in these patients. Duodenal fluid obtained from this patient and one other with Whipple's disease exhibited a relative increase in glycine conjugates of cholate and deoxycholate-^{14}C (G:T ratio) (9), the latter obtained following intravenous administration of taurocholate-^{14}C (Table II, Fig. 6). However, values in Whipple's disease were lower than those observed in any patient with an ileal disorder (Table II). The validity of these relatively limited observations in two patients with Whipple's disease was supported by values obtained for the G:T ratio following treatment and clinical remission, which decreased to a level comparable to that in normal controls (9) (Fig. 6). These results were not attributed to preferential absorption of glycine-conjugated bile salts, since the G:T ratio of deoxycholate-^{14}C was relatively unchanged throughout 72 hr of study. Although the precise mechanisms involved remain speculative, an enhanced demand for conjugation of the recirculating unconjugated bile salts may be operative, in part (see also the discussion of "blind-loop" syndrome). Further study of bile salt metabolism using quantitative and qualitative methods should provide considerable additional information in these disorders.

B. Altered Intestinal Microflora, "Blind-Loop" Syndrome

The pathophysiology and clinical setting of the "blind-loop" syndrome have been reviewed recently by Donaldson (30). It is clear that a resident bacterial flora in the proximal small intestine has a major role in the development of the absorptive and luminal defects which have been observed repeatedly in these patients (20,31–35), who have a variety of basic illness, and in experimental animals (31,36) in which an antiperistaltic pouch has been constructed either in the proximal or middle jejunum. However, the presence and extent of the absorptive defects, i.e., vitamin B_{12}, folate, xylose, and lipid, as well as the extent of luminal metabolic alteration of bile salts have varied in individual patients. This variability is probably related to

qualitative and quantitative differences in microbial populations and strains and also to such factors as luminal *p*H and substrate availability. A variety of microorganisms are known to be present in the feces and within the colon of normal individuals (37,38). In general, obligate anaerobes such as bacteroïdes constitute the overwhelming majority of these microbes (38). Coliform and gramnegative anaerobes, characteristically numerous in the cecum, are relatively sparse in the ileum and rarely if ever found in the jejunum (39,40). Nevertheless, it would appear that the small intestinal lumen in normals has a distinctive flora consisting chiefly of small numbers of grampositive anaerobes which are most readily cultured from distal ileal aspirates but are also present in luminal fluid obtained from proximal jejunum (39). Several of the organisms identified from these several sites are capable of enzymatic hydrolysis of conjugated bile salts, and three of these have been shown to reduce cholic acid to deoxycholic acid (14). The organisms involved in these reactions have been enumerated recently by Hill and Drasar (14)

TABLE III. Distribution of Taurocholate Amidase and Cholate-7-dehydroxylase Among Various Bacterial Genera

Species	Number of strains tested	Percentage showing taurocholate amidase	Percentage possessing cholic acid-7-dehydroxylase
Aerobic bacteria			
Pseudomonas sp.	58	0	0
Facultative anaerobic bacteria			
E. coli	75	0	0
Proteus mirabilis	1	0	0
Staph. aureus	47	21	0
Strep. pyogenes	2	0	0
Strep. faecalis	109	50	2
Strep. salivarius	30	0	0
Strep. viridans	12	0	0
Anaerobic bacteria			
Bacteroides sp.	237	48	8
Clostridium sp.	16	94	12
Bifidobacterium	89	38	0
Veillonella sp.	47	30	8
Microaerophilic bacteria			
Lactacillus sp	12	0	0
Fungi			
Candida albicans	28	0	0
Candida sp.	6	0	0
Saccharomyces sp.	4	0	0
Torulopsis sp.	7	0	0

From Hill and Drasar (14).

(Table III). A detailed discussion of these reactions is contained in Chapter 5. The physiological significance of the small bowel flora in normals can, at this time, only be inferred from the observations reported in the initial paragraphs of this chapter (Fig. 1). As stated previously, duodenal and proximal jejunal luminal contents contain only conjugated bile salts when obtained from normal subjects or patients with ileal disorders without severe stricture formation. However, it is possible, but unsupported, that bacterial modification of bile salts may be initiated in the more distal regions of the small intestine of normal individuals, where bacteria capable of such reactions have been identified (39). In contrast, patients who exhibit the findings attributable to the "blind-loop" syndrome characteristically show very significant increases in the microflora of luminal contents obtained from the proximal small intestine (14,32–34), usually consisting of organisms capable of both modifications (hydrolysis and reductions). Thus significant amounts of unconjugated bile salts have been present in the proximal luminal fluid of these patients (31–35). For example, a recent report (35) of six patients has shown that the concentration of conjugated bile salts in luminal fluid obtained from the upper jejunum was less than 5.0 mmoles/liter, values in normals ranging between 5–10 mmoles/liter. In two other reports of similar patients, the luminal bile salt concentration attributable to deconjugates was 22.5 and 3.7% in one (33) and 50–100% in the other (19). Significant quantities of free deoxycholate have been measured in the luminal contents of these and other patients with this syndrome (31,33). However, the frequency and degree to which dehydroxylation follows deconjugation are not known. In a single patient who exhibited malabsorption of vitamin B_{12}, xylose, and fat associated with a subtotal gastrectomy (Billroth II) performed 3 years previously, taurocholate-24-^{14}C was injected intravenously and the residual total ^{14}C radioactivity and its composition were determined in proximal luminal (efferent limb) samples at 3, 24, and 72 hr. Although the total residual ^{14}C radioactivity was comparable to normal values at 24 and 72 hr, the composition of this radioactivity had changed materially by the 3-hr period (initial sampling); 22% was comprised of taurocholate-^{14}C and 78% was present as unconjugated cholic acid-^{14}C. Luminal samples obtained at 24 and 72 hr showed 5–10% of the ^{14}C radioactivity present in glycocholate-^{14}C and the remainder in cholic acid-^{14}C. Thus a bacterial flora was present (cultures were not obtained) capable of deconjugation but presumably not producing significant 7α-dehydroxylation in this patient with "blind-loop" syndrome. It is possible that deoxycholate was formed and then reabsorbed by passive diffusion processes in the afferent loop at a more rapid rate than the unconjugated trihydroxy bile salt (37). This sequence has been suggested by Tabaqchali *et al.* (35) as an explanation for the relatively greater concentration of unconjugated trihydroxy bile salts as compared

with dihydroxy unconjugates in luminal aspirates obtained from their patients. In addition, these authors (35) have demonstrated an increased relative conjugation of luminal bile salt conjugates with glycine as compared with taurine (an increased G:T ratio). In light of the discussion in preceding paragraphs of this chapter, it would seem likely that this increase in G:T ratio may be attributable to an increased demand for conjugation with glycine and taurine and a consequent decrease in the relative availability of taurine. In their entirety, these observations have demonstrated an abnormal resident microbial flora capable of deconjugation and dehydroxylation of bile salts in the proximal intestine of patients who exhibit the "blind-loop" syndrome. Such alterations are demonstrable readily by quantitative and qualitative measurements of luminal bile salt concentration. Precise identification of the organisms involved and the extent of bile salt modification require cultural methods and assay of the composition of residual radioactivity following *in vivo* (Figs. 4–6) and *in vitro* (14,33) incubations with sterol-labeled conjugated bile salts. Such observations should provide information which is pertinent not only to an understanding of the pathophysiology of this disorder but also to its management, since appropriate antibiotic therapy can be recommended only when the organism in question has been shown operative in producing these metabolic alterations. At this time, similar observations of 7α-dehydroxylation of the primary dihydroxy bile salt, chenodeoxycholic acid, have not been obtained, although organisms capable of this transformation (38) are known to exist in the intestinal flora of normals (39) and have been shown to be present in luminal fluid from these patients (14,34). The questions related to the mechanisms involved in the production of fat malabsorption exhibited by these patients and the interrelationships of these qualitative and quantitative alterations in bile salt metabolism have been reviewed (30) and are discussed in Chapter 5.

REFERENCES

1. L. Lack and I. M. Weiner, *J. Pharmacol. Exptl. Therap.* **139,** 248 (1963).
2. T. S. Low-Beer and M. P. Tyor, unpublished observations.
3. K. W. Heaton, W. I. Austad, L. Lack, and M. P. Tyor, *Gastroenterology* **55,** 5 (1968).
4. B. Borgström, A. Dahlquist, G. Lundh, and J. Sjövall, *J. Clin. Invest.* **36,** 1521 (1957).
5. A. F. Hofmann, L. J. Schoenfield, B. A. Kottke, and J. R. Poley, *in* "Methods in Medical Research" (R. E. Olson, ed.), Year Book Medical Publishers, Chicago (1970), pp. 149–179.
6. S. Lindstedt, *Acta Physiol. Scand.* **40,** 1 (1957).
7. H. Danielsson, P. Eneroth, K. Hellström, S. Lindstedt, and J. Sjövall, *J. Biol. Chem.* **238,** 2299 (1963).
8. J. Wollenweber, B. A. Kottke, and C. A. Owen, Jr., *J. Lab. Clin. Med.* **69,** 584 (1967).
9. J. T. Garbutt, K. W. Heaton, L. Lack, and M. P. Tyor, *Gastroenterology* **56,** 711 (1969).

10. M. M. Stanley and B. Nemchausky, *J. Lab. Clin. Med.* **70,** 627 (1967).
11. W. E. Meihoff and F. Kern, Jr., *J. Clin. Invest.* **47,** 261 (1968).
12. A. Norman and R. Grubb, *Acta Pathol. Microbiol. Scand.* **36,** 537 (1955).
13. A. Norman and M. S. Shorb, *Proc. Soc. Exptl. Biol. Med.* **110,** 552 (1962).
14. M. J. Hill and B. S. Drasar, *Gut* **9,** 22 (1968).
15. T. S. Low-Beer, M. P. Tyor, and L. Lack, *Gastroenterology* **56,** 721 (1969).
16. J. Sjövall, *Acta Physiol. Scand.* **46,** 339 (1959).
17. W. G. M. Hardison and I. H. Rosenberg, *New Engl. J. Med.* **277,** 337 (1967).
18. B. W. Van Deest, J. S. Fordtran, S. B. Morawski, and J. D. Wilson, *J. Clin. Invest.* **47,** 1314 (1968).
19. C. L. Krone, E. Theodor, M. H. Sleisenger, and G. H. Jeffries, *Medicine* **47,** 89 (1968).
20. G. M. McLeod and H. S. Wiggins, *Lancet* **1,** 873 (1968).
21. L. Lack and I. M. Weiner, *Am. J. Physiol.* **200,** 313 (1961).
22. J. E. Glasser, I. M. Weiner, and L. Lack, *Am. J. Physiol.* **208,** 359 (1965).
23. M. R. Playoust, L. Lack, and I. M. Weiner, *Am. J. Physiol.* **208,** 363 (1965).
24. B. Borgström, G. Lundh, and A. F. Hofmann, *Gastroenterology* **45,** 229 (1963).
25. W. I. Austad, L. Lack, and M. P. Tyor, *Gastroenterology* **52,** 638 (1967).
26. J. Hirsh, E. H. Ahrens, Jr., and D. H. Blankenhorn, *Gastroenterology* **31,** 274 (1956).
27. J. M. Dietschy, H. S. Solomon, and M. D. Siperstein, *J. Clin. Invest.* **45,** 832 (1966).
28. S. M. Kurtz, T. D. David, Jr., and J. M. Ruffin, *Lab. Invest.* **11,** 653 (1962).
29. W. O. Dobbins, III, and J. M. Ruffin, *Am. J. Pathol.* **51,** 225 (1967).
30. R. M. Donaldson, Jr., *Fed. Proc.* **26,** 1426 (1967).
31. R. M. Donaldson, Jr., *J. Clin. Invest.* **44,** 1815 (1965).
32. S. Tabaqchali and C. C. Booth, *Lancet* **1,** 12 (1966).
33. I. H. Rosenberg, W. G. Hardison, and D. M. Bull, *New Engl. J. Med.* **276,** 1391 (1967).
34. B. S. Drasar, M. J. Hill, and M. Shiner, *Lancet* **2,** 1237 (1966).
35. S. Tabaqchali, J. Hatzioannou, and C. C. Booth, *Lancet* **1,** 12 (1968).
36. Y. S. Kim, N. Spritz, M. Blum, J. Terz, and P. Sherlock, *J. Clin. Invest.* **45,** 956 (1966).
37. R. M. Donaldson, Jr., *New Engl. J. Med.* **270,** 938, 994 (1964).
38. S. L. Gorbach, L. Nahas, P. I. Lerner, and L. Weinstein, *Gastroenterology* **53,** 845 (1967).
39. S. L. Gorbach, A. G. Plaut, L. Nahas, and L. Weinstein, *Gastroenterology* **53,** 856 (1967).
40. M. H. Kalser, R. Cohen, I. Arteaga, E. Yawn, L. Mayoral, W. R. Hoffert, and D. Frazier, *New Engl. J. Med.* **274,** 500 (1966).

Chapter 5

BILE ACIDS AND THE INTESTINAL ABSORPTION OF FAT AND ELECTROLYTES IN HEALTH AND DISEASE*

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Publication of a book devoted entirely to the chemistry, physiology, and metabolism of bile acids indicates a renascence in interest in these polyfunctional detergents. Here we will summarize present views on the physical and physiological properties of bile acids in relation to their chemical structure which bear on their participation in the intestinal absorption of fat, their enterohepatic circulation, and their influence on electrolyte and water absorption by the colon. Several of these topics are considered in detail elsewhere in this book, as well as in recent reviews (1–4). This chapter will focus on our own studies but will also emphasize areas in which information is needed.

I. INTRODUCTION

A. Occurrence of Bile Acids in Vertebrates

Bile acids are considered to have at least three major functions in mammals: (a) transport of lipids in micellar form—cholesterol in bile and lipolytic products, cholesterol, and fat-soluble vitamins in small intestinal content

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during digestion (1,2); (b) regulation of cholesterol biosynthesis—in liver (5) and in small intestine (6); and (c) regulation of electrolyte and water transport—in large intestine and possibly in small intestine (7). The first of these functions, the transport of lipids in micellar form, may be demonstrated *in vitro,* since bile acid solutions possess a striking ability to disperse polar lipids in micellar form; in this chapter, we shall frequently refer to the dispersant properties of bile acids. However, demonstration of the regulatory functions of bile acids requires intact tissues, at least presently.

Conversion of cholesterol to bile acids requires multiple enzymatic steps (8,9), including (a) hydroxylation at the 7- or 12-position or both, (b) oxidation at the 3-position, (c) reduction at the 3-position with saturation of the $\Delta^{5,6}$ double bond in the B ring to give an A/B *cis* juncture, and (d) shortening and oxidation of the side chain. The elegant work of Haslewood (4, 10,11), now spanning 20 years, has shown that the intermediates considered or demonstrated to occur in mammals during the conversion of cholesterol to bile acids serve as end products of cholesterol metabolism in lower vertebrates. The scheme of biochemical evolution constructed by Haslewood permits the postulate that progressive changes in the bile acid molecule are associated with superior dispersant (or regulatory) properties. It should be of considerable interest to compare dispersant and regulatory properties of primitive bile acids with those of modern bile acids. In this chapter, we shall discuss criteria for examining the dispersant properties of a bile acid. It seems possible to test also the influence of any bile acid on electrolyte and water transport by the perfused colon (7). However, no simple method has been described for assessing the influence of a given bile acid on cholesterol biosynthesis, although very recent work has suggested that changes in the level of hepatic hydroxymethylglutaryl–coenzyme A reductase (12) may be used to assess the influence of administered bile acids.

Bile acids may compose but a small fraction of the biomass of biologically occurring detergents. Other detergents with transport or regulatory functions or both may occur in the chordate invertebrates as well as in the hemichordates, arthropods, echinoderms, etc. An acyl dipeptide, lauryl sarcosyl taurate, has been isolated from the digestive juice of the common crab (13). This compound is assumed to have a transport function, but its dispersant properties have not been compared with those of bile acids or typical ionic detergents. It is not known whether the compound possesses regulatory functions.

B. The Enterohepatic Circulation of Bile Acids

In the past decade, the mammalian ileum has been shown to transport conjugated bile acids unidirectionally in the absence of any electrochemical

gradient (14,15). The demonstration of this "active-transport" site, which is completely absent in the jejunum, offers an explanation for the efficient enterohepatic cycling of bile acids—a physiological process which permits a large circulating pool of bile acids despite a relatively small synthesis rate. The efficient ileal absorption results in a high bile acid concentration in the small intestine and a relatively low concentration in the large intestine. We have little understanding of the biochemical mechanisms responsible for such "active transport."

C. Influence of Conjugation

The bile alcohols are insoluble at any *p*H, and the bile acids whether C_{27} or C_{24} are water soluble only above *p*H 6 (1). Hepatic enzymes convert the bile alcohols to sulfates (in an ester linkage) and the bile acids to taurine conjugates (in a peptide linkage) in lower vertebrates and most carnivores, to glycine conjugates (also in peptide linkage) in higher mammals which are herbivores, and to both glycine and taurine conjugates in higher mammals which are omnivores (4). The process of conjugation, whether with sulfate, glycine, or taurine, forms compounds which are soluble under the relatively acid conditions present in the upper small intestine during digestion (16). The synthesis of alkyl taurates (fatty acid linked in peptide bond with taurine) by the detergent industry is a similar chemical process also resulting in the formation of detergents soluble under acid conditions.

The significance of the evolution from ester sulfates to peptide conjugates is unclear. The detergent properties of paraffin-chain sulfates are similar to those of paraffin-chain sulfonates; presumably, the dispersant properties of bile alcohol sulfates and taurine-conjugated bile acids do not differ greatly. Conjugation with glycine forms a molecule with a more acidic carboxyl group than that of the parent free acid (17); the neighboring amide group induces an electron shift (18) which causes the pK_a of a given glycine conjugate to be about a pK_a unit below that of its parent free bile acid. The lower pK_a associated with glycine conjugation is probably chiefly responsible for the greater solubility of glycine-conjugated bile acids under relatively acid conditions (19). Solutions of glycine-conjugated bile acids are more sensitive to precipitation by divalent cations than are solutions of taurine-conjugated bile acids (1); these differences probably derive solely from the difference between the carboxyl group and the sulfonate group *per se* and not from the presence of the bile acid nucleus.

In addition to changing the physical properties of bile acids, conjugation also alters their physiological properties. On the basis of extremely limited evidence, it seems likely that the bile acid pool of animals with exclusively taurine conjugates is maintained chiefly by active absorption from the ileum.

Since free bile acids are rapidly absorbed from the jejunum (14,20–22), whereas taurine-conjugated bile acids are absorbed to a negligible extent (23,24), conjugation may be considered to prevent jejunal absorption and thus maintain the bile acid concentration in the jejunum during digestion. In animals with exclusively glycine-conjugated bile acids, it seems probable that some passive jejunal absorption of glycine dihydroxy bile acids occurs (21,22); however, glycine trihydroxy conjugates, i.e., glycocholate, may be absorbed chiefly in the ileum. There is no information on the intestinal absorption of bile alcohol sulfates.

D. Bacteria–Bile Acid Relationships

The small intestine is characterized by a relatively high concentration of bile acids, a low concentration of bacteria, and little evidence of bacterial degradation of bile acids. Whether bile acids influence the bacterial flora of the intestine is unknown, but evidence consistent with this hypothesis has been reported: (a) bacterial proliferation has been reported in clinical conditions in which there is a reduced intraluminal concentration of bile acids (25); (b) in the colon, the bile acid concentration is low, the bacterial density is high, and there is striking evidence of bacterial deconjugation and 7α-dehydroxylation of bile acids (26); and (c) in patients with ileal resection, the concentration of bile acids is increased in colonic contents and bacterial 7α-dehydroxylation is decreased (27). The demonstration that secondary bile acids (i.e., bacterial metabolites) are a normal constituent of human bile and may be more powerful inhibitors of electrolyte and water transport than primary bile acids (7) (see below) indicates that bile acid–bacteria interactions are of physiological significance in the healthy mammal; a corollary is that bile acid–bacteria interactions must be considered in any scheme for the biochemical evolution of bile acids. Potential secondary bile acids which are uncommon must be examined in terms of their deleterious effect on the host; prevalent secondary bile acids must be examined in terms of enhanced fitness of the host. Again, the parameters for examination include the transport and regulatory effects; in addition, for potential secondary bile acids which are uncommon, toxic effects must also be considered.

II. TRANSPORT FUNCTION OF BILE ACIDS DURING FAT DIGESTION AND ABSORPTION

The chemical events in fat digestion in nonruminants are quite simple (28,29). Triglyceride is hydrolyzed to 2-monoglyceride and fatty acid by pancreatic lipase; the process occurs extremely rapidly under rather fixed conditions (37°C *p*H 5.5–6.5, Na+0.15 M). Bile acids increase the rate of

hydrolysis in a manner that is poorly understood, although a number of actions have been suggested: (a) bile acids together with biliary lecithin emulsify dietary triglyceride; (b) bile acids may promote the adsorption of pancreatic lipase to the oil–water interface and may also influence the conformation of the enzyme (30); (c) bile acids may bind to the lipolytic products formed at the oil–water interface and displace lipase to a new interface; (d) bile acids may remove lipolytic products from the interface, exposing unsplit triglyceride molecules; (e) bile acids disperse the lipolytic products in micellar form, enhancing their diffusion and convection to the cell lumen interface; (f) bile acids enhance the ionization of fatty acids present in the bile salt–lipolytic product micelle, inhibiting re-esterification of fatty acid and thus driving lipolysis in the direction of complete hydrolysis.

In this section, we shall consider the last three items, i.e., the surface and bulk interactions of lipolytic products with bile acids. We shall concentrate on the dispersion of lipolytic products in micellar form by bile acid solutions. This subject is complex and difficult to present clearly because it involves the physical chemistry of surface and bulk properties, which has received relatively little attention from physical chemists. We have termed the events occurring during fat digestion as physicochemical events because the chemical events influence and are influenced by the physical properties of the participating molecules.

We shall consider first the types of behavior displayed by lipids in water. We shall then examine the behavior of triglyceride and its lipolytic products (diglyceride, monoglyceride, fatty acid, soap, and fatty acid–soap mixtures) in water. We must compare the properties of long-chain unsaturated lipids with their long-chain saturated homologues. In addition, we must also consider medium-chain, i.e., C_8 to C_{12}, lipids, which are having increasing application in medical therapy (31). The principles developed here will be applied to the description of the interactions of these lipolytic products with bile acid solutions. Finally, to appreciate the distinctive properties of bile acid solutions, we must compare them with solutions of typical ionic detergents in both the presence and absence of lipolytic products.

A. Behavior of Individual Lipolytic Products in Water

1. Principles

Small (32) has proposed a working classification of lipids according to their behavior in aqueous systems which promises to be of great value to the biologist. He proposes that all lipids which have a sufficiently hydrophilic group to allow formation of a stable film at interfaces be termed "polar lipids" or "amphiphiles" ("liking both phases"). Small divides polar lipids into three groups according to their behavior in water: (a) Insoluble non-

swelling amphiphiles—compounds which have extremely low solubilities in water, which do not interact with water appreciably in bulk, and which separate as an immiscible liquid or solid phase (generally crystals). Examples are long-chain fatty acids, triglycerides, and sterols. (b) Insoluble swelling amphiphiles—compounds with appropriately arranged polar and nonpolar regions such that although their molecular solubility is very low, they hydrate in water to form liquid crystalline phases (33). The apparently paradoxical term "liquid crystalline" is used to describe the phase which is crystalline by X-ray diffraction since it has order in one or two dimensions; however, it is a liquid macroscopically and hydrodynamically. Commonly, this phase is composed of indefinitely long bimolecular leaflets of swelling amphiphile which have their paraffin chains inward and the polar head at the surface of the leaflet; the water molecules lie between the leaflet. In another arrangement (hexagonal), the molecules are arranged in cylinders packed hexagonally, again with the polar heads oriented outward toward the water molecules which fill the spaces between the cylinders. Examples of such insoluble swelling amphiphiles are lecithin, monoglycerides, or lipid extracts of myelin, from which the term "myelin figure" arises. (c) Soluble amphiphiles—compounds with a finite molecular solubility but which aggregate spontaneously to form polymolecular aggregates called "micelles" at a relatively low concentration called the "critical micellar concentration" (34–36). Typical soluble amphiphiles, historically termed "amphipathic compounds" or "amphipaths," are soaps and ionic detergents. Bile acids are to be considered as a special type of soluble amphiphile.

It cannot be stressed too strongly that the formation of a liquid crystalline phase or a micellar phase can only occur above a certain critical temperature. This temperature is that at which the paraffin chains become liquid, and it is related to the energy required to break the intermolecular bonds at the polar end of the molecule as well as the short-range forces binding the paraffin chains when they are present in crystalline form. Thus at this critical temperature, which is termed the "transition temperature" for swelling amphiphiles, water penetrates the anhydrous crystal and a liquid crystalline phase results. The transition temperature may be observed microscopically (37,38) or, better, detected by a number of spectrophotometric techniques (*cf.* 39,40) which indicate marked changes in molecular packing when a crystalline suspension of swelling amphiphiles is heated slowly in water. For soluble amphiphiles, the critical temperature is called the "critical micellar temperature (CMT)" or "Krafft point." At this point, that soap or detergent which is present above its molecular solubility changes from a crystalline excess to a micellar solution. Although the CMT or Krafft point is not greatly influenced by the amphiphile concentration, the presence of electrolyte (41) or other amphiphilic additives (37,42) may have a marked effect. Therefore,

it is best to refer to the critical micellar temperature or Krafft point of the entire system rather than to that of the compound.

To describe the behavior of a lipid in water, it is customary to use a phase diagram in which the physical state or molecular arrangement of the lipid or the system is shown in relation to the composition of the mixture (Fig. 1). Composition is usually expressed in percentage by weight (abscissa), and the ordinate is temperature. In two-component systems, called "binary

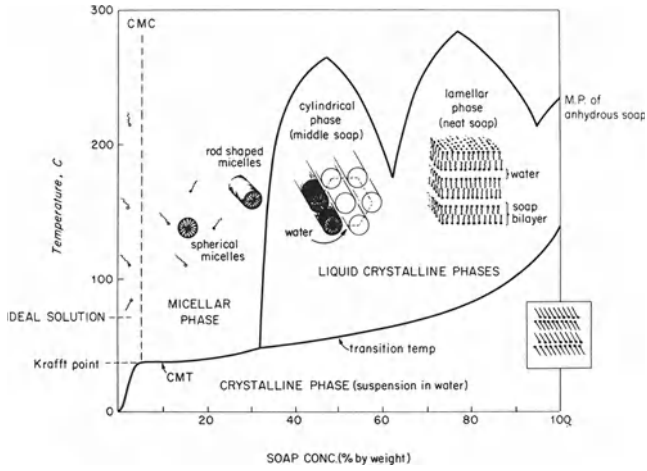

Fig. 1. The fatty acid soap–water phase diagram of McBain (58) modified (1) to show the molecular arrangement in relation to aqueous concentration (abscissa) and temperature (ordinate). Ideal solution, i.e., true molecular solution, is to the left of the vertical dashed line, indicating the critical micellar concentration (CMC), which varies little with temperature. At concentrations above the CMC, provided that the temperature is above the critical micellar temperature (CMT), a micellar phase is present. At high concentrations, the soap exists in a liquid crystalline arrangement, provided that the solution is above the transition temperature of the system, i.e., the temperature at which a crystalline phase becomes liquid crystalline. The Krafft point is best defined (D. M. Small, personal communication) as the triple point, i.e., the concentration and temperature at which the three phases (true solution, micelles, and solid crystals) coexist, but in the past the Krafft point has been equated with the CMT. The diagram emphasizes the requirement for micelle formation: (a) a concentration above the CMC, (b) temperature above the CMT, and (c) a concentration below that at which the transition from micelles to liquid crystals occurs. Modified from Hofmann and Small (1).

systems," if one is interested only in very low concentrations, the abscissa could be expressed in molarity. Physical state or molecular arrangement is defined by light microscopy, X-ray diffraction, light scattering, or other appropriate techniques; if two or more phases are present, they may be separated and analyzed. In practice (e.g., 43), one prepares a series of mixtures in sealed vials and examines them at different temperatures after equilibrium has been obtained. When one is interested in three components, one uses triangular coordinates to plot the composition of mixtures (Fig. 2). Triangular coordinates may be used because the sum of the three components is constant. To show changes with temperature, one constructs the triangular phase diagram for each temperature and then places the triangles above each other; the result is a prism (44). For four components at one temperature, one constructs a tetrahedron (45). It seems impossible to show

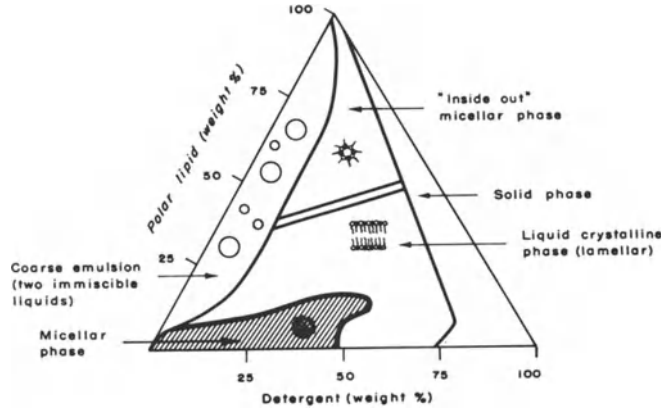

Fig. 2. The ternary phase diagram for water (lower left corner), detergent or fatty acid soap (lower right corner), and a polar lipid such as hexanol (upper corner) at a single temperature (37). Each corner corresponds to 100% of the individual component, although the water corner is not named as such. The hatched area in the aqueous corner indicates that micelles form at extremely low concentration for the system; no CMC is apparent. A liquid crystalline phase forms at higher concentrations of detergent and water alone (base) or polar lipid is added to a micellar solution. The base of the triangle indicates the phase equilibria present in a water–detergent system at a single temperature and is to be contrasted with the graph of phase equilibria over a range of temperatures shown in Fig. 1. To show the phase equilibria present in relation to temperature, one would construct a prism with temperature as the altitude. For systems which are in water, such as intestinal content (99% water) or bile (90% water), one is interested chiefly in the aqueous corner of the phase diagram (for further discussions of such systems, see references 32 and 44). Redrawn from Lawrence (37).

the effect of temperature when four components are present, since it is inconvenient to show the tetrahedrons above each other. One can hold one component constant in concentration, which is equivalent to sectioning the tetrahedron parallel to one side, and then plot the resultant triangles above each other. The result is also a prism.

Ideally, then, to describe the physicochemical events occurring during fat digestion, we should first construct the phase diagram for the binary systems of fatty acid–water, monoglyceride–water, and soap–water. We should then proceed to ternary systems, but since water is always present we are chiefly interested in the systems of fatty acid–soap–water, fatty acid–monoglyceride–water, and soap–monoglyceride–water. Finally, we should mix all four components and construct the tetrahedron. Such a tetrahedron would give us a description of the behavior of all possible mixtures of the lipolytic products in the absence of bile acids. To have physiological relevance, the water should be replaced by 0.15 M NaCl, and, since such a solution consists of two components, one is concerned with phase equilibria which cannot be represented in three dimensions. The discouraging complexity of this formulation can be circumscribed by working with systems which simulate those present physiologically. Thus since intestinal content is between 98–99.5% water, one can work with mixtures of lipolytic products whose total concentration may be held constant at 1%, i.e., systems containing 99% water. In addition, one may work at constant physiological electrolyte concentration and temperature. Thus if one has a system of two components, e.g., water and fatty acid, one is interested in an extremely small range of concentration; if one is only interested in phase equilibria at 37°C, the area of interest is described by a short segment parallel to the abscissa, if temperature is the ordinate. Where one has three components such as fatty acid, monoglyceride, and water, one may examine the behavior of varying molar ratios of fatty acid and monoglyceride, keeping the water concentration constant at 99%. One thus moves across a line in the aqueous apex of the triangular phase diagram (water–fatty acid–monoglyceride). A line describes the equilibria at a single temperature, but changes with temperature are shown using mole fraction as the abscissa and temperature as the ordinate. Such a graph represents a section of the prism previously described, which had temperature at its altitude (44).

It should be noted that *p*H is not usually considered an independent variable in such systems. In systems containing fatty acids and soap mixtures, the bulk *p*H is influenced by the buffering capacity of the ionizable lipids which are present, and this buffering capacity depends on the concentration and temperature of the system. Customarily, one measures the *p*H at equilibrium. Although body temperature is fixed, it is also often useful to vary temperature experimentally in order to distinguish effects of, for example,

saturation and unsaturation from chain length. Medium-chain compounds (C_6 to C_{10}) may be liquid or solid at 37°C, and their physical state must be assessed in attempting to assign changes in physical properties to chain length alone.

A detailed consideration of the behavior and solubility of lipolytic products in aqueous systems not containing bile acids has two justifications. First, digestion is an aqueous process and significant intestinal absorption of certain lipolytic products may occur in the absence of bile acids, despite their low solubility. It is a reasonable assumption that such absorption occurs from a molecular solution, or at lest a nonmicellar solution, and we therefore seek information on molecular solubility or types of aggregation or both in aqueour systems. Second, behavior of lipolytic products in the absence of bile acids provides a framework from which to predict the behavior of these compounds when bile acids are added.

2. Monoglycerides

Long-chain unsaturated monoglycerides are swelling amphiphiles at body temperature (47,48). They have low solubilities in water and physiological saline; no precise values are available, but the solubility of 1-monoolein has been reported to be less than 5×10^{-6} M. When long-chain unsaturated monoglyceride is incubated with buffer or saline, it hydrates and forms myelinics (49). The complete phase diagram has been published for the mono-olein–water system, but recent experiments of Krog and Larsson (50) suggest that both the types of phases present and their boundaries will be influenced by *p*H and ionic strength. The transition temperature of a given unsaturated monoglyceride is probably 10–30°C below its anhydrous melting point (38) (Table I). At experimental temperatures below its transition temperature, an unsaturated monoglyceride behaves as an insoluble amphiphile; i.e., it is crystalline when suspended in water (49).

The predominant form of monoglyceride present in intestinal content during fat digestion is the 2-isomer, which is unstable, slowly isomerizing to the 1-isomer. Few studies have compared the physical properties of the 1- and 2-isomers, but glyceryl monoethers, which are nearly isosteric and should have fairly similar physical properties, are prepared without difficulty. It would seem both feasible and interesting to examine the phase equilibria as well as molecular solubility of glyceryl 1- and 2-monounsaturated ethers.

Saturated long-chain monoglycerides behave similarly to unsaturated long-chain monoglycerides, provided that the experimental temperature is above their transition temperature. The transition temperature for saturated long-chain 1-monoglycerides is 17–27°C below their anhydrous melting point (38), and Δ_t, the difference between the anhydrous melting point and the transition temperature, is probably greater for 2-monoglycerides than for

TABLE I. Transition Temperatures (°C) for Monoglycerides and Fatty Acids in Water[a]

	Stable β-form		Metastable α-form	
	m.p.	T_{pen}	m.p.	T_{pen}
1-Monolaurin	62.5	40.0	44	<20
1-Monomyristin	69.0	49.5	56	34
1-Monopalmitin			63	47
2-Monopalmitin	68	48.5	58	34
1-Monostearin	81.5	65	74	60.5
2-Monostearin	73	53.5	55	—
1-Monostearyl glyceryl ether	68.5	59.5		
2-Monostearly glyceryl ether	68.9	54.5		

[a] Data from Lawrence's group (37,113) have been combined with unpublished data from this laboratory. In these experiments, a crystal of monoglyceride in water was observed microscopically and heated slowly. The temperature at which water penetrated the surface of the crystal to form a liquid crystalline phase is called the "temperature of penetration" (T_{pen}). Note that the anhydrous melting point is lower and the difference between the anhydrous melting point and T_{pen} greater for 2-isomers. Lawrence (37) discusses the relationship of T_{pen} to phase equilibria in ternary systems, and Larsson (47) and Lutton (48) give complete monoglyceride–water phase diagrams.

1-monoglycerides (50) (Table I). At body temperature, long-chain saturated monoglycerides are crystalline and essentially insoluble (49). The effect of temperature is to change them from insoluble amphiphiles to swelling amphiphiles. Thus saturated and unsaturated monoglycerides behave similarly at appropriate experimental temperatures; at body temperature, however, they behave very differently.

Medium-chain monoglycerides seem to be a special type of swelling amphiphile. They differ significantly from long-chain saturated homologues by forming dispersions in water or 0.15 M NaCl at body temperature (47,49). The solubility is not great, and the form of the excess is unclear, although it is probably lamellar in structure. Mono-octanoin has been reported to have a solubility of 4 mM (Na^+ 0.15 M, *p*H 6.3), We did not observe micelle formation by any of the medium-chain monoglycerides, using azobenzene solubilization as a criterion for micelle formation. No studies have been reported on medium-chain 2-monoglycerides, but these are known to isomerize rapidly to the 1-isomer (51).

3. Diglycerides and Triglycerides

Long-chain diglycerides and triglycerides behave similarly in water and in physiological saline, and may be considered together. Both have a negligible solubility (14); precise values have not been reported to our knowledge. If liquid at experimental temperature, they form an unemulsified immiscible phase which macroscopically appears as oil droplets. If solid, they remain as undispersed crystals. Presumably, their solubility should decrease

as the chain length increases, and their solubility, as that of any homologous series, should be described by an equation of the following type (52):

$$\log \text{solubility} = a + bn$$

where n is the number of carbon atoms and b is a term which has been shown to describe the molar free enthalpy of transferring a methyl group from a hydrocarbon environment to an aqueous environment; a is an empirical constant relating largely to interactions involving the nonparaffinic part of the molecules, which would be the ester bonds in the case of triglycerides and ester bonds plus the alcoholic group in the diglycerides. Whether there are significant differences in the solubility of 1,2- and 1,3-diglycerides does not appear to have been examined.

4. Fatty Acids, Soaps, and Fatty Acid–Soap Mixtures

a. Fatty Acids. Long-chain saturated fatty acids are insoluble in water and physiological saline. Several studies (e.g., 53,54) on the solubility of homologous fatty acids in water show a linear decrease in log solubility with chain length described by the equation (for 20°C)

$$\log \text{solubility} = -0.65n + 2.82$$

where n is the number of carbon atoms in the paraffin chain. Restated, the log solubility increases linearly as the chain length shortens. Appreciable solubilities (10^{-4} or 0.1 mM) occur with medium-chain fatty acids, i.e., decanoic and shorter (Fig. 3). There is no break in the log plot associated with the fatty acid being above its anhydrous melting point at the experimental

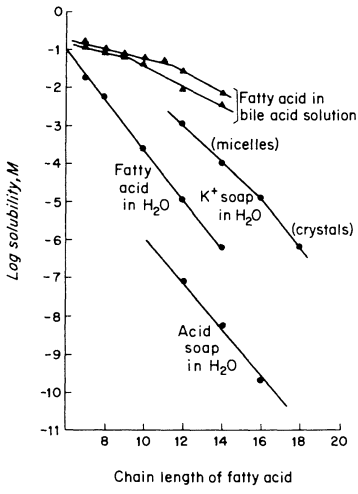

Fig. 3. Log solubility of acid soap ($HA \cdot K^+A^-$), fatty acid (HA), potassium soap (K^+A^-), and fatty acid (HA) in micellar bile acid solution at 37°C (▲) or 25°C (△) in relation to chain length. The solubility of fatty acid in bile acid solutions seems to decrease more rapidly with increasing chain length at experimental temperatures below the fatty acid's melting point, which agrees with Fig. 9, which suggests that the solubility of a given fatty acid in bile acid solution is greater at experimental temperatures above the fatty acid's melting point than below. The lower three solubility curves are plotted from the data of Lucassen (54), and data for the upper two curves were taken from the report from Verkade's laboratory (68).

temperature. Unsaturation appears to increase aqueous solubility several orders of magnitude for a given chain length. Simmonds *et al.* (55) have reported values of the order of 10^{-5} M for oleic acid in physiological saline, and values as high as 10^{-4} M have been claimed for linoleic acid (56). A reasonable figure for stearic acid solubility in water would be of the order of 10^{-9} M (53,54). We are unaware of systematic studies on the effect of ionic strength or counter-ion concentration on fatty acid solubility; presumably, the effect of physiological electrolyte concentration is relatively small.

The transition temperature, i.e., the melting point, of fatty acids when suspended in water is very close to their anhydrogenous melting point (38). Fatty acids do not interact with water and thus behave as di- and triglycerides, i.e., as insoluble amphiphiles irrespective of experimental temperature.

b. Soaps. Soaps are termed "soluble amphiphiles" because, under appropriate conditions, clear aqueous solutions may be prepared which contain relatively high total concentrations of soap. As discussed, soap molecules have a finite molecular solubility and above this concentration aggregate to form polymolecular aggregates called "micelles" (34–36). The narrow concentration range over which micelle formation occurs is called the "critical micellar concentration (CMC)." Most micelles composed of soaps or typical ionic detergents are considered to be spherical; the polar heads of the molecules are outward toward the bulk of the aqueous phase, and the hydrocarbon chains form an oily liquid center of the micelle. The molecules of any given micelle are considered to be exchanging rapidly and continuously with the molecules of other micelles (57) as well as with those dispersed in true molecular solution; micelles should be considered as flickering clusters.

Micelle formation occurs spontaneously in any soap solution provided that (a) the solution is above the CMC of the system and (b) the temperature is above the critical micellar temperature of the system. Micellar solutions are clear to casual inspection, although the formation of micelles is readily detected by conventional light-scattering techniques. The formation of micelles is signaled by a decreased rate of change in colligative properties with concentration, as well as by the appearance of physical properties associated with macromolecules. At high aqueous concentrations, the micelles coalesce to form large liquid crystalline aggregates (Fig. 1). The transformation from a micellar solution to a liquid crystalline dispersion is signaled by an abrupt increase in viscosity and the appearance of anisotropy, i.e., birefringence. As discussed, it is customary to plot these changes in molecular arrangement with concentration and temperature by phase diagrams. James McBain, the discoverer of the micelle, spent many years constructing the phase diagrams for fatty acid soap–salt–water systems which are of practical importance in soap making (58).

Although soaps are called "soluble amphiphiles," the formation of micelles is in fact a manifestation of the low molecular solubility of the soap molecule. Micelle formation can only occur above the critical micellar temperature of the system. Below this temperature, soaps form a crystalline phase (which may or may not contain water depending on the history of the system). This means that the classification of a given lipid as a soluble or insoluble amphiphile is conditional upon the experimental temperature.

Lucassen (54) has shown that the molecular solubility of the soap anion is about 5×10^{-2} moles greater than that of the corresponding fatty acid. Assuming that dimerization does not occur, one may describe the solubility of potassium soaps by the following equation:

$$\log K_{KS} = -0.65n + 5.4$$

where K is the solubility product of potassim soaps and n refers to the number of carbon atoms in the chain. The equation for the CMC of potassium soaps is similar, except that the proportionality constant for the concentration of potassium counter-ions is different:

$$\log \mathrm{CMC}_{KS} = 0.41n - 0.57[K^+] + 2.52$$

This equation indicates that the log CMC falls linearly with increasing chain length and electrolyte concentration. Thus addition of electrolyte lowers the CMC but lowers the concentration of soap anion even more greatly. The addition of electrolyte thus tends to salt out the soap solution rather than cause micelle formation. Micelle formation can be induced by raising the experimental temperature. Addition of electrolytes lowers the CMC but raises the critical micellar temperature, and the latter effect is greater (41).

c. Fatty Acid–Soap Mixtures. The *p*H of small intestinal content during fat digestion is 5–7, and in long-chain soap–water mixtures below *p*H 7 a major fraction of the soap is present in protonated form (59).

Therefore, to consider the behavior of lipolytic products in water or physiological saline in the absence of bile acids, one must discuss the behavior of fatty acid–soap systems (54,59). Such systems are extremely complicated. Although Lucassen (54) has described clearly such systems for homologous saturated potassium soaps, the systems of unsaturated fatty acid–soap where sodium ion is present in physiological concentrations have not been studied. There are a number of rules which apply to such systems, based on the work of Lucassen: (a) For all long-chain fatty acids, saturated or unsaturated, clear solutions are impossible above 0.001 M if as much as one-fourth of the soap has been protonated. (b) Changes in bulk *p*H associated with acidification are complicated and are influenced by soap concentration and by the chemical nature of the precipitate which occurs. (c) The precipitate will generally be a fatty acid or an acid soap having the composition $K^+H^+(soap)_2$;

a shift in the chemical form of precipitate from fatty acid to acid soap may be signaled by a more rapid fall in bulk *p*H, since potassium ions are precipitating from solution and hydrogen ions are being added. (d) The term "*p*K*a*" is probably meaningless in such heterogeneous systems; the bulk *p*H at which the total fatty acid of the system is half neutralized depends on the soap concentration. With more concentrated systems, and for long-chain soaps (greater than C_{14}), the *p*H corresponding to neutralization of half of the total fatty acid present will be 7–9, and for medium-chain soaps 6–8 at higher concentrations and near 5 at extremely dilute concentrations. (e) In the absence of added electrolyte, the bulk *p*H resulting from protonating a given fraction of the soap present increases with increasing chain length of soap. Thus the addition of 0.75 mole of hydrochloric acid (as titrant) to 0.001 M soap solutions (containing 1 mole of soap) gives the following bulk *p*H: C_{10} *p*H 4.9; C_{12} 6.2; C_{14} 7.8. (f). Finally, the solubility of acid soaps [i.e., $K^+H^+(soap)_2$,...] can also be described by a solubility product. The solubilities of these acid soaps are considerably below that of either the fatty acid or the soap, consistent with their molecular weight being twice as great.

Application of these principles, based on partially acidified soap–water mixtures, to the events of fat digestion is not straightforward. If one equilibrates oleic acid (0.1–10 mM) in buffers from *p*H 5 to 7 (37°C, Na^+ 0.15 M), one finds that below *p*H 7 the solubility (i.e., is the sum of oleic acid) as well as oleic ions is low, less than 10^{-3} M; the only change observed with *p*H is an increase in the degree of emulsification above *p*H 6.6 (60). Saturated fatty acids under similar conditions have very low solubilities noted, and, when examined over the *p*H range 5–7, the total solubility of palmitate and stearate actually decreases with increasing *p*H, presumably because of the effect of the high cation concentration. The behavior of medium-chain soap–fatty acid mixtures under conditions of physiological electrolyte concentration has not been examined in detail. At *p*H 6.3, the total solubility of laurate is considerably greater than that of higher saturated homologues, but still low. Decanoate has a solubility less than 0.004 M and also does not form micelles.

To summarize, under the conditions present in small intestinal content during fat digestion (37°C, *p*H 5–7, Na^+ 0.15 M), the only group of substances possessing appreciable molecular solubility is medium-chain fatty acids (C_8–C_{12}). Low but probably physiologically significant solubilities are demonstrated by long-chain unsaturated fatty acids—especially polyunsaturated fatty acids such as linoleic—and medium-chain monoglycerides. Long-chain unsaturated monoglycerides form polymolecular liquid crystalline aggregates which contain water, but their molecular solubility is very low. Long-chain saturated fatty acids and monoglycerides and all long-chain di- and triglycerides show no interaction with water, and their molecular

solubilities are negligible. An increase in *p*H will have no effect on any of the above classes except fatty acid–soap mixtures. With medium-chain fatty acids, the solubility (defined as the total concentration of all molecules irrespective of the degree of ionization) will probably increase with *p*H at values similar to those observed in small intestinal content (*p*H 6–7). With long-chain unsaturated fatty acids, molecular solubility also increases with rising *p*H, but the *p*H at which the solubility increase begins is probably inversely proportional to saturation; i.e., linoleic acid solubility increases at about *p*H 6 but oleic acid solubility does not begin to increase until about *p*H 7. These *p*H-mediated increases in solubility could influence the site of fat absorption in conditions in which the intraluminal concentration of bile acids was reduced, since the *p*H of the small intestine increases distally (61).

B. Behavior of Individual Lipolytic Products in Bile Acid Solutions

Before considering the behavior of lipolytic products in bile acid solutions, we will compare briefly certain aspects of bile acid solutions with those of typical ionic detergents. Micellar aggregation is signaled by an abrupt increase in the solvent properties of detergent solutions for water in soluble molecules. The ability of micellar solutions to dissolve water-insoluble materials is called "solubilization" and is explained by the ability of the micelle to incorporate appropriately shaped additives into its center or between the amphipathic molecules composing the micelle (34–36). The sudden increase in solubility when micellar aggregation occurs provides a simple method for determining the critical micellar concentration of a detergent. The solubilized additive is believed to be exchanging rapidly between other micelles and those few molecules in true molecular solution (57). In the simplest possible system for characterizing solubilization, one has a detergent, a solute, and water, and again the equilibrium should be described by phase diagrams using triangular coordinates. The behavior of lipids in micellar solution is predictable to a considerable extent from their behavior in water. Insoluble amphiphiles will often have low micellar solubility and, when the micelle is saturated, will form a separate phase of immiscible liquid or crystals. Swelling amphiphiles will participate in micelle formation, lowering the CMC and forming mixed micelles. At high concentrations, they will form a separate liquid crystalline phase, which will incorporate the detergent molecules in it. Finally, soluble amphiphiles, as would be expected, form mixed micelles, and the CMC of the system is usually intermediate between the CMC of the two components. The possible systems are discussed in complementary reviews by Lawrence (37,62) and Small (32).

The bile acid molecule is shaped very differently than a typical ionic detergent (1). The body is ridged and kinked at the *cis* juncture of the A/B

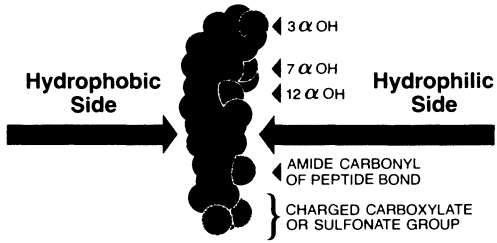

Fig. 4. Corey–Pauling–Koltun model of conjugated bile acid molecule photographed from the side to show the spatial arrangement of hydrophobic and hydrophilic regions. The hydroxy groups influence temperature–solubility relationships, since monohydroxy and unsubstituted cholanoic acids are insoluble at body temperature (1). The slightly curved aspect of the A/B ring portion of the steroid nucleus (top) is caused by the *cis* configuration of the A/B ring juncture; A/B trans bile acids (allo bile acids) have inferior dispersant properties (66), indicating the importance of the steric configuration of the A/B ring juncture of the cholanoic acid.

ring. The polar ionic group is located quite distantly from the body, which possesses a hydrophobic side and a hydrophilic side containing the hydroxyl group. Bile salts may be considered planar amphipaths, in contrast to typical ionic detergents, which are polar amphipaths (Fig. 4).

The aggregation of bile acids in aqueous solution to form micelles containing two to 24 molecules is discussed in detail elsewhere. The lack of a liquid hydrocarbon interior is probably responsible for micellar bile acid solutions having much poorer solvent properties for insoluble amphiphiles than typical ionic detergent solutions (19). A significant difference between 5β bile acids (A/B *cis*) and typical ionic detergents is that 5β bile acids at high concentrations in water do not form a condensed liquid crystalline phase.* In the binary phase diagram of bile acid–water, a liquid crystalline phase does not appear even at extremely high bile acid concentrations, whereas with typical ionic detergents, a liquid crystalline phase appears from concentrations of 20–50% by weight, depending on molecular structure and experimental conditions (1,2,32).

The number and position of hydroxy groups on the bile acid nucleus strongly influence the behavior of bile acids in solution. Bile acids appear to differ from typical anionic detergents in having a critical micellar tem-

*5α bile acids (A/B *trans*) form liquid crystalline phases at fairly low concentrations in water. Thus the steric configuration of the A/B juncture appears to be a major determinant of the phase equilibria observed in bile acid–water systems.

perature, i.e., the temperature where solubility reaches the critical micellar concentration, which is much more dependent on concentration (1,66). Thus 5α bile acids (A/B *trans*) with no hydroxy groups are insoluble in water at 100°C; compounds with one hydroxy group (only those with substitution at the 3- and 7-position have been examined) have critical micellar temperatures between 15–100°C; and the physiologically occurring di- and trihydroxy bile acids have critical micellar temperatures below 0°C, provided that the electrolyte concentration is 0.15 M or below. The critical micellar temperature of a given bile acid, as that of any anionic detergent, is also dependent on the type of counter-ion (66). Hydroxy keto bile acids appear to have a critical micellar temperature below 0°C, but few systematic studies have been done.

Besides influencing the critical micellar temperature, the number and position of hydroxy groups also influence the critical micellar concentration. The critical micellar concentration of dihydroxy bile acids is significantly below that of trihydroxy acids (1,63), but no values have been reported for monohydroxy acids. Conjugation with glycine or taurine does not appear to have any particular effect on those properties of bile acids which are related to the steroid nucleus, provided that experiments are carried out at *p*H sufficiently alkaline that all bile acid molecules present are ionized (64).

1. Behavior of Bile Acids in Water

The principles developed when describing the behavior of lipolytic products in water can now be applied to a description of their behavior in bile acid solution. Donald Small is largely responsible for proposing that bile acids disperse lipids by apposing the hydrophobic side of the steroid nucleus to the side of the paraffin chain of the lipid molecule (65). The lipids of bile and intestinal content which are most effectively dispersed by bile acid solutions are all swelling amphiphiles, i.e., lipids which spontaneously form a liquid crystalline phase in water. Dispersion of these large aggregates to small bile acid–polar lipid micelles, a major physical or chemical event in bile formation and fat digestion, involves complex energy relationships which must consider lipid–lipid interaction, bile acid–bile acid interaction, and bile acid–lipid interaction.

2. Diglycerides and Triglycerides

The diglycerides and triglycerides, insoluble amphiphiles, have very low solubilities in bile acid solution. As in water, such lipids when incubated with bile acid solutions remain as oil droplets or crystals, depending on their melting point relative to the experimental temperature. No systematic studies on the solubility of homologous tri- and diglycerides in bile acid solutions have been carried out.

3. Monoglycerides

As was noted, there is a striking difference in the behavior of monoglycerides in water compared to that of di- and triglycerides. Monoglycerides interact with water to form a liquid crystalline state, provided that the hydrocarbon chains are liquid at the experimental temperature. This difference between monoglycerides and higher glycerides is also present in bile acid solutions. Monoglycerides have a high micellar solubility in bile acid solutions, in contrast to the low solubility of the di- and triglycerides (19). The monoglycerides participate in micelle formation, and the critical micellar concentration of systems containing monoglyceride and bile acid in water is considerably below that of bile acid–water systems (19).

The phase equilibria present in the monoglyceride–bile acid–water system should be described by triangular phase diagrams, but at present only the broad outlines of the diagram have been constructed (62,66). The micellar region is quite large, and its border signals the formation of liquid crystalline aggregates containing monoglyceride, water, and bile acid whose molecular arrangement is unknown at present. Transition from a micellar dispersion to a liquid crystalline dispersion occurs when between one and three molecules of long-chain monoglyceride are present per molecule of micellar bile acid (19), depending on the chain length of the monoglyceride (49).

The sizes of the micellar region for 1- and 2-monoglycerides are probably very similar, although the only experiments bearing on this have been simple solubilization experiments which showed that the micellar solubilities of 1- and 2-mono-olein in dilute micellar bile acid solutions were identical (49).

With saturated monoglycerides, it is necessary to consider both chain length and melting point (or transition temperature) relative to the experimental temperature. We have studied this problem several ways. First, we

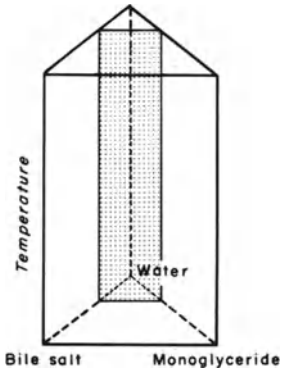

Fig. 5. Experimental design to assess phase equilibria present in bile salt (bile acid)–monoglyceride–water system at a fixed water concentration, but varying temperature, indicated by the stippled plane (46). Since intestinal content is probably greater than 98% water, one can hold water concentration constant and vary bile acid/monoglyceride ratios.

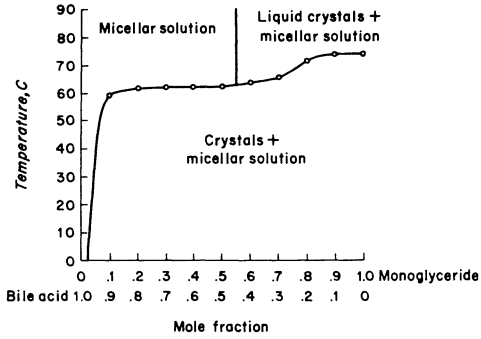

Fig. 6. Phase equilibria for the system indicated in Fig. 4. For clarity, the behavior of a single saturated long-chain monoglyceride is shown in Fig. 6 and that of a series of homologous, saturated monoglycerides in Fig. 7. Eleven mixtures were used, the sum of micellar bile acid and monoglyceride concentrations equaling 40 mM. The point indicated for monoglyceride alone is the transition temperature in water and is 15–30°C below the anhydrous melting point (Table I). When the temperature of the system reaches the transition temperature of a given monoglyceride, a marked increase in the micellar solubility of the monoglyceride is observed. At higher monoglyceride/bile acid ratios, a liquid crystalline phase of undefined composition and molecular arrangement occurs.

have examined the solubility of medium- and long-chain monoglycerides at experimental temperatures well above their transition temperature so that they would be liquid crystalline in the systems under study. Here solubility in micellar bile acid solution increased as the chain length decreased (49). To gain further information on the behavior of long-chain saturated monoglycerides in bile acid solutions, we studied the behavior of samples containing different ratios of monoglyceride/bile acid at fixed water concentration. As noted, such an experimental design corresponds to a line in the triangular phase diagram which is in the aqueous tip and parallel to the water base (Fig. 5). By observing the samples at a number of temperatures, we obtain a section through the prism whose base is the bile acid–monoglyceride–water system and whose altitude is temperature. The results (Figs. 6 and 7) indicated that (a) saturated monoglycerides have low micellar solubilities until the experimental temperature is increased to the transition temperature, where the solubility increases strikingly; (b) the presence of bile acids has only a slight effect on the transition temperature, i.e., the difference between the

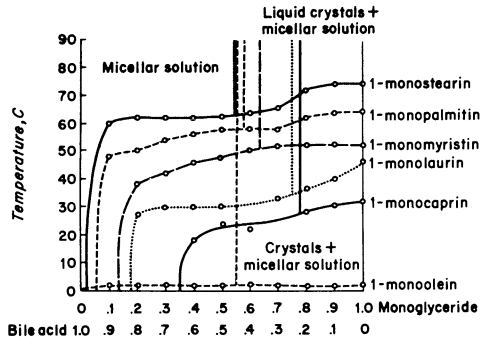

Fig. 7. Phase equilibria for homologous, saturated 1-monoglycerides and 1-mono-olein. For the saturated monoglycerides, the phase diagrams are similar. At temperatures at which the monoglyceride is solid crystalline, it has a low but measurable micellar solubility (vertical lines on left), which decreases with increasing chain length (49). When the experimental temperature is increased to that at which the monoglyceride changes from crystalline to liquid crystalline, there is an abrupt increase in micellar solubility (horizontal lines on left). The temperature at which the transition from crystalline to liquid crystalline occurs is not apparently affected to any great extent by bile acids. At higher monoglyceride/bile acid ratios, the micellar solubility of the monoglyceride is exceeded, and a liquid crystalline phase occurs (vertical lines on right). The saturation ratio (19), i.e., moles of micellar monoglyceride/moles of micellar bile acid, decreases with increasing chain length at experimental temperatures above the transition temperature, also. The phase equilibria for 1-mono-olein are similar, except that a solid crystalline phase does not appear. 1-Mono-olein and 1-monostearin behave identically at sufficiently high experimental temperatures.

anhydrous melting point and the transition temperature is chiefly influenced by the presence of water and not the additional presence of bile acids; (c) as noted, the solubility decreases with increasing chain length.

It was noted previously that fatty acid solubility in water was not influenced by the physical state of the fatty acid at the experimental temperature. The striking influence of physical state on monoglyceride solubilization suggests that bile acids can only disperse large liquid aggregates when the short-range intramolecular forces have already been disrupted by the combined effect of heat and water.

We have also examined the transition temperature of monoglyceride–bile acid mixtures in equimolar proportions (Table II). In these experiments, we used all the common 5β bile acids but observed no marked difference among any of the bile acids, indicating again that the water–monoglyceride interaction is the critical variable in determining the temperature at which micellar solubility increases.

We have also compared the behavior of monoglycerides in solutions of

TABLE II. Temperature (°C) at Which Liquid Crystalline Phase Becomes Micellar Phase for Lipolytic Products in Solutions of Bile Acids or Anionic Detergents[a]

Detergent solution	Glyceryl-1-monopalmitate	Sodium palmitate	Δ
Cholyl taurine	54	32	−22
Cholyl glycine		32	
Deoxycholyl taurine	50	35	−15
Deoxycholyl glycine	50	35	−15
Chenodeoxycholyl taurine	52	40	−12
Chenodeoxycholyl glycine	52	35	−17
Allodeoxycholyl taurine	52	75	+23
Lauryl *N*-methyl taurine	48	52	+4
Octyl benzene sulfonate	43	54	+11
Lauryl sulfate	54	54	0

[a] Suspensions of monoglycerides in the micellar solution were heated slowly in capped glass vials, and the temperature at which the mixture became isotropic was recorded; temperatures agreed within 3°C whether approached from above or below. The number of moles of monoglyceride was equivalent to the number of moles of micellar bile acid or detergent, and the concentration of the mixture was varied from 300 mM to 10 mM by dilution with bile acid or detergent solution at its CMC. The sodium palmitate–detergent mixtures were 20 mM in sodium palmitate, 20 mM in micellar detergent, and 0.11 M in NaCl.

typical ionic detergents without bile acid solution. We synthesized a lauryl taurate, which differs from taurocholate in possessing an acyl nucleus instead of a steroid nucleus. We then varied the molar ratio of 1-monoolein to detergent or bile salt to see at which molar ratio the liquid crystalline phase would occur (60). The results (Fig. 8) indicate that the micellar area is much smaller in the lauryl taurate–mono-olein–water system than in the bile acid–mono-olein–water system. This observation agrees well with the fundamental work of Lawrence and his colleagues, which has shown that in the phase equilibria of detergent–polar additive–water systems, the size of the micellar zone for a given additive is related to the concentration at which liquid crystalline aggregates form in the binary system of detergent and water (37,62). Thus those detergents which form a liquid crystalline phase at relatively low concentrations will have a smaller micellar zone than those detergents which do not form a liquid crystalline phase until higher concentrations. Since bile acids do not form a liquid crystalline phase in the binary system of bile acid–water (32), the micellar zone of the bile acid–monoglyceride–water system is much larger than that of the lauryl taurate–monoolein–water system.

4. Fatty Acids, Soaps, and Fatty Acid–Soap Mixtures

a. Fatty Acids. The solubility of nonionized unsaturated fatty acid in bile acid solution is considerably greater than that in water and probably

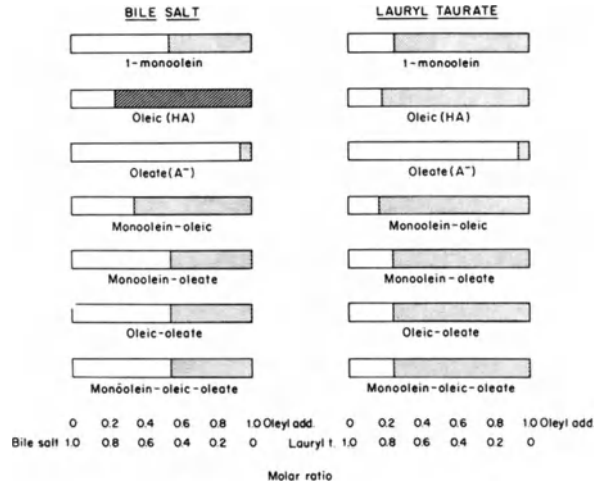

Fig. 8. Phase equilibria for oleyl homologues varying in polar group in bile salt solution. Compounds possessing a small micellar area (clear) form an immiscible oil phase (diagonal hatching) when present in excess in bile acid solution or in buffer in absence of bile acid. Compounds with high micellar solubility form a liquid crystalline phase when present in excess in bile acid solutions and form liquid crystalline phases in buffer alone. Since these experiments were carried out at a single temperature, the phase equilibria correspond to a line parallel to the base in the area shown in Fig. 5. From Hofmann (60), with the publisher's permission.

somewhat greater than that in solutions of typical ionic detergents for a given micellar concentration. A reasonable figure for oleic acid would appear to be about 1 mole of micellar oleic acid per mole of micellar bile acid, and figures of 0.8 for linoleic acid and 1.4 for vaccenic have been reported (67). This figure (the "saturation ratio") is lower than that of hexadecyl unsaturated monoglycerides, for which values between 1.5–2.0 have been observed.*

Added fatty acid, as monoglyceride, reduces the CMC of bile acid solutions (19). The experiments reported were carried out at *p*H 6.3, where some fatty acid ionization occurs, and it seems probable that the reduction in CMC in the presence of fatty acids would be considerably less if the experiments were carried out at a *p*H sufficiently acid to repress fatty acid ionization.

*Since oleic acid begins to ionize at *p*H 5.5 in bile acid solution, experiments designed to determine the solubility of a given fatty acid (completely protonated) should be carried out below *p*H 5. At this *p*H, the fatty acid separates as an emulsified oil (assuming that the experiment is done above its melting point), and equilibrium in such systems is only reached after many, many hours, even with vigorous shaking.

The solubility of saturated fatty acids in water increases regularly with decreasing chain length, as discussed. The solubility of a series of homologous fatty acids in bile acid solution also decreases with increasing chain length (68) (Fig. 3). The melting point of saturated fatty acids in bile acid solutions is only one or two degrees below the anhydrous melting point. Saturated fatty acids, as unsaturated fatty acids, do not form a liquid crystalline phase when present in excess; the excess is either an immiscible oil phase or a suspension of crystals depending on the experimental temperature (66).

In order to describe the behavior of saturated fatty acids in bile acid solutions, we have mapped out roughly the phase diagram for saturated fatty acids–bile acids–water systems using the same experimental design as described for saturated monoglycerides. We varied the ratio of fatty acid/conjugated bile acid, keeping the sum of the two components constant at 40 mM. The counter-ion concentration was kept constant by the addition of NaCl, and water was present at a fixed concentration of 99%. Again we con-

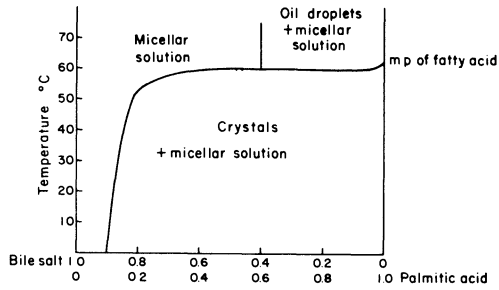

Fig. 9. Phase equilibria for the bile salt (bile acid)–fatty acid–water system at constant water concentration in relation to temperature (see Fig. 5). Six mixtures varying in molar ratios of bile salt (bile acid) and palmitic acid with total concentration of micellar bile acid plus palmitic acid equal to 40 mM were examined. Fatty acid has a finite solubility in the micellar bile acid solution, the excess being crystalline at body temperature. At 50–60°C, there is a marked increase in micellar solubility, and the fatty acid melts. At higher fatty acid/bile acid ratios, the micellar solubility is exceeded, and an immiscible oil phase occurs. The melting point of fatty acid in the presence of water is nearly identical to that in the anhydrous state (38), in contrast to the behavior of monoglyceride (Table I). As shown in Fig. 3, the size of the micellar area decreases with increasing chain length. Unsaturated fatty acids (not shown) behave similarly to saturated fatty acids, but their micellar solubility is greater, and at most experimental temperatures a crystalline phase will not occur.

struct a rectangle which is a slice through the prism having the base bile acid–fatty acid–water and the altitude temperature. The results (Fig. 9) indicate that fatty acids have low micellar solubility in bile acid solution until they liquify at a temperature a degree or two below their anhydrous melting point. At that temperature, there is a marked increase in micellar solubility.

No studies have compared the solubilization of a given fatty acid by different bile acids. Although trihydroxy bile acids have a lower saturation ratio than dihydroxy acids, it seems unlikely that this difference would be of physiological significance.

Fatty acids have a lower solubility in typical ionic detergent solutions than in bile acid solutions, for a given micellar concentration. To paraphrase, the micellar zone in the ternary phase diagram of this system (ionic detergent–fatty acid–water) is smaller than that in the system bile acid–fatty acid–water. Small has constructed the sodium oleate–oleic acid–water phase diagram (32); the micellar zone is extremely small because of the formation of liquid crystalline phases of oleic–sodium oleate at very low oleic acid/sodium oleate ratios. In unpublished experiments carried out several years ago, we compared the solubility of lauric acid in 40 mM solutions of sodium taurodeoxycholate and sodium glycodeoxycholate with that in sodium octyl benzene sulfonate. Lauric acid at concentrations of 1, 5, and 10 mM was completely soluble in these bile acid solutions at *p*H 6.3. By contrast, a 5 mM concentration of lauric acid in sodium octyl benzene sulfonate solution was completely turbid.

Last, it should be pointed out that no information exists on the influence of fatty acid structure on its micellar solubility in bile acid solutions. It would be of interest to compare branched-chain fatty acids, cyclopropane fatty acids, hydroxy fatty acids, etc.

b. Soaps. Sodium oleate forms isotropic micellar solutions in water, but when sodium ion is present at physiological concentration, viscoelastic gels form even when the sodium oleate concentration is as low as 10 mM (60). The addition of bile acids to such gels converts them to isotropic non-viscous solutions; cholate–oleate micelles form which are much smaller than the aggregates formed by oleate alone (69). The broad outlines of the system (sodium cholate–sodium oleate–water) have been constructed by Small (32); the micellar area is quite large. The behavior of other unsaturated fatty acid soaps in bile acid solutions has not been examined.

Saturated fatty acid soaps dissolve in water at their critical micellar temperature to form micellar solutions. The addition of sodium ion raises the critical micellar temperature of this system, as discussed. One can measure the critical micellar temperature in systems composed of varying molar ratios of two ionic detergents, and the phase equilibria present in such

experiments have been clarified by Lecuyer and Dervichian (46), who noted that determination of the critical micellar temperature of such a system was equivalent to constructing the phase diagram described by a line parallel to the water phase in the ternary system (first soap–second soap–water). Since only the molar ratio of the soaps was being varied, the experiments were carried out at constant water concentration. One then examined the phase equilibria present at different temperatures and thus created a rectangle which was, in fact, a slice through the prism formed by examining the phase equilibria in the first soap–second soap–water systems at different temperatures (cf. Fig. 5).

We have carried out similar experiments with saturated fatty acid soaps as the first soap and bile acids (as the sodium salt) as the second soap (66). The bile acids behave as typical ionic detergents with critical micellar temperatures well below 0°C (Fig. 10). For any saturated fatty acid soap, its critical micellar temperature decreases as the chain length shortens. Thus, at 37°C, more of a short-chain soap will be solubilized for a given amount of bile acid present, or, to paraphrase, the molar ratio of soap/bile acid will be much higher for short-chain soaps at 37°C. One carries out these experiments by incubating a series of different molar ratios of soap/bile acid. The bile acid is micellar, and the soap is crystalline. Over a few degrees of temperature range, the crystalline soap completely dissolves. No satisfactory quantitative description of these experiments has, as yet, been proposed.

It is tedious to examine a complete series of molar ratios for each bile acid conjugate, and we have therefore examined the critical micellar temperature of a 1:1 molar ratio of sodium palmitate and all of the commonly occurring conjugated bile acids (Table II). All bile acids behave similarly with critical micellar temperatures between 30–40°C. By contrast, the critical micellar temperature of alledeoxycholyl taurine (the 5α-epimer) was 75°C, indicating that this 5α bile acid could cocrystallize with sodium palmitate, whereas the common 5β bile acid did not. The typical ionic detergents, such as sodium dodecyl sulfate and sodium octyl benzene sulfonate, also had higher critical micellar temperatures with sodium palmitate than bile acids, but these detergents have much higher critical micellar temperatures in water than bile acids. Medium-chain soaps have not been examined in such experiments, but their critical micellar temperatures are well below room temperature, and such soaps should have an extremely high solubility in bile acid solutions.

c. Fatty Acid–Soap Mixtures. As discussed, nonionized fatty acid has a low solubility in bile acid solutions, whereas ionized soap has an extremely high solubility in bile acid solution. Accordingly, it would be predicted that increasing the ratio of soap/fatty acid would increase the amount of chemical fatty acid dissolved for a given micellar concentration of bile acid and further

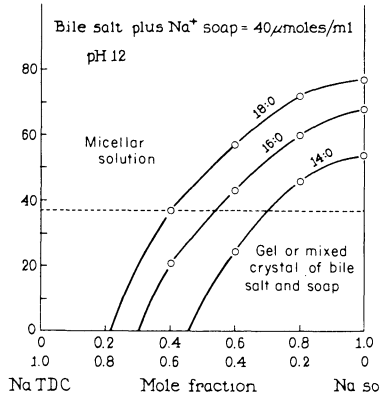

Fig. 10. Phase equilibria of the bile acid (as sodium salt)–fatty acid soap–water phase diagram at constant water concentration in relation to temperature. Mixtures with varying molar ratios of bile acid/sodium soap (total concentration 40 mM) were incubated, and the temperature at which the system became clear was plotted; solutions were buffered to *p*H 12. The curves indicate the critical micellar temperature of the system and have also been termed "mixed Krafft points" (46). The CMT of the bile acids is extremely low.

that ionization in such a system with various possibilities for molecular aggregation might be very complex. Again, we are interested in constructing the system soap–fatty acid–bile acid–water and noting the size of the micellar region as the ratio of soap/fatty acid is varied.

The solubility of unsaturated fatty acid in bile acid solution rises above *p*H 6.5, suggesting that fatty acid ionization is occurring at a *p*H close to that considered to be present in small intestinal content during fat digestion (60). In order to determine the extent to which fatty acid ionizes when present as a micellar solute in bile acid solutions, we have carried out experiments in which micellar solutions of conjugated bile acid, well above their critical micellar concentration containing fatty acid as a micellar solute were titrated, and the bulk *p*H corresponding to 50% ionization of the fatty acid was determined (60) (Table III). With oleic acid as a micellar solute, the bulk *p*H corresponding to 50% ionization was above 6.5 and was not significantly influenced by temperature, addition of monoglyceride, or the type of bile acid employed. The solubility of long-chain saturated fatty acids is too low to permit study of their ionization by such a technique, but one can examine the ionization of solubilized medium-chain fatty acid and short-chain fatty acid and one observes that the bulk *p*H corresponding to 50% ionization decreases toward the intrinsic pK_a of the fatty acid as its chain length is shortened. The interpretation of these experiments is also not simple because as one shortens the fatty acid chain length one varies the distribution of the fatty acid molecule between the molecular phase and the micellar phase, and no information is available on the fraction present in either form or the rate of exchange.

If the conjugated bile acid used to dissolve the fatty acid, which was titrated, was replaced by a typical anionic detergent, the bulk *p*H correspond-

TABLE III. Ionization of Fatty Acids Present as Micellar Solute[a]

	Detergent (mM)	Fatty acid (mM)	Bulk *p*H at which 50% ionization occurs
A. Oleic acid in micellar solutions of bile acids and detergents			
Varying detergents			
CTAB (cationic)	16.0	4.8	5.2
HDPC (cationic)	16.0	5.0	5.4
Lauryl taurine (anionic)	20.0	4.0	7.4
ABC (anionic)	20.0	4.0	7.2
TDC (anionic)	16.0	5.0	6.6
Varying bile salts			
Deoxycholyl taurine	16.0	5.0	6.6
TC-TDC (1:1)	20.0[b]	8.0	6.4
Deoxycholyl glycine	16.0	5.0	6.6
SIC	16.0[b]	5.0	6.6
Varying ratios of bile salt to oleic acid			
TDC	12.0	5.0	6.65
	16.0	5.0	6.60
	20.0	5.0	6.50
	24.0	5.0	6.45
B. Homologous fatty acids (5.0 mM) in micellar bile acid solution (16 mM)			
Oleic acid (18:1)	6.6		
Myristic acid (14:0)	6.6		
Lauric acid (12:0)	6.0		
Capric acid (10:0)	6.0		
Hexanoic acid (6:0)	4.9		
Propionic acid (3:0)	4.8		

[a] All solutions were isotropic before and after titration, which was carried out as described. The increased ionization of short-chain fatty acids probably reflects a greater fraction of these being present in true molecular solution. The influence of micellar charge on the pK_a of solubilized fatty acid has been treated quantitatively by Shankland (69). All solutions were isotropic 0.15 M in total Na^+ concentration.

[b] For bile salt mixtures, total concentration of bile salts is given. CTAB, cetyl trimethylammonium bromide; HDPC, hexadecyl pyridinium chloride; lauryl taurate, sodium lauryl taurate; TC-TDC, sodium taurocholate–sodium taurodeoxycholate; ABS, sodium *p*-(*n*-octyl) benzene sulfonate; SIC, a mixture of sodium taurocholate, sodium taurodeoxycholate, sodium taurochenodeoxycholate, sodium glycocholate, sodium glycodeoxycholate, and sodium glycochenodeoxycholate composed to resemble human small intestinal content during fat digestion and absorption (19).

ing to 50% ionization was about 0.5 pK_a units higher. If the bile acid was replaced by a cationic detergent, the bulk *p*H corresponding to 50% ionization was much lower, 5.2. Thus the bulk *p*H corresponding to 50% ionization of the fatty acid was strikingly influenced by the charge on the detergent molecule dispersing the fatty acid.

Shankland (69) has recently extended these experiments in considerable detail. He has shown that the enhanced ionization of fatty acid in the bile acid micelle is completely explicable using polyelectrolyte titration theory. The apparent shift in pK_a for any ionizable compound present in micellar form can be related to micellar charge, which, in turn, is the product of the number of charged molecules and a factor related to the binding of counterions. The bile acid micelle is highly charged, since the fraction of counterions which is bound is small. However, the size of the bile acid micelle is so small that the micellar charge is less. Hence the difference between the true pK_a of a fatty acid were it water soluble (5.0) and that observed in micellar bile acid solutions (6.5) is less (1.5) than that between the true pK_a and that observed in a micellar solution of an anionic detergent, such as lauryl sulfate ($7.0 - 5.0 = 2.0$) (*cf.* 70).

The alteration in the ionization of fatty acids by bile acids results in the following: (a) An increase in unsaturated fatty acid solubility at a pH above about 6.3. In the absence of bile acid, fatty acid solubility does not begin to rise significantly until bulk pH is above 7 (60). (b) Decreased re-esterification of fatty acids during pancreatic lipolysis, since ionized fatty acid cannot be esterified to glyceryl by pancreatic lipase (71).

Since saturated fatty acids are insoluble in bile acid solutions, and since saturated fatty acid soaps are only soluble in terms of the mole fraction of a soap–bile acid mixture having a critical micellar temperature of 37° C, one would anticipate saturated fatty acid–soap mixtures to have negligible solubility in bile acid solutions. Some years ago, we compared the behavior of sodium, palmitate, and stearate at pH 5.8, 6.2, 6.6, and 7.0 in buffer or buffer containing bile acid. In the absence of bile acid, the saturated fatty acids remained as unwetted crystals. When bile acid was added, the solubility increased measurably but only very slightly.

C. Behavior of Mixtures of *All* Lipolytic Products

We have now considered the behavior of each of the individual lipid classes in water and in bile acid solutions. We now wish to compare the behavior of appropriate mixtures of lipid classes in water with that in bile acid solution. We are interested in two major types of mixture: (a) the mixture of lipolytic products, fatty acid, 2-monoglyceride, and soaps and (b) the mixture of lipolytic products plus their precursors—di- and triglycerides. We are also interested in the behavior of long-chain unsaturated compounds versus that of long-chain saturated compounds versus that of medium-chain compounds.

1. In Water

Experiments (60) were carried out in which oleic acid, 1-mono-olein,

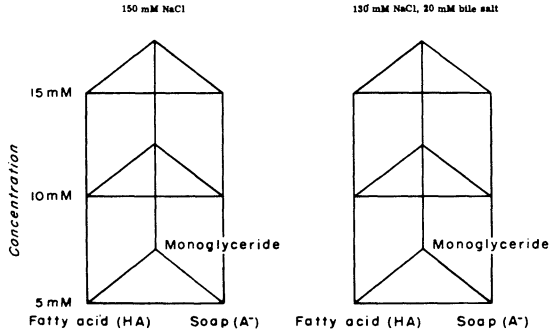

Fig. 11. Experimental design to assess the behavior of mixtures of lipolytic products in 150 mM NaCl (left) for 20 mM bile acid solution (right). In these experiments, the water concentration was constant at 99%.

and sodium oleate mixtures were incubated in 0.15 ml of NaCl at a total lipid concentration of 5, 10, and 15 mM (Fig. 11). Molar ratios for mixtures were selected by the use of triangular coordinates. All mixtures were grossly turbid at 5 mM. In those containing high ratios of fatty acid, the excess was present as oil droplets. In those containing predominantly 1-mono-olein, the excess was present as an opaque viscous phase which contained water droplets in a continuous viscous-oil phase. Those containing sodium oleate appeared to be fine emulsions (Fig. 12).

2. In Bile Acid Solution

These mixtures at 5, 10, and 15 mM total concentration were incubated in a 20 mM solution of sodium taurocholate–sodium taurodeoxycholate. The critical micellar concentration of this system should be 2–4 mM. All mixtures at 5 mM were clear micellar solutions. At 10 and 15 mM, samples containing oleic acid were turbid, with oil droplets of oleic acid present. Those containing chiefly oleic acid, but some mono-olein, were faintly opalescent, indicating large aggregates, possibly very large micelles, or liquid crystalline aggregates.

These experiments confirm and extend the observations made on a simple model system. Of the lipolytic products, the least soluble in bile acid solutions are fatty acids. Monoglyceride and soap have much higher solubilities, indicating that the formation of a clear micellar phase is enhanced *in vivo* by alkalinization (from pancreatic bicarbonate) or by having an appreciable fraction of fatty acid present as monoglyceride (which occurs because of the positional specificity of pancreatic lipase).

One is then interested in the ternary phase diagram in which lipolytic products (fatty acid, 2-monoglyceride, and soap) are treated as one com-

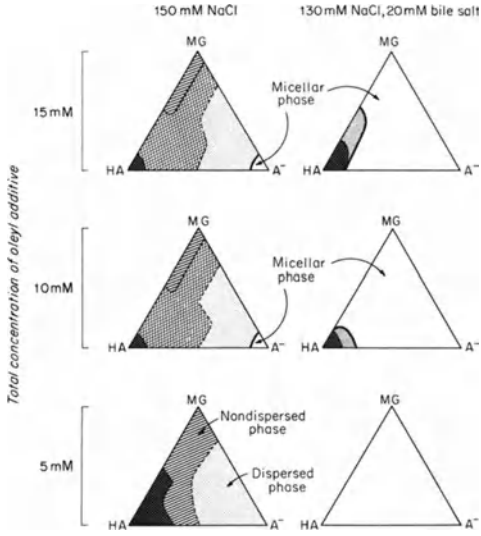

Fig. 12. Solubility and behavior at 37°C of mixtures of oleic acid (HA), sodium oleate (A^-), and mono-olein (MG); the experimental design is as indicated in Fig. 11. Solid black line separates dispersions of large aggregates from dispersions of micellar size—turbid dispersions from clear dispersions. In 150 mM NaCl, fatty acid is present as oil droplets (black with white stippling) and mono-olein as a nondispersed liquid crystalline phase (horizontal hatching) or a viscous water-in-oil emulsion (cross-hatching). Increased ratios of sodium oleate result in a dispersed phase (white with black stippling), and at 10 and 15 mM sodium oleate alone is present in micellar form. In bile salt, fatty acid is also present as oil droplets (black with white stippling), and at higher concentrations mono-olein and fatty acid form a dispersed liquid crystalline phase (white with dots). In 20 mM bile salt, most of the lipid mixtures are now present in micellar solution (clear). From Hofmann (60), with the publisher's permission.

ponent, with bile acid the second and water the third. Such has not been constructed, but experiments have been carried out in which an equimolar mixture of the lipolytic products was taken as a single solute and incubated with different molar ratios of bile acid at constant water concentrations. As noted, this corresponds to moving along a line in the triangular diagram parallel to the water base. This experiment showed that the micellar region of the triangular phase diagram (lipolytic products–bile acid–water) is large and is much larger than a similar diagram carried out in which bile acid is replaced by lauryl taurate (Fig. 8). The large micellar area in the system lipolytic products–bile acid–water is related to (a) the failure of bile acids to

form a liquid crystalline phase in the binary system bile acid–water, and this in turn appears related to the A/B *cis* configuration; (b) the enhanced ionization of fatty acid in bile acid solutions.

No studies have been carried out on mixtures of medium-chain fatty acid, soap, and monoglyceride.

If all mixtures of long-chain unsaturated lipolytic products are insoluble in 0.15 M NaCl, all mixtures of long-chain saturated lipolytic products should also be insoluble. Such is true, but the form of the excess, i.e., whether liquid, solid, or liquid crystalline, depends on the experimental temperature relative to the transition temperature and critical micellar temperatures of the pure components—and, in addition, whether any eutectics occur. We have examined this question by incubating various molar ratios of palmitic acid (m.p. 62° C), glyceryl-2-monopalmityl ether (m.p. 63° C) (as a 2-monoglyceride analogue), and sodium palmitate in 0.15 M MaCl and 0.15 M NaCl plus 20 mM bile acid. In saline, all mixtures were insoluble, but melting of the paraffin chain, as judged by microscopic appearance, occurred at a temperature close to the anhydrous melting point for the fatty acid, at a temperature of about 20° C below the anhydrous melting point for the 2-monoglyceride analogue, and at a temperature (the critical micellar temperature) some 100° C below the melting point of the anhydrous soap. Only very small eutectics were observed. When bile acid was added, those samples containing a predominance of monoglyceride and/or soap became micellar. The temperature at which this occurred was below the apparent transition temperature in saline, but the difference in transition temperature, i.e., transition temperature in 0.15 M NaCl minus that in bile acid solution, was small (5–15° C) in samples containing fatty acid. In samples in which fatty acid was not present, the difference was greater—16–40° C. These experiments indicate then that mixtures of saturated long-chain fatty acid, monoglyceride, and soap can be dispersed in bile acid solution at physiological temperatures, provided that sufficient monoglyceride is present and/or sufficient ionization occurs. Figure 13 summarizes schematically the effect of temperature on the solubility of long-chain saturated lipolytic products in micellar bile acid solutions. Unfortunately, the application of these *in vitro* data to *in vivo* data showing poor absorption of high melting point triglycerides is impossible, since most feeding experiments have failed to distinguish defective lipolysis from defective micellar dispersion.

Finally, we wish to examine the entire system, i.e., all lipolytic products and their precursors—di- and triglycerides—in water versus bile acid solutions. In the absence of bile acids, the concentration of all lipids in the aqueous phase is very low. In principle, the concentrations of fatty acid (as soap) should rise as the *p*H of the aqueous phase is increased; this effect would be greater for polyunsaturated fatty acids because of their greater

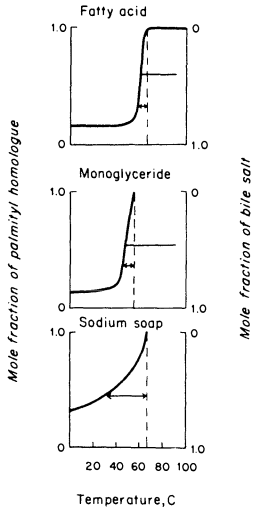

Fig. 13. The effect of temperature on the solubility in micellar bile acid solution of fatty acid (top), monoglyceride (center), and sodium soap (bottom). The data of Figs. 6, 8, and 10 have been plotted with the coordinates switched and the axes reversed. Fatty acid solubility increases markedly at a temperature close to the melting point of the anhydrous acid. Monoglyceride solubility increases at a temperature close to that of the transition temperature of monoglyceride in water. Thus, for both fatty acids and soap, the temperature at which a marked increase in solubility occurs is determined chiefly by lipolytic product–water interaction. With sodium soaps, the temperature range over which the solubility increases is much broader, and a significant depression is caused by bile acids. For a given acyl radical, the temperature at which solubility increases is lowest for soap, intermediate for monoglyceride, and highest for fatty acid.

aqueous solubility. In the presence of bile acids, the aqueous phase contains monoglyceride and fatty acid, and the partitioning of fatty acid between the two phases is *p*H dependent. The presence of monoglyceride and fatty acid in the aqueous phase has been confirmed in model systems by Borgström (72) and in studies in man in which digestive content was obtained by intubation (73). Recently, Freeman (67) has examined the distribution of a number of fatty acids between an oil phase of triglyceride and a micellar phase of 4 mM sodium glycodeoxycholate. The micellar phase/oil phase distribution ratio was in reasonable agreement with the solubility relationships previously discussed (mono-olein $\gg$ lauric acid > myristic acid > palmitic acid = stearic acid), but oleic acid and palmitic acid had similar micellar phase/oil phase distribution ratios. Further, oleic acid competed with other fatty acids for solubilization in the micellar phase and thus reduced the micellar phase/oil phase distribution ratio for other fatty acids. In contrast, monoolein, which greatly favored the micellar phase, increased the micellar solubility of other fatty acids; lysolecithin was even more effective than monoolein in this respect.

This incomplete, but nonetheless lengthy, description of model systems bearing on the physicochemical events of fat digestion indicates the overwhelming complexity of the phenomena and how much work remains to be done. The challenge to a physiologist and physical chemist, who all too frequently turn out to be the same person, will be to examine those systems which are most useful to clarify physiological events or alternatively permit the formation of quantitative relationships or, better yet, both.

D. Lipid Digestion and Absorption

1. Triglyceride

Fat digestion, by definition, is the transformation of triglyceride to a micellar solution of fatty acids and 2-monoglyceride and should be identical *in vivo* and *in vitro*. Here we shall summarize present views of the role of bile acids in fat digestion. Triglyceride is ingested in emulsified or nonemulsified form and is presumably hydrolyzed to a slight extent in the stomach, depending on its fatty acid composition and probably its state of emulsification. Conceivably, gastric hydrolysis should be greater if duodenal reflux occurs during digestion, but such does not occur to any appreciable extent in man (74). The limited figures which are available suggest that only limited gastric lipolysis occurs, in agreement with recent studies on human gastric lipase which indicate that the enzyme hydrolyzes long-chain triglycerides quite slowly (75). Gastric lipase does cleave the medium-chain fatty acids of human milk triglyceride readily, and milk fat, in contrast to other dietary lipids, could be appreciably hydrolyzed (perhaps 20–30% of its ester bond) by the gastric lipase in the human stomach (75). The extent of emulsification of ingested triglycerides in the stomach or small intestine has not been quantified.

In the small intestine, pancreozymin causes the gallbladder to contract, and bile, a micellar solution of bile acids, lecithin, and cholesterol, is secreted into the duodenum. Pancreozymin also causes discharge and continued synthesis of pancreatic lipase which adsorbs to the oil–water interface, liberating 2-monoglycerides and fatty acids (76). Whether bile acids adsorb to the interface and if so how they spatially orient with respect to lipolytic products and lipase is unknown. At concentrations below the CMC, bile acids will adsorb to monolayers of lipolytic products (77), but no information is available on the interaction of bile acid solutions above their CMC with monolayers of lipolytic products. Somehow, the lipolytic products are transferred to the bulk phase, where they form mixed micelles with bile acid molecules (Fig. 14).

The molecular arrangement of the bile acid–lipolytic product micelle is unknown but is probably similar to that of the bile acid–lecithin micelle, the structure of which, based on nuclear magnetic resonance studies, is a cylindrical bimolecular leaflet of lecithin molecules coated on the sides by bile acid molecules, their hydrophobic backs apposed to the paraffin chains of the phosphatide (65). All of the molecular species of the micelle are considered to be in rapid exchange with those of other micelles, as well as the concentration of molecularly dispersed lipolytic products and bile acids (at their CMC) in the bulk phase surrounding the micelles. Benzene molecules exchange rapidly between bile acid–monoglyceride micelles, a mean micellar

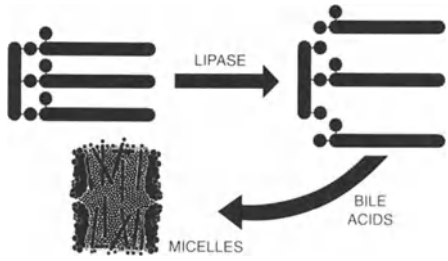

Fig. 14. Schematic depiction of selected physical and chemical events during fat digestion. The 1- and 3-ester linkages of triglyceride (upper left) are cleaved by lipase, forming 2-monoglyceride and fatty acid. These lipolytic products leave the oil–water interface and are dispersed in the aqueous phase as mixed bile acids–lipolytic product micelles. A proposed molecular arrangement of the bile acid–lipolytic product micelle is shown in cross-section; this model is based on studies of the bile acid–lecithin micelle (65). In this model, the hydrophobic back of the bile acid molecule apposes the paraffinic chains of the lipolytic products, and the hydroxy groups of the bile acid molecule are toward the aqueous phase. The paraffin chains of the interior of the micelle are liquid, thus permitting other water-insoluble molecules such as cholesterol and fat-soluble vitamins to dissolve in the micelle. Indeed, the solvent capacity of the bile acid–lipolytic product micelle is contributed chiefly by the paraffin chains of the lipolytic products.

residence time having been estimated to be on the order of milliseconds (T. Nagakawa and A. F. Hofmann, unpublished observations). No information exists on the rates of exchange of the bile acid and lipolytic products.

The micellar and oil phases of human small intestinal content may be isolated by ultracentrifugation (Fig. 15). The composition of the micellar phase, as would be predicted from the phase equilibria and solubility relationships discussed previously, is fatty acid (partially ionized), monoglyceride, and bile acid (72). The oil phase contains higher glycerides and a considerable amount of fatty acid. The partition of fatty acid between the oil and micellar phases is *p*H dependent; the fraction of fatty acid present in the oil phase increases at more acid *p*H values (67,72).

The mechanism by which the lipolytic products pass from the micellar phase into the mucosal cell is unknown, and an extensive discussion of the possibilities is inappropriate here. Since lipolytic products are extensively absorbed whereas taurine-conjugated bile acids are not absorbed in jejunal perfusion experiments (23), and since exchange rates of lipolytic products between micelles are thought to be quite rapid (78), molecular collision of lipolytic products with the cell membrane, resulting in reversible or partially reversible adsorption, has been proposed as the mechanism of fat absorp-

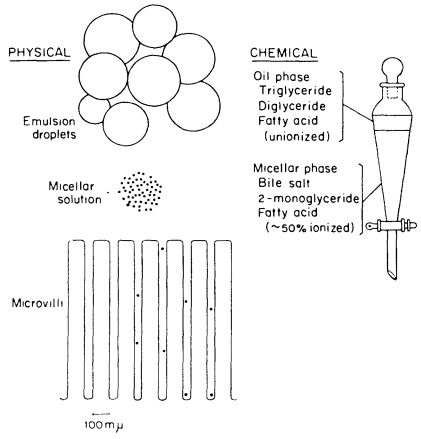

Fig. 15. Schematic representation of the physical and chemical states of lipids in intestinal content during fat digestion and absorption. An oil phase composed of higher glyceride and some nonionized fatty acid is in equilibrium with an aqueous micellar phase of bile acid, monoglyceride, nonionized fatty acids, and fatty acid soaps. Nonpolar lipids—e.g., fat-soluble vitamins, cholesterol, and hydrocarbons—are partitioned between the two phases. The emulsion droplets, micelles, and microvilli are drawn approximately to scale. Each black dot represents a single micelle. From Hofmann and Small (1), with the publisher's permission.

tion (55,79). No direct evidence exists on this fundamental question as yet. The micelle is thus considered to provide a high "driving" concentration of materials which would be insoluble in the aqueous phase in the absence of bile acids (80). Micelle formation may also be considered to increase the area of ingested lipids (28). Bile acids probably also greatly increase the rate of transfer of compounds to the bulk phase from the oil–water interface; recently described techniques (81) which permit exquisitely refined measurement of interfacial barriers to diffusion should allow this hypothesis to be tested and allow definition of exchange rates for solutes between the oil phase and the micellar phase.

Compounds whose oil–micellar phase partition coefficient is extremely high, or, to paraphrase, whose solubility in the micellar phase is extremely low are absorbed to a negligible extent. The triether analogue of triglyceride, glycerol triether, is a typical example of such a compound, and its nonabsorbability (82,83) has been considered to indicate that absorption of oil droplets is insignificant quantitatively and, as a corollary, that the triglyceride is not absorbed intact. In contrast, compounds which are dispersed in the aqueous phase only when micelles are present, such as cholesterol, are dependent on the presence of micelles for absorption (2,55). Together these observations indicate that (a) the micelle is the final common path of most lipids having negligible water solubility, (b) nonspecific absorption of lipids by mechanisms such as pinocytosis seems unimportant quantitatively in mature vertebrates, and (c) the micellar phase not only enhances the rate of compounds which are partitioned into it but also the micellar/oil phase distribution ratio could be a major determinant of the rate at which various lipids are absorbed. Although dispersion in a micellar phase may be necessary for the absorption of lipids, it is probably not sufficient, since specificity factors

in uptake have been demonstrated to be present, at least for certain sterols (84).

As discussed, the major determinant of the distribution coefficient between the aqueous phase and the oil phase of relatively nonpolar lipids such as cholesterol is the concentration of lipolytic products in the aqueous phase, since the paraffin chains of the lipolytic products, forming the lipid core of the micelle, are responsible for its solvent capacity (23,73). Micelle formation and the associated appearance of new solvent properties result from a cooperative effect of bile acids and lipolytic products (Fig. 16). Bile acids alone aggregate to form micelles, but these micelles have extremely poor solvent properties. Lipolytic products alone form very large liquid crystalline aggregates which probably have excellent solvent properties for lipids. When mixed, micelles of intermediate size are formed. The size is sufficiently small to permit rapid diffusion, and the lipolytic products in the center of the micelle provide a core of liquid hydrocarbon with useful solvent properties.

In ruminants, the fatty acids which are released by bacterial lipolysis in the lumen are subsequently hydrogenated to form saturated fatty acids (85). Lysolecithin, formed by the action of pancreatic phospholipases, forms mixed micelles with bile acids which, together, promote the dissolution of the saturated fatty acids to form a micellar solution. There does not appear to be an oil phase as is present in nonruminants.

2. *Other Lipids*

The role of bile acids in the absorption of other lipids such as cholesterol, fat-soluble vitamins, and fat-soluble drugs is similar to that described for fat (2). Bile acids are considered to influence the rate of hydrolysis of ingested cholesterol esters by interacting with cholesterol esterase and protecting it from tryptic digestion (86). Bile acids also influence the cleavage rate of β-carotene (87); conceivably, this effect is mediated on the surface of the cell.

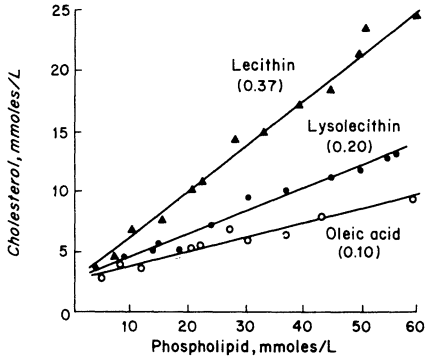

Fig. 16. Influence of oleic acid, lysolecithin, or lecithin concentration on cholesterol solubility in bile acid solution at a fixed concentration. The increment in cholesterol solubility with added polar lipids is indicated by the slope of the line, which is given in parentheses. Data from Neiderhiser and Roth (114), with their kind permission.

Bile acids are considered obligatory for the absorption of lipids such as cholesterol which, because of their insolubility in water, can only reach quantitatively significant concentrations in the aqueous phase in micellar form.

E. Other Effects of Bile Acids

It seems unlikely that bile acids directly influence intracellular events during absorption of triglyceride and other lipids (88), although bile acids must influence intracellular events indirectly since they regulate the flux of lipid-soluble molecules into the cell. Recent experiments suggest that the aqueous phase of intestinal content in bile fistula animals contains free fatty acid but does not contain monoglyceride, and experiments (R. G. H. Morgan and A. F. Hofmann, unpublished observations) with doubly labeled triglycerides have suggested that the glyceryl moiety of ingested triglyceride is not absorbed in the absence of bile. These experiments suggest that a micellar phase of bile acids is essential for the entrance of monoglyceride. The normal pathway of triglyceride synthesis in the cell is acylation of monoglyceride, and in its absence the glycerol or triglyceride synthesis is derived from glycerol phosphate, triglyceride synthesis proceeding by the phosphatidic acid pathway (89). Thus bile acids appear to regulate the triglyceride synthesis pathway in the mucosal cell. Whether bile acids have a similar regulatory role in the lecithin synthesis pathway has not been investigated.

Recent experiments (90) have suggested that bile acids at concentrations below the CMC may influence the permeability of the jejunal cell and increase the absorption rate of molecules which are absorbed by passive nonionic diffusion, i.e., by solution in the lipid membrane of the cell.

F. Disturbances in Fat Digestion

Normal micelle formation, i.e., fat digestion, requires active pancreatic lipase and bile acids to be present above their critical micellar concentration. Disturbances in fat digestion are caused by (a) decreased pancreatic lipase activity or (b) decreased bile acid concentrations or both (Table IV).

The reasons for bile acid deficiency are more complex, since normal bile acid concentration depends not only on bile acid synthesis but also on the conservation of the bile acid pool by efficient intestinal absorption. In addition, there is a manyfold excess of pancreatic lipase, whereas the concentration of bile acids during digestion is only about five times the critical micellar concentration; i.e., there is only a fivefold excess of bile acids. In addition to the reasons listed for bile acid deficiency, an increase in the trihydroxy/dihydroxy ratio of bile acids, if present, would cause a higher CMC so that less bile acids would be present for a given concentration of bile acids.

TABLE IV. A Classification of Fat Maldigestion

Disturbance	Examples in disease
A. Impaired lipolysis	
1. Decreased secretion of pancreatic lipase into intestine	
Defective pancreozymin stimulation	Intestinal mucosal disease such as sprue
Decreased lipase synthesis	Pancreatitis, pancreatic resection, genetic defects
Defective delivery	Duct obstruction, cystic fibrosis
2. Normal secretion into intestine but intraluminal disturbances reducing concentration	
Acid denaturation	Gastrin-secreting islet cell tumor causing gastric hypersecretion
B. Impaired micellar dispersion	
1. Decreased secretion of bile acids into intestine	
Decreased synthesis	Hepatic parenchymal disease
Defective delivery	Obstruction to bile flow or fistula, possibly defective pancreozymin stimulation
2. Bile acid malabsorption interrupting the enterohepatic cycle	
Defective intestinal absorption	Ileal disease or resection
Intraluminal binding preventing intestinal absorption	Intraluminal binding preventing ileal absorption, e.g., cholestyramine or neomycin administration
3. Normal secretion into intestine but intraluminal disturbances reducing concentration	
Dilution	Gastric hypersecretion
Precipitation	Jejunal acidification or bacterial deconjugation
Sequestration	Cholestyramine or neomycin administration
Deconjugation with rapid absorption of free bile acids	Blind-loop syndrome

The events in fat digestion and absorption in bile acid deficiency states are under active investigation. It seems generally agreed that (a) even if the rate of lipolysis is impaired, lipolysis goes to completion (91), and (b) fat is absorbed moderately well in the absence of bile, but nonpolar solutes such as cholesterol and fat-soluble vitamins are absorbed negligibly, if at all, when micelles are not present (2). As noted, recent work (Morgan and Hofmann, unpublished observations) suggests that the distribution ratio of fatty acids between an aqueous phase lacking micelles and an oil phase is higher than that of long-chain 2-monoglycerides and that as a result, in the absence of

bile acid micelles, fatty acid is absorbed significantly better than monoglyceride. Medium-chain triglycerides are well absorbed in the absence of bile (31). Presumably, octanoic acid has an aqueous phase/oil phase partition ratio in the absence of bile acids as great as that of long-chain fatty acids in the presence of bile acids. The 2-mono-octanoin formed, as noted, isomerizes rapidly to the 1-isomer, which is readily cleaved by the pancreatic lipases (57).

Very limited information suggests that bacterial overgrowth may be associated with and even result from decreased jejunal concentrations of bile acids. In patients with severe bile acid malabsorption resulting from ileal resection, jejunal bile acid concentrations are reduced, causing fat maldigestion and fat malabsorption (92). In such patients, the unabsorbed fatty acids are converted by bacteria to hydroxy fatty acids, indicating that a bile acid deficiency in the jejunum influences the chemical events occurring in the colon by providing substrate for bacterial biotransformations (93).

The events occurring in fat digestion in absence of pancreatic lipase will not be discussed.

G. The Enterohepatic Circulation of Bile Acids

1. The Intact Enterohepatic Circulation

The enterohepatic circulation of bile acids is characterized by a large pool of bile acids (2,15,26) which cycles many times (probably six to ten) each day. The size of the pool is determined by the efficiency of intestinal absorption and by the rate of hepatic synthesis of bile acids from cholesterol (94). The efficiency of absorption is high—in health probably greater than 98%—and the amount of bile acids not absorbed is balanced by hepatic synthesis (Fig. 17). Bile acids are excreted only in feces, and their nucleus is considered invulnerable to bacterial attack; therefore, the measurement of fecal bile acids either by chemical estimation or isotope dilution techniques indicates hepatic synthesis (94). The pool size may be estimated directly by isotope dilution, and an indirect estimate of pool size can be obtained by measuring jejunal bile acid concentration during digestion of a test meal.

Bile acid synthesis appears to be regulated by a negative feedback mechanism (5), although details are poorly understood, and even this concept has been disputed (95). Events which decrease the return of bile acids to the liver, such as bile fistula, ileal bypass or resection, or cholestyramine administration, are associated with increased bile acid synthesis. Conversely, events causing increased return of bile acids to the liver, such as bile acid feeding or the infusion of bile acids into the intestine, decrease bile acid synthesis (5). A significant exception to these observations is starvation, where both cholesterol and bile acid synthesis are greatly reduced despite the lack of enterohepatic cycling of bile acids. The negative feedback of bile acids is considered

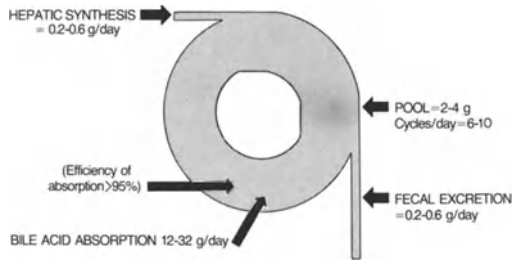

Fig. 17. Schematic depiction of the enterohepatic circulation of bile acids in man. In the steady state, fecal excretion equals hepatic synthesis, and accordingly hepatic synthesis may be estimated from fecal bile acid excretion. The pool size may be estimated only by isotope dilution techniques, which also give the hepatic synthesis rate (94). The mass of bile acid secreted into the small intestine is equal to the pool multiplied by the number of cycles per day; this cannot be estimated by any simple means in intact man at present. The diagram does not show the complexity of intestinal absorption of bile acids, which involves passive absorption of the glycine dihydroxy bile acids from the jejunum and active absorption of all conjugated bile acids from the ileum, as well as passive absorption of probably predominantly unconjugated bile acids from the colon.

to involve the enzyme hydroxymethylglutaryl–coenzyme A reductase in cholesterol biosynthesis and cholesterol-7α-hydroxylase in the conversion of cholesterol to bile acids (5,96).

The efficient intestinal absorption of bile acids involves both active and passive absorption, but little information on the relative sites and mechanisms of absorption and on their contribution to the entire enterohepatic cycle of bile acids exists. Although the contribution of passive and active absorption of bile acids in the rat small intestine has been measured (20), no data are available for other species. The major site of absorption in all vertebrates appears to be the ileum, where an active transport site exists (14,15). Free bile acids are absorbed passively in the jejunum by nonionic diffusion, dihydroxy acids being absorbed more rapidly than trihydroxy acids (21,22). Perfusion studies in the human jejunum have suggested that glycine dihydroxy bile acids may be absorbed to some extent, and additional evidence for jejunal absorption of bile acids has been obtained in patients and animals with ileal resection (97,98). No information exists on the importance of jejunal bile acid absorption in health in man. Taurine-conjugated bile acids do not appear to be absorbed in the human jejunum (24).

The colon probably has the capacity to absorb any unconjugated bile acid, although in health the bile acids present in the colonic lumen should be predominantly deoxycholic acid and lithocholic acid (26). The absorptive

capacity of the colon is considerably less than that of the ileum (99) but could be sufficient to influence significantly the amount of bile acids synthesized by the liver (15). In all probability, the amount of bile acids absorbed by the colon is influenced most by the rate of bacterial dehydroxylation of bile acids, since the conversion of cholic acid to deoxycholic acid results in partial precipitation from solution and/or binding to bacteria, and the conversion of chenodeoxycholic acid to lithocholic acid causes complete precipitation, since lithocholic acid, whether free or conjugated, is insoluble below 50° C(1).

2. The Interrupted Enterohepatic Circulation

The enterohepatic circulation of bile acids may be interrupted by biliary obstruction or biliary fistula; the events occurring in fat digestion in the absence of bile acids have been discussed. Of more interest are the disturbances in bile acid and fat metabolism occurring when the enterohepatic circulation of bile acids is interrupted by ileal disease or resection.

Bile acid malabsorption has now been clearly documented in patients with ileal resection, as evidenced by the rapid fecal excretion of orally or parenterally administered labeled cholate or taurocholate (100–102); increased hepatic synthesis of bile acids occurs, which is commonly 5–10 times the synthetic rate in health and may be as much as 20 times greater (103). Two situations may be distinguished (100): In some patients, the increased synthesis together with residual absorption successfully compensates for the loss of ileal absorption, and the bile acid pool, measured indirectly by the jejunal concentration of bile acids during digestion, is not significantly below levels observed in control subjects. Such patients have been termed "compensated." In the second group of patients, the increase in synthesis is as great or almost as great but is still inadequate to compensate for the loss of absorptive capacity. The bile acid pool falls, and the concentration of bile acids in the jejunum during digestion is reduced. These patients have been termed "decompensated."

Detailed studies on patients with ileal resection have now clarified some of the interrelated pathophysiological disturbances. The "compensated," patients have resections of less than 100 cm and often have some ascending colon remaining (27,104). Even though the ratio of primary/secondary fecal bile acids is markedly increased, the total amount of secondary bile acids formed in these patients is considerably greater than normal. The large amount of bile acids passing into the colon appears to influence the bacterial alterations of bile acids, since 7-dehydroxylation is reduced absolutely as well as relatively (93); indeed, in many of these patients, deoxycholic acid is absent from both bile and feces. In addition, the glycine/taurine ratio of the biliary bile acids may be high (105) and, if so, can be returned toward normal by oral taurine supplementation, suggesting that a relative taurine deficiency is pres-

ent in some patients. Steatorrhea is mild, less than 20 g/day, and is probably attributable to decreased intestinal surface and rapid intestinal transit. Bile acid concentrations are 2–8 mM in the aqueous supernatant of centrifuged stool, indicating that unusually high concentrations of bile acids are present in solution in the colonic lumen (93). The high bile acid concentrations could cause alterations in colonic flora and might explain the apparent reduction in 7α-dehydroxylation. The bile acid pool in such patients is presumably maintained by increased synthesis, increased passive jejunal absorption because of the increased glycine/taurine ratio, and increased passive colonic absorption because of the high concentration of bile acids in the colon which remain in solution at the observed *p*H (6–7) because bacterial 7α-dehydroxylation is reduced. As noted previously, cholic and chenodeoxycholic acids, the primary bile acids which are the major constituents of feces in these patients, are more soluble than their respective secondary bile acids. These patients have a watery diarrhea (500–800 g/day) attributable to increased passage of water into the colon; colonic secretion of water may occur

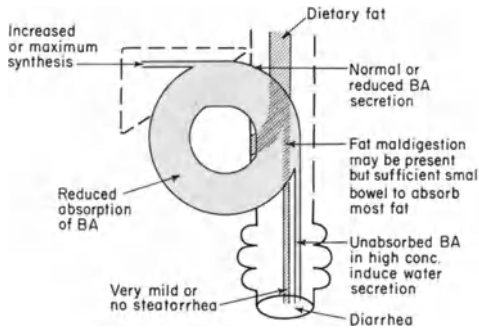

Fig. 18. Schematic depiction of the enterohepatic circulation of bile acids in patients with slight impairment in bile acid absorption. Decreased absorption causes increased hepatic synthesis, which restores the bile acid pool size to normal. Fat digestion is essentially normal, and fat malabsorption is not present. The increased passage of bile acids into the colon causes sodium and water secretion, manifested by diarrhea which is responsive to cholestyramine. The figure does not show the complexity of the intestinal absorption in these patients. Since they have predominantly glycine-conjugated bile acids, jejunal absorption may be increased; further, since the concentration of bile acids in colonic content is increased, passive absorption from the colon probably also is increased and contributes to the conservation of the bile acid pool. Since the increased synthesis, as well as alterations in the site of intestinal absorption, results in maintenance of the bile acid pool at essentially normal levels, such patients have been termed "compensated." The syndrome has been termed 5,6 acid diarrhea or "cholanorrheic diarrhea."

because of the increased passage of bile acids into the colon (Fig. 18). Fecal frequency and fecal weight respond strikingly to the cholestyramine, 16 g/day and although fecal fat usually doubles, the increase in steatorrhea is not of nutritional significane (104). In such patients, the symptoms of the diarrhea are more troublesome than the consequences of bile acid sequestration, suggesting that bile acid excess in the colon is less desirable than bile acid deficiency in the jejunum. If this reasoning is correct, future therapeutic efforts may well be aimed at repressing the derepression of the hepatic cell rather than at sequestration of bile acids in the colonic lumen.

The situation in the decompensated patients is less well understood. These patients, in the author's experience, have greater than 100 cm resection and often have an ileal transverse colostomy. The bile acid synthesis rate is quite similar to that in the compensated patients, but in an occasional patient it seems to be lower. The bile acids of bile contain a normal or high proportion of deoxycholic acid, and fecal bile acids are predominantly lithocholic acid and deoxycholic acid. Jejunal concentrations of bile acids are markedly reduced, and up to one-half of the bile acids which are present may have

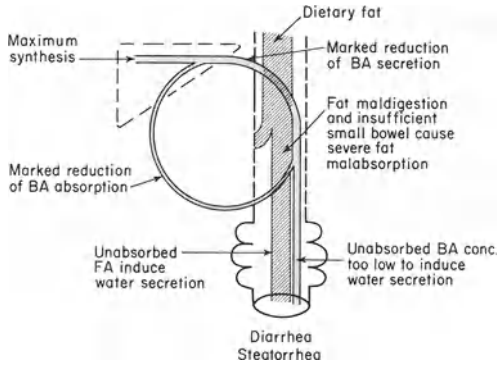

Fig. 19. Schematic depiction of the enterohepatic circulation of bile acids in patients with severe impairment in bile acid absorption. Decreased absorption causes increased hepatic synthesis, which is inadequate to restore the bile acid pool size to normal. The concentration of bile acids in the jejunum during digestion is below the critical micellar concentration, and the resultant fat digestion, as well as decreased intestinal surface area, causes fat malabsorption. The unabsorbed fatty acids or their bacterial degradation products, hydroxy fatty acids, or both induce water secretion. Bacterial changes in the colon appear to remove bile acids from solution, so that these patients have a fatty acid diarrhea and not a bile acid diarrhea (115). These patients have been termed "decompensated."

precipitated from solution, probably attributable to the high proportion of glycine dihydroxy bile acids present in these patients. These patients have moderate to severe steatorrhea attributable to the decreased bile acid concentration as well as the severe reduction in surface area. The bile acids contain a high concentration of secondary bile acids, indicating that bacterial dehydroxylation is taking place to an extent probably greater than normal (93). Detailed bacteriological studies have not been carried out. In these patients, cholestyramine administration greatly worsens the steatorrhea, causing negative caloric balance. In contrast to the compensated patients, removal of fat from the diet causes a significant reduction in fecal water in a manner not understood. Since removal of fat from the diet abolishes the diarrhea and steatorrhea it seems possible that the unabsorbed fatty acids are a major cause of catharsis in these patients (93)(Fig. 19). If so, future therapeutic efforts may well be aimed at bile acid replacement, possibly by a nonionic detergent, since it would appear that if efficient fat absorption could be achieved, the diarrhea of these patients would be greatly improved. Why these patients do not have bile acid diarrhea is unclear, since bile acid malabsorption is present and synthesis rates are greatly increased. Preliminary data suggest that the bacterial transformations of bile acids are so complete that the concentration of bile acids in solution in the colon is too low to inhibit the absorption of salt and water (93).

III. INFLUENCE OF BILE ACIDS ON WATER AND ELECTROLYTE TRANSPORT IN THE INTESTINAL TRACT

Nearly 100 years ago, Tappeiner, while studying the site of bile acid absorption in the canine intestine, observed that the presence of bile acids in the lumen seemed to cause secretion of water (106). During the past 2 years, work in several laboratories (7,107) has suggested that bile acids may influence water and electrolyte movements in the small and large intestine, in both health and disease.

A. The Stomach

The concentration of bile acids in gastric contents during fasting or after eating is very low in health, indicating competency of the pylorus. In patients with gastric ulcer along the proximal lesser curvature, the concentration of bile acids is elevated after meals, with a mean concentration of 0.65 $\pm$0.34 S.E. reported by Rhodes *et al.* (74). Normally, the mucosa of the stomach is remarkably impermeable to HCl, but after exposure to taurocholic

acid or bile the functional gastric mucosal barrier to acid diffusion is greatly decreased (108). This observation, considered together with the evidence for increased reflux in patients with gastric ulcer, as well as other observations (109) showing that the mucosa of some of such patients is more permeable than that of healthy controls, suggests that bile reflux into the stomach in patients with gastric ulcer could influence hydrogen ion movements and could conceivably also cause ulceration or delay healing in pre-existing ulceration.

B. The Small Intestine

Little information exists on the influence of bile acids on electrolyte and water movements in the small intestine. Forth *et al.* (107) noted that deoxycholic acid, but not conjugated bile acids, inhibited water and electrolyte absorption; systematic examination of the effect of nucleus substitution and presence and type of conjugation was not carried out. No information is available at present on the influence of bile acids on water movements in the human jejunum and ileum.

C. The Large Intestine

Forth *et al.* reported that deoxycholic acid inhibited the absorption of water and sodium by the rat colon, and work in our own laboratory using the technique of colonic perfusion (7) has now confirmed and extended these observations in man. In man, the addition of dihydroxy bile acids to colonic perfusates causes secretion of sodium and water and a reduction in bicarbonate absorption. The effect is concentration dependent, elicited by deoxycholic acid at 3 mM and chenodeoxycholic acid at 5 mM, whether these dihydroxy bile acids are free or conjugated. The trihydroxy bile acid, cholic acid, has no detectable influence on water and sodium movements at 10 mM whether free or conjugated. Since the effect appeared related to concentration rather than extent of absorption, it was considered to be attributable to a membrane effect. Similar results were elucidated in the dog, although the amount of induced secretion was less than in man. Lithocholic acid has not been studied in man because of its potential toxicity (110,111). The secretion induced by bile acids was isotonic and resembled the composition reported for diarrheal secretion. These experiments suggested that if bile acids influence colonic water movements in health, deoxycholic acid, the major fecal bile acid and the most potent secretagogue, should be largely responsible. In patients with ileal resection and diarrhea, as noted, 7α-dehydroxylation is reduced and chenodeoxycholic acid may be the only dihydroxy bile acid in feces. In such patients, this acid would appear to be the bile acid contributing to the diarrhea.

No information is available at present on the effects of the many other secondary bile acids present in feces in small concentrations on water and electrolyte movement in the colon. In addition, no information is available on the role of bile acids in constipation, irritable bowel syndrome, nonspecific diarrhea, antibiotic diarrhea, or the diarrhea of the germ-free animal. Of interest is the response of the diarrhea of the germ-free animal to an anion exchange resin similar to cholestyramine (112). Studies are needed to define the structure–activity relationships for bile acids and the induction of water and sodium secretion by the colon. In addition, information is needed on the composition, concentration, and physical state of bile acids in colonic contents and feces in health and disease.

REFERENCES

1. A. F. Hofmann and D. M. Small, *Ann. Rev. Med.* **18,** 433 (1967).
2. A. F. Hofmann, *in* "Handbook of Physiology" (C. F. Code, ed.), Vol. 5, p. 2507, Williams & Wilkins, Baltimore (1968).
3. C. H. Gray, D. C. Nicholson, and R. V. Quincey, *in* "Handbook of Physiology" (C. F. Code, ed.), Vol. 5, p. 2483, Williams & Wilkins, Baltimore (1968).
4. G. A. D. Haslewood, *in* "Bile Salts," Methuen, London (1967).
5. S. Shefer, S. Hauser, I. Bekersky, and E. H. Mosbach, *J. Lipid Res.* **10,** 646 (1969).
6. J. M. Dietschy, *Gastroenterology* **57,** 461 (1969).
7. H. S. Mekhjian, S. F. Phillips, and A. F. Hofmann, *J. Clin. Invest.*, **50,** 1569 (1971).
8. S. Bergström and H. Danielsson, *in* "Handbook of Physiology" (C. F. Code, ed.), Vol. 5, p. 2391, Williams & Wilkins, Baltimore (1968).
9. H. Danielsson and T. T. Tchen, *in* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. II, p. 118, Academic Press, New York and London (1968).
10. G. A. D. Haslewood, *Biol. Rev.* **39,** 537 (1964).
11 G. A. D. Haslewood, *in* "Handbook of Physiology" (C. F. Code, ed.), Vol. 5, p. 2375, Williams & Wilkins, Baltimore (1968).
12. B. Hamprecht, *Naturwissenschaften* **56,** 398 (1969).
13. A. G. A. Van den Oord, doctoral thesis, University of Utrecht, Netherlands, Schotanus & Jens, Utrecht (1965).
14. J. M. Dietschy, *J. Lipid Res.* **9,** 297 (1968).
15. I. M. Weiner and L. Lack, *in* "Handbook of Physiology" (C. F. Code, ed.), Vol. 3, p. 1439, Williams & Wilkins, Baltimore (1968).
16. A. F. Hofmann and B. Borgström, *Fed. Proc.* **21,** 43 (1962).
17. P. Ekwall, T. Rosendahl, and N. Löfman, *Acta Chem. Scand.* **11,** 590 (1957).
18. J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, p. 466, Academic Press, New York (1958).
19. A. F. Hofmann, *Biochem. J.* **89,** 57 (1963).
20. J. M. Dietschy, H. S. Solomon, and M. D. Siperstein, *J. Clin. Invest.* **45,** 832 (1966).
21. D. M. Switz, I. G. Hislop, and A. F. Hofmann, *Gastroenterology,* **58,** 999 (1970) (Abst.).
22. I. G. Hislop, A. F. Hofmann, and L. J. Schoenfield, *J. Clin. Invest.* **46,** 1070 (1967) (Abst.).
23. B. Borgström, G. Lundh, and A. F. Hofmann, *Gastroenterology* **45,** 229 (1963).
24. W. J. Simmonds, A. F. Hofmann, and E. Theodor, *J. Clin. Invest.* **46,** 874 (1967).

25. G. A. Martini, E. A. Phear, B. Ruebner, and S. Sherlock, *Clin. Sci.* **16,** 35 (1957).
26. H. Danielsson, *Advan. Lipid Res.* **1,** 335 (1963).
27. A. F. Hofmann and J. R. Poley, *Gastroenterology* **56,** 1168 (1969) (Abst.).
28. J. R. Senior, *J. Lipid Res.* **5,** 495 (1964).
29. F. H. Mattson and R. A. Volpenhein, *J. Biol. Chem.* **239,** 2772 (1964).
30. G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta* **105,** 121 (1965).
31. J. R. Senior, T. B. Van Itallie, and N. J. Greenberger, eds., "Medium Chain Triglycerides," University of Pennsylvania Press, Philadelphia (1968).
32. D. M. Small, *J. Am. Oil Chemists' Soc.* **45,** 108 (1968).
33. D. M. Small and M. Bourges, *J. Mol. Crystals* **1,** 173 (1966).
34. K. Shinoda, T. Nakagawa, B. Tamamushi, and T. Isemure, "Colloidal Surfactants," p. 310, Academic Press, New York (1963).
35. G. S. Hartley, *in* "Actualités Scientifiques Industrielles," p. 1, Hermann, Paris (1936).
36. P. Mukerjee, "Advances in Colloid Interface Science," p. 241, Elsevier, Amsterdam (1967)
37. A. S. C. Lawrence, *in* "Surface Activity and Detergency" (K. Durham, ed.), p. 158, Macmillan, London (1961).
38. A. S. C. Lawrence, K. Hume, C. B. Capper, and A. Bingham, *J. Phys. Chem.* **68,** 3470 (1964).
39. D. Chapman, P. Byrne, and G. G. Shipley, *Proc. Royal Soc. A* **290,** 115 (1966).
40. T. J. Flautt and K. D. Lawson, *in* "Ordered Fluids and Liquid Crystals," p. 26, Advances in Chemistry Series No. 63, American Chemical Society, Washington, D. C. (1967).
41. H. Nakayama and K. Shinoda, *Bull. Chem. Soc. Japan* **40,** 1797 (1967).
42. H. Nakayama, K. Shinoda, and E. Hutchinson, *J. Phys. Chem.* **70,** 3501 (1966).
43. L. Mandell, K. Fontell, and P. Ekwall, *in* "Ordered Fluids and Liquid Crystals," p. 89, Advances in Chemistry Series No. 63, American Chemical Society, Washington, D.C. (1967).
44. A. S. C. Lawrence and J. T. Pearson, *in* "Chemistry, Physics and Application of Surface Active Substances" (J. T. G. Overbeek, ed.), p. 709, Gordon & Breach, New York (1967).
45. W. H. Admirand and D. M. Small, *J. Clin. Invest.* **47,** 1043 (1968).
46. H. Lecuyer and D. G. Dervichian, *Kolloid-Zeitschrift* **197,** 115 (1964).
47. K. Larsson, *Z. Phys. Chem. (Frankfurt)* **56,** 173 (1967).
48. E. S. Lutton, *J. Am. Oil Chemists' Soc.* **42,** 1068 (1965).
49. A. F. Hofmann, *Biochim. Biophys. Acta* **70,** 306 (1963).
50. N. Krog and K. Larsson, *Chem. Phys. Lipids* **2,** 129 (1968).
51. A. Boudreau and J. M. deMan, *Can. J. Biochem.* **43,** 1799 (1965).
52. E. L. Skau and R. E. Coucher, *J. Phys. Chem.* **58,** 460 (1954).
53. D. N. Eggenberger, F. K. Broome, A. W. Ralston, and H. J. Harwood, *J. Org. Chem.* **14,** 1108 (1948).
54. J. Lucassen, *J. Phys. Chem.* **70,** 1824 (1966).
55. W. J. Simmonds, T. J. Redgrave, and R. L. S. Willix, *J. Clin. Invest.* **47,** 1015 (1968).
56. A. F. Mabrouk and L. R. Dugan, Jr., *J. Am. Oil Chemists' Soc.* **38,** 9 (1961).
57. T. Nakagawa and K. Tori, *Kolloid-Zeitschrift* **194,** 143 (1964).
58. J. W. McBain, Proc. Roy. Soc. London Obituary Notices, Cambridge University Press, p. 529 (1952–1953).
59. H. L. Rosano, K. Breindel, J. H. Schulman, and A. J. Eydt, *J. Colloid Interface Sci.* **22,** 58 (1966).

60. A. F. Hofmann, *in* "Molecular Association in Biological Systems," Vol. 84, p. 53, American Chemical Society, Washington D. C. (1968).
61. J. S. Fordtran and T. W. Locklear, *Am. J. Digest. Dis.* **11,** 503 (1966).
62. A. S. C. Lawrence, B. Boffey, A. Bingham, and K. Talbot, *in* "Chemistry, Physics and Application of Surface Active Substances" (J. T. G. Overbeek, ed.), p. 673, Gordon & Breach, New York (1967).
63. M. C. Carey and D. M. Small, *J. Colloid Interface Sci.* **31,** 382 (1969).
64. A. Norman, *Acta Chem. Scand.* **14,** 1295 (1960).
65. D. M. Small, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta* **176,** 178 (1969).
66. A. F. Hofmann, thesis, University of Lund, 32 pp. (1964).
67. C. P. Freeman, *Brit. J. Nutr.* **23,** 249 (1969).
68. P. E. Verkade and W. Meerburg, *Rec. Trav. Chim. Pays* **74,** 263 (1955).
69. W. Shankland, *J. Colloid Interface Sci.*, **34,** 9 (1970).
70. P. Mukerjee and K. Banerjee, *J. Phys. Chem.* **68,** 3567 (1964).
71. B. Borgström, *J. Lipid Res.* **5,** 522 (1964).
72. B. Borgström, *J. Lipid Res.* **8,** 598 (1967).
73. A. F. Hofmann and B. Borgström, *J. Clin. Invest.* **43,** 247 (1964).
74. J. Rhodes, D. E. Barnardo, S. F. Phillips, R. A. Rovelstad, and A. F. Hofmann, *Gastroenterology* **57,** 241 (1969).
75. M. Cohen, R. G. H. Morgan, and A. F. Hofmann, *Gastroenterology* **60,** 1 (1971).
76. F. H. Mattson and L. W. Beck, *J. Biol. Chem.* **219,** 735 (1956).
77. K. D. Dreher, J. H. Schulman, and A. F. Hofmann, *J. Colloid Interface Sci.* **25,** 71 (1967).
78. T. Nakagawa and K. Tori, *Kolloid-Zeitschrift* **194,** 143 (1964).
79. S. Mishkin and J. I. Kessler, *Biochim. Biophys. Acta* **202,** 222 (1970).
80. K. J. Mysels, *in* "Pesticidal Formulations Research. Physical and Colloidal Chemical Aspects," Advances in Chemistry Series No. 86, American Chemical Society, Washington, D.C. (1969).
81. W. Higuchi, *Fed. Proc.* **29,** (1970).
82. F. Spener, F. Paltauf, and A. Holasek, *Biochim. Biophys. Acta* **152,** 368 (1968).
83. R. G. H. Morgan and A. F. Hofmann, *J. Lipid Res.* **11,** 223 (1970).
84. B. Borgström, *J. Lipid Res.* **9,** 473 (1968).
85. A. K. Lough, *in* "Proceedings of the Third International Symposium on the Physiology of Digestion and Metabolism in the Ruminant" (A. T. Phillipson, ed.), Oriel Press, Newcastle-upon-Tyne (1970).
86. J. Hyun, H. Kothari, J. Mortenson, C. R. Treadwell, and G. V. Vahouny, *J. Biol. Chem.* **244,** 1937 (1969).
87. J. A. Olson, *J. Lipid Res.* **5,** 402 (1964).
88. W. J. Simmonds, *J. Clin. Nutr.* **22,** 266 (1969).
89. J. M. Johnston, *in* "Handbook of Physiology" (T. H. Wilson, ed.), Vol. 3, p. 1353, Williams & Wilkins, Baltimore (1968).
90. S. Feldman and M. Gibaldi, *J. Pharm. Sci.* **58,** 425 (1969).
91. L. K. Knoebel and J. M. Ryan, *Am. J. Phys.* **204,** 509 (1963).
92. G. M. McLeod and H. S. Wiggins, *Lancet,* p. 873 (1968).
93. A. F. Hofmann, J. R. Poley, H. S. Mekhjian, and S. F. Phillips, *J. Clin. Invest.* **49,** 44a (1970) (Abst.).
94. A. F. Hofmann, L. J. Schoenfield, B. A. Kottke, and J. R. Poley, *in* "Methods in Medical Research," Yearbook Publishers, Chicago, Vol. 12, p. 149 (1970).
95. H. J. Weis and J. M. Dietschy, *J. Clin. Invest.* **48,** 2398 (1969).

96. S. Shefer, S. Hauser, I. Bekersky, and E. H. Mosbach, *J. Lipid Res.*, **10,** 646 (1969).
97. R. H. Dowling and D. M. Small, *J. Clin. Invest.* **46,** 26a (1968) (Abst.).
98. R. Abaurre, S. G. Gordon, J. G. Mann, and F. Kern, Jr., *Gastroenterology* **57,** 679 (1969).
99. H. S. Mekhjian, S. F. Phillips, and A. F. Hofmann, *Clin. Res.* **17,** 307 (1969) (Abst.).
100. A. F. Hofmann, *Gastroenterology* **52,** 752 (1967).
101. W. E. Meihoff and F. Kern, Jr., *J. Clin., Invest.* **47,** 261 (1968).
102. M. M. Stanley and B. Nemchausky, *J. Lab. Clin. Med.* **70,** 627 (1967).
103. P. Samuel, G. M. Saypol, E. Meilman, E H. Mosbach, and M. Chafizedeh, *J. Clin. Invest.* **47,** 2070 (1968).
104. A. F. Hofmann and J. R. Poley, *New Engl. J. Med.* **281,** 397 (1969).
105. W. I. Austad, M. P. Tyor, and L. Lack, *Gastroenterology* **52,** 638 (1962).
106. A. J. F. H. Tappeiner, *Wien. Akad. Sitzber.* **77,** 281 (1978).
107. W. Forth, W. Rummel, and H. Glassner, *Naunyn-Schmiedebergs Arch. Pharmakol. Exptl. Pathol.* **254,** 364 (1966).
108. H. W. Davenport, *Gastroenterology* **54,** 175 (1968).
109. M. A. Chapman, J. L. Werther, and H. D. Janowitz, *Gastroenterology* **55,** 344 (1968).
110. N. B. Javitt and S. Emerman, *J. Clin. Invest.* **47,** 1002 (1968).
111. R. H. Palmer and Z. Hruban, *J. Clin. Invest.* **45,** 1255 (1966).
112. T. Asano, *Am. J. Physiol.* **217,** 917 (1969).
113. A. S. C. Lawrence and M. P. McDonald, *Mol. Crystals* **1,** 205 (1966).
114. D. H. Neiderhister and H. P. Roth, *Gastroenterology* **58,** 26 (1970).
115. A. F. Hofmann and J. R. Poley, *Gastroenterology* **62,** 918 (1972).

Chapter 6

EXPERIMENTAL CHOLELITHIASIS*

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I. INTRODUCTION

A. Background

Gallstones have galled man (and beast) for at least 35 centuries, and they continue today to be a major source of morbidity, if not mortality. Recent figures (1,2) indicate a gallstone prevalence that increases with age, approximating 10% in men and 20% in women in the age group 55–64. An estimated 15 million people in the United States have (though do not necessarily suffer from) gallstones, and each year one-third of a million have their stones removed surgically. It is of course hoped that work in experimental cholelithiasis will help to elucidate the mechanism of human cholelithiasis and develop medical approaches to the prevention and dissolution of gallstones.

Bile salts‡ assume an important place in any considerations of the etiol-

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‡The terms "bile acid" and "bile salt" are used somewhat interchangeably, except that the latter usually refers to the ionized species, whereas the former is a generic term but

ogy of cholelithiasis because of their role in cholesterol solubilization. Individual bile salts, however, have a variety of physiological and pharmacological effects: some increase bile flow, others diminish it; some solubilize cholesterol, others do not; some prevent stones, others cause them. The purpose of this review is to collate those reports bearing on the experimental production of gallstones and to interpret them, where possible, in relation to current concepts of biliary physiology. A more general review of the literature on the causes, complications, and treatment of gallstones can be found in the classic monograph of Rains (3).

B. Composition of Human Gallstones

Since the purpose of research on gallstone formation is to elucidate the human disease, it is pertinent to summarize briefly our knowledge of the composition of human gallstones. The chemical content varies considerably. In gallbladder stones, cholesterol or its monohydrate is usually the predominant component, but calcium carbonate (as calcite or aragonite) and pigment (as calcium bilirubinate or other pigments) are also major constituents and occur in varying proportions. In stones forming in the hepatic and common ducts, pigment predominates and cholesterol is a minor constituent. All gallstones contain proteins, mucopolysaccharides, salts of bile acids and fatty acids, and various other anions and cations in smaller amounts. They contain a considerable amount of water and are generally relatively friable. Morphologically, stones vary from amorphous sludge, microliths, and viscous, perhaps liquid crystalline, semisolid material to large, highly structured concretions. The "pure" cholesterol stones on cross-section reveal a radiating crystalline structure, while "mixed" cholesterol–pigment stones show, in addition, concentric layers of obviously differing compositions. Stones of one or more types may coalesce, and gross variations in chemical composition may occur within one stone or among different stones. Most calculi, even of the "pure" cholesterol type, have at least a visible fleck of pigment at the center, lending importance to the concept of a nidus for stone formation. Chemically, the centers contain greater amounts of certain bile acids, pigment, and protein than do the outer parts (4). Pigment stones in the Orient frequently form around ascaris worms or other parasites. Other

may, in context, imply the un-ionized species. Trivial names used in this chapter and their systematic nomenclature are cholic acid, 3α,7α,12α-tri-hydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-monohydroxy-5β-cholanoic acid; dehydrocholic acid, 3,7,12-triketo-5β-cholanoic acid; cholestanol (=dihydrocholesterol), 5α-cholestan-3β-ol; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid. Other abbreviations: EFA, essential fatty acids; ACTH, adrenocorticotropic hormone; PAS, para-amino salicylate.

stones have formed around pins, vegetable fibers, seeds, sutures, epithelial cells, and various organisms (typhoid bacilli, *Escherichia coli,* and actinomyces). Most stones, particularly mixed stones, appear to have a mucopolysaccharide matrix which can be visualized after dissolving the major constituents or by the use of appropriate stains. Further information and references can be obtained in the excellent reviews of Womack *et al.* (5), Rains (3), and Bouchier and Freston (6).

C. Stages in Gallstone Formation

It is clear that gallstone formation is a complex process involving several stages and that the process can be influenced at several points. It is convenient to consider four major stages, which are more or less interdependent and overlapping but which may simplify consideration of experimentally induced cholelithiasis.

1. Nidus Formation

As Small (7) has so simply and clearly stated, in gallstone formation "the first and basic change in bile is a change of physical state from a simple one-phase liquid to a two-phase system containing both liquid and undissolved solid." It is generally conceded that most if not all stones form about a nidus. This nidus may be exogenous (with respect to bile) organic or inorganic material, such as parasites, bacteria, mucin spherules, cellular debris, or suture material, or it may be a precipitate formed from the endogenous constituents of bile. This latter "change in . . . physical state . . . can be brought about only by a change in the composition of the solution" (7). Compositional changes may result from secretion of an abnormal (and unstable) bile by the liver, from alterations in hepatic bile through physiological or pathological secretion or absorption by the biliary tract mucosa, or from chemical (usually enzymatic) processes secondary to stasis and/or microbial activity. It is this stage of nidus formation, particularly when the nidus is formed from the precipitation of endogenous biliary solutes, which has been the subject of greatest interest in the experimental literature; it seems to be a necessary and requisite condition for stone formation.

Hultén (8) has recently published some simple and extremely fascinating observations on gallbladder morphology and stone formation. From plastic casts and cystoscopic examination of the interiors of distended gallbladders, he demonstrated that the mucosa has many small, closely packed, lobulated niches, in which are formed small (1–2 mm) spherules of mucoid gel. Cholesterol and pigment could be seen as precipitates within some of the spherules occupying these niches, which are presumed to be the site of water and electrolyte reabsorption. Contraction of the gallbladder musculature apparently displaced these spherules, which then conglomerated to form stones, or which

could be seen sticking to the surface of pre-existing stones and obviously participating in stone accretion. These studies have important implications and serve to emphasize some of the differences between stones formed in the gallbladder and in the ductular system. It remains to be shown whether these gel spherules can serve as nidi themselves and whether the gel phase has any direct effect on the solubility of cholesterol and other biliary constituents.

2. Nidus Retention

Considering the variety of substances which can serve as foci for stone formation, it seems probable that potential nidi of mucin spherules, desquamated cells, bacteria, chemical precipitates, and other microscopic particles occur with considerable frequency in normal bile but dissolve, break up, and/or are flushed out by the flow of bile. The occasional dissolution of even large stones has now been amply documented both radiologically and by direct observation at laparotomy. Formation of clinically significant gallstones therefore requires that the nidus remain in the biliary tract long enough for significant organization and enlargement to occur. (Of course, even extremely small stones and sludge can cause symptoms in the common duct.)

There are several important factors affecting nidus retention. First, bile flow may exert a flushing action. This may be impaired by diminished bile secretion or biliary tract obstruction. Second, mucus may either hinder or facilitate excretion of nidi. Third, abnormal patterns of gallbladder contraction may occur as a result of physiological factors (e.g., fasting versus continuous nibbling, pregnancy, or thyroid and steroid hormone status) or pathological processes (infection or inflammation). Finally, the rate of stone enlargement, which depends in part on the particular constituent being deposited, is important, since particles can only be flushed out before they reach a certain size. Thus quantitative aspects of the rate of stone formation may determine the qualitative presence or absence of stones. Several of these factors are discussed in greater detail in Section II.

3. Structural Organization

Except for those stones composed chiefly of amorphous pigment precipitates or sludge, such as those commonly found in the common duct, gallstones appear to have a definite, though poorly understood structural organization. Considerable evidence now points to the importance of an organic matrix in both natural and experimentally induced gallstones. This protein or mucopolysaccharide scaffold is probably important in the deposition of calcium carbonate, but its relationship to other major stone constituents is not clear. Hultén (8) believes that structural organization and crystallization of constituents may occur within individual or aggregated

mucoid spherules. In keeping with this concept, Kleeberg (9,10) has demonstrated that structures resembling gallstones in many details can be produced by the precipitation of mineral salts within the colloid matrix of gelatin *in vitro,* and the occurrence of laminated structures is of particular interest. It seems likely, however, that at least part of gallstone organization depends on the fluctuating composition of bile and its saturation with respect to cholesterol. More work is necessary to determine the importance of the protein and polysaccharide components.

4. Stone Enlargement

During this stage, the stone grows from micro- to macroscopic size by the deposition of the major stone constituents. For cholesterol stones, at least, this appears to require a bile that is supersaturated with respect to cholesterol. The capacity of bile to hold cholesterol in solution varies from species to species (11), and this accounts for the difficulty in producing cholesterol gallstones in some species and the relative ease with which they can be produced in others.

The main factors that determine the cholesterol-holding capacity of bile are the relative concentrations of cholesterol, phospholipids, and bile salts, and when these are plotted on triangular coordinates it becomes possible to predict with relative certainty whether or not cholesterol can be maintained in solution (7). It would appear that cholesterol deposition will not occur unless the bile is saturated or supersaturated with cholesterol. This can result, as suggested by Small (7), either from formation of abnormal or supersaturated hepatic bile (see Section IIIB) or by alterations in normal bile by reabsorption of bile salts in the gallbladder (12,13) (see Section IIIA). In addition, Fitz-James and Burton (14) have called attention to another method by which supersaturated bile, with subsequent precipitation of cholesterol crystals, may occur. It is well known from radiological and direct observational studies that fresh, relatively dilute hepatic bile may become layered over old, concentrated bile in the gallbladder, leading to zones of varying specific gravities. Fitz-James noted that these zones also varied in their ratios of bile salt/cholesterol and that if such bile specimens were incubated *in vitro,* fine precipitates of cholesterol could be observed in the zones with the lowest bile salt/cholesterol ratios. He assumed that bile salts and cholesterol diffused at different rates from more concentrated to less concentrated bile, leading to local areas of supersaturation and cholesterol precipitation. Such factors, which could lead to the formation of cholesterol nidi or to the deposition of cholesterol on pre-existing nidi, seem plausible, but their importance in human or experimental cholelithiasis remains to be determined.

II. STASIS AND INFECTION

A. History

The early history of experimental gallstone production has been reviewed by Hospers (15), who cites 40 references, starting with Blachstein in 1891. Calculi were generally produced by introducing foreign bodies into the gallbladder, by producing stasis, by infecting the gallbladder, or by inducing hypercholesterolemia in rabbits, dogs, cats, or guinea pigs. Almost all were described as being small pigment stones or precipitates, with little or no cholesterol. The methods used did not produce typical gallstones and lacked consistency; chemical analyses were infrequent and unsophisticated. The reports, generally only of historical interest, are summarized below.

In 1900, Miyake reported the production of faceted stones containing cholesterol, calcium, and pigment in dogs (and one rabbit) following ligation of the cystic duct (16). Aoyama (1914) produced "cholesterol stones" in rabbits and guinea pigs following cystic duct ligation and cholesterol feeding or injections (17), but Iwanaga (1922) could not reproduce the stones and bound only amorphous precipitates (18). Hansen (1927) reported pigment stones in rabbits with narrowed cystic ducts; simultaneous cholesterol feeding produced concretions containing cholesterol crystals (19). Whitaker (1927) produced small, uncharacterized gallstones in cats anesthetized for 5–15 days, in which fasting and dehydration resulted in stasis and concentration of bile (20). Westphal and Gleichmann (1931) combined partial cystic duct occlusion, cholesterol feeding, and injections of charcoal and bacteria into the gallbladder to produce bilirubin stones up to 1 cm in diameter (21). Cascao de Anciaes (1933) produced pigment precipitates in dog galblladers by cystic duct ligation; these dissolved during prologed ligation (22). When chemical, mechanical, or infectious inflammation of the mucosa was induced, the precipitates were found to contain cholesterol. Similar precipitates occurred with vagal stimulation in the absence of cystic duct ligation. Aronson (1940) noted the incidental finding of gallstones containing calcium, pigment, cholesterol, phosphorus, and residue in a dog with a partial stricture of the common duct (23). They could not be reproduced when stasis was created in a cannula equipped with a glass diverticulum (24). Simendinger (1944) found that thyroid function was important for adequate gallbladder function and that hypothyroid dogs, in addition to nonvisualizing gallbladders, developed thick, viscous bile containing a brown precipitate (25).

In 1957, Imamoglu *et al.* (26) reported that 58% of 50 patients with cholelithiasis had a narrow (less than 3 mm) opening in the ampula of Vater. An autopsy series confirmed the impression that patients with gallstones had smaller than normal openings. These investigators then attempted to produce

gallstones in dogs, rabbits, and monkeys by constricting the common bile duct. Seven of eight rabbits, two of eight dogs, and one of two monkeys developed bilirubin or mixed bilirubin–cholesterol stones. Similar stones were even produced in cholecystectomized rabbits and dogs with common duct strictures (27,28). Stones in the gallbladder could be produced by cystic duct ligation (29). Since cholangiographic studies in postpartum women had been noted to result in frequent nonvisualization or delayed gallbladder emptying, the effects of progesterone and estrogen on experimental gallstone formation were investigated. Rabbits receiving progesterone and diethylstilbestrol together, but neither one separately, developed gallstones similar to those produced previously by stasis (28,30). Ricci *et al.* (31) have also reported gallstones following the production of common duct strictures in dogs; they emphasized the histological changes in the gallbladder, which may be related to stone formation.

Lindelöf and van der Linden (32) found that neither increased bile flow induced by cholecystokinin or common duct strictures altered the incidence of cholestanol-induced cholelithiasis (see Section IVBI).

B. Pathophysiology

Stasis, from any cause, facilitates gallstone formation in several ways. Crystallization of constituents, compaction of stone elements, and retention and enlargement of small stones are all more or less dependent on some degree of stasis. Stasis also predisposes to infection, with multiple consequences. First, infection may result in deconjugation of bilirubin, with precipitation of calcium bilirubinate. Second, it may result in *p*H changes and/or deconjugation of bile salts, with their subsequent reabsorption from the biliary tract. Finally, it may, by producing inflammation, interfere with gallbladder function or the patency of the ampulla. It is therefore not surprising that experiments involving the production of stasis have not contributed greatly to our knowledge of the mechanism of gallstone formation. They have, however, helped to delineate a variety of physiological and pathological conditions which predispose to gallstone formation.

Stasis is, by definition, a diminution in the normal rate of bile flow, which appears to be necessary for the removal of potential gallstone nidi. This flow is initially determined primarily by bile salt secretion from the hepatocyte, but secretion by ductular cells and reabsorption of fluid and electrolytes by biliary tract epithelium are also important factors. In species with gallbladders, flow in this organ also depends on its filling passively and then emptying by muscular contraction. In all cases, flow can be diminished by mechanical obstruction, and most of the early work with stasis mentioned

above involved mechanical interference with bile flow. The physiology and pathology of bile secretion, however, represent an important area that is not well enough understood.

The control of bile (and bile salt) secretion is complex (33) and subject to a variety of metabolic, endocrine, neurogenic, and vascular influences. Except for one report (25), it is only in the last 10 years that metabolic and endocrine determinants of bile flow have even been implicated in the pathogenesis of gallstones. Simendinger (25) produced pigment precipitates in hypothyroid dogs, and hypothyroidism is known to decrease bile salt secretion (34,35) and hence flow. Any other condition resulting in an increased loss of bile salts would tend to reduce the bile salt pool and thus also decrease biliary flow (36). Clinically, such situations occur with ileal resection or disease (37) and the administration of cholestyramine, which may be associated with an increased incidence of cholelithiasis. Moreover, patients with cholelithiasis have been found to have a diminished bile salt pool (38). Experimentally, unsaturated fats, which increase the fecal excretion of bile salts, are associated with an increased incidence of amorphous pigment stones in hamsters (see Section IIIA), although there are no direct data bearing on bile flow.

Estrogens induce biliary stasis in rats, although the mechanism is not entirely clear (39–42). They decrease the hepatic transfer maximum of bile salts (40) and probably inhibit the rate of basal bile secretion (42), but there is also evidence to suggest that a major factor in estrogen cholestasis is enhanced back-diffusion of water and electrolytes (40,43). The increased incidence of gallstones in women and the role of estrogens in the jaundice and pruritis of pregnancy are consistent with an estrogen-induced functional cholestasis. Experimental gallstones can be produced more readily in some female animals, or under the influence of estrogens (30,44), but in other species estrogens enhance bile flow (44). The effects of estrogens on liver blood flow and other factors influencing biliary secretion have not been investigated.

Gallbladder stasis is presumably extremely important in the genesis of cholesterol stones, but the factors involved in adequate filling and emptying of the gallbladder are poorly understood. Even with appropriate stimuli, the gallbladder does not empty completely, and the effects of various eating patterns, anatomical variations, and neurogenic and hormonal stimuli on the mixing and emptying of bile have not been systematically investigated. The only experimental observations bearing on this point are those of Lindelöf (32), who found that injections of cholecystokinin did not inhibit cholestanol-induced cholelithiasis. However, this system may not be a good one to use for evaluating the effects of increased flow, since the stimulus for gallstone formation is so strong.

III. CHOLESTEROL STONES

A. Hamsters

A major contribution to the study of gallstone formation has come from the work of Dam and colleagues (45). While studying the effects of fat-free diets, Dam and Christensen (46) noted the occurrence of gallstones with a high (50–90%) cholesterol content in hamsters maintained on a fat-free, 20% casein, 74.3% sucrose, vitamin-supplemented diet. Replacing sucrose with sorghum or ground oats prevented stone formation, but including linoleic acid, lard (2%), or cholic acid (0.2%) did not (47). [Later studies have indicated that linoleic acid can inhibit stone formation (48–50).] In further studies, soybeans, dried yeast, and copper sulfate (partially) prevented stone formation, whereas sitosterol, lecithin, inositol, cystine, and taurine did not (51). Adding 0.1% hyodeoxycholic acid to the diet afforded a high degree of protection against cholesterol stone formation, whereas adding cholic, deoxycholic, dehydrocholic, and lithocholic acids did not (52). Antibiotics such as sulfaguanidine, oxytetracycline, neomycin, and nystatin also did not affect stone formation (53). Hanel *et al.* found that gallstone production was not associated with an increase in liver cholesterol synthesis, as measured by the incorporation of acetate-2-^{14}C into cholesterol in slices (54) or *in vivo* (55). These results, however, are at variance with later studies showing an increased rate of cholesterol synthesis (50,56).

The cholesterol content of the stones can be altered by dietary manipulations. When hamsters maintained on a lithogenic diet for 63 days were transferred to a nonlithogenic diet containing yeast, some cupric sulfate, more lard, and no sucrose, the stones diminished in size and their cholesterol content decreased from 50% to 0.7% (57). Adding 2% lard, soybean oil, or codliver oil (in order of increasing fatty acid unsaturation) to the lithogenic diet decreased the number of animals with cholesterol stones from 77% to 38.5, 7.7, and 0%, respectively (58). At the same time, the number of animals with amorphous pigment stones rose from 0% 2.5, 10.3, and 20.5%, respectively. The addition of unsaturated fats therefore not only reduced the total number of animals with gallstones but greatly reduced the amount of cholesterol incorporated into the stones. The pigment stones contained large amounts of the calcium salts of glycine-conjugated dihydroxy bile acids and considerable ash (59); they will be discussed further in Section IVA.

The importance of the type of carbohydrate was studied in hamsters receiving diets containing 2% lard (60). Glucose (72.3%) gave 100% cholesterol stones, whereas sucrose gave predominantly amorphous pigment stones; only two of seven contained cholesterol. Rice starch and lactose (72.3%) completely prevented lithiasis. In a second series, when rice starch was sub-

stituted in varying amounts for the sugars, cholesterol stones formed with both glucose and sucrose at the 72.3% and 60.3% levels, whereas amorphous pigment stones occurred with both sugars at concentrations of 36% or less.

The explanation for this carbohydrate effect may be related to the fact that the composition of gallbladder bile is affected by the nature of the dietary carbohydrate (48,61,62). Gallbladder bile from hamsters fed 74.3% glucose had higher concentrations of cholesterol, lower concentrations of bile salts, and, in general, lower concentrations of phospholipids than did that from hamsters fed the preventive (rice starch) or curative (10% lard, 20% casein, 36% yeast, 28.3% rice) diet. Animals fed lactose, however, had values for all three constituents that were in the same range as those fed glucose and still did not form stones, suggesting the importance of additional factors in this form of stone prevention. Hamsters placed on the glucose diet at 16 weeks of age instead of at 5–6 weeks of age had lower cholesterol, higher phospholipid, and similar bile salt concentrations. These animals did not form cholesterol stones but had a marked propensity to form amorphous pigment stones. Limited analyses of fistula bile have revealed no great differences (63). White mice reared on the glucose, fat-free diet did not form stones (64); their bile contained about the same amount of cholesterol but more bile salts and much more phospholipid than did the hamster bile.

Codliver oil and various treated oil fractions also inhibited stone formation and decreased cholesterol incorporation, thus favoring amorphous pigment stone formation (65). Codliver oil caused moderate increases in the bile acid/cholesterol and phospholipid/cholesterol ratios of gallbladder bile, particularly in females (62). The curative diet produced a high phospholipid/cholesterol ratio, largely due to the increase in biliary phospholipid from the 10% dietary lard. Substitution for butter with a dietetic margarine rich in linoleic acid increased both the bile acid/cholesterol and phospholipid/cholesterol ratios, concomitant with a reduction in the incidence of cholesterol gallstones. There was, however, some overlapping of these two groups, suggesting that other factors, such as the fatty acid composition of the phospholipids, might also be important in determining these changes. The incidence of amorphous pigment stones was low in both the margarine and butter groups (48).

In a study on the effects of hormones, testosterone-treated males and estrogen-treated females had a lower incidence of amorphous pigment stones than gonadectomized animals but may have had a higher incidence of cholesterol stones; the total incidence was similar (66). Progesterone, on the other hand, lowered the incidence of cholesterol stones in females. Deoxycorticosterone had no clear effect on stone formation. Desiccated thyroid lowered and methyl thiouracil raised the ratio of cholesterol/amorphous pigment stones.

Jensen and Dam (56) have shown that hamsters on the glucose diet incorporate more intraperitoneally injected acetate-1-^{14}C into total body cholesterol (1.2%) than do those on the rice starch (0.15%) or curative (0.4%) diets. The specific activity of total body sterol was also increased (25 counts/sec/mg versus 4.0 and 11.4), although the total body cholesterol (mg/100 g body weight) was also greater in the glucose-fed hamsters. There was no significant difference in the elimination of labeled cholesterol from the body pool. These findings are in conflict with earlier studies (54,55) but have been supported recently by Muroya *et al.* (49,50). These workers found a fourfold increase in the incorporation of acetate-2-^{14}C into cholesterol in liver slices or homogenates from glucose-fed hamsters compared with animals on the starch diet. The increased incorporation could be prevented by adding ethyl linoleate but not ethyl palmitate to the diet, and the incorporation varied directly with the presence or absence of gallstones. Mevalonate incorporation was not enhanced, indicating that the effect depended on reactions occurring prior to mevalonate synthesis. The increased incorporation of acetate apparently results in both an increased total body cholesterol (56) and an increased biliary excretion of cholesterol (61,67).

In recent experiments, Dam *et al.* (68) have shown that 1% cholesterol *decreased* the incidence of cholesterol stones in hamsters fed a lithogenic diet containing butterfat but increased the incidence of amorphous pigment stones in females. Cholesterol added to a diet containing margarine, with its high content of linoleic acid, did not alter the low incidence of cholesterol stones but again markedly increased the incidence of amorphous pigment stones in females. It was postulated that the marked reduction in cholesterol stone formation might be a result of inhibition of endogenous cholesterol synthesis by exogenous cholesterol or stimulation of bile acid formation and bile flow. The former is consistent with the previous studies on the incorporation of acetate-2-^{14}C into cholesterol; both are of course possible.

Several other groups have confirmed and extended the observations of Dam, Christensen, and colleagues. Fortner (69) produced gallstones (48–93% cholesterol) in Syrian hamsters raised on Dam's glucose diet. Large amounts of mucoid material were found in the gallbladders of both control and experimental animals, but in the experimental animals stones and smaller crystalline masses were frequently found embedded in the translucent, gelatinous, mucoid substance. Vitamin A deficiency, and to a lesser extent vitamin D deficiency, increased the number of animals with stones. Van der Linden *et al.* (70) demonstrated that stones do not form in the common ducts of cholecystectomized hamsters. Drews (71) produced gallstones in hamsters and noted that portal inflammation, focal areas of necrosis, and fatty changes in the periphery of the liver lobule appeared prior to the development of gallstones.

Bergman and van der Linden (72) found that relatively large amounts of *d*-thyroxine (5 mg %) favored the production of amorphous gallstones and fatty livers in intact hamsters. The changes could also be produced by *l*-thyroxine and could not be prevented by propylthiouracil (73). Lipotrophic factors (choline, tryptophan, methionine, and α-tocopherol), however, markedly inhibited stone formation without influencing the fatty livers. Cholestyramine (3%) seemed to protect against cholesterol gallstone formation and fatty degeneration of the liver in hamsters previously fed *d*-thyroxine and then the basic lithogenic diet (74). These results are difficult to interpret, but the authors pointed out that cholestyramine also binds thyroxine in addition to bile salts.

A second line of investigation was pursued by Hikasa *et al.* (75–77). They had noted that the adrenals, liver, and heart contained more essential fatty acids (EFA) than other organs and developed the hypothesis that the rate of cholesterol catabolism to steroid hormones and bile acids might depend in part on the extent to which cholesterol was esterified with EFA (76). Thus the amount of cholesteryl arachidonate in the adrenals of rats on different diets correlated well with the serum level of corticosterone. In a large group of humans, the serum tetraenoic acid level also correlated well with the urinary 17-hydroxycorticosteroid excretion following ACTH stimulation. Since a decreased bile acid/cholesterol ratio in bile has long been associated with cholesterol stone formation, they reasoned that patients with cholelithiasis might be deficient in EFA, with consequent impairment of cholesterol catabolism. In support of this hypothesis were the observations that patients with gallstones had subnormal serum tetraenoic acid levels and ACTH responses. [These patients have also been found recently to have smaller than normal bile salt pools (38).] Hikasa therefore analyzed liver biopsy specimens from patients with cholesterol gallstones and found that they contained larger amounts of palmitoleic acid, oleic acid, and their eicosatrienoic derivatives and smaller amounts of arachidonic, docosanoic, and docosahexaenoic acids than liver biopsy specimens from other surgical patients. They also found that the total cholesterol content and the fraction esterified were increased but that the fraction esterified with EFA was decreased. These findings suggested a deficiency in the supply or utilization of EFA, which might result in a decrease in bile acid excretion and thus contribute to cholesterol stone formation.

To test this hypothesis, they raised rats, mice, and hamsters on diets deficient in EFA and produced changes in the composition and amounts of fatty acids, bile acids, and cholseterol in liver and bile similar to those found in humans with cholelithiasis. Furthermore, cholesterol gallstones occurred regularly in the mice and hamsters. Linoleic acid, however, which was depressed in the experimental animals, was normal in the human liver biopsy

specimens, so that a simple deficiency of EFA could not be invoked as an explanation for human cholesterol stones. Adding linoleic acid to the experimental diet, in the presence of a relative deficiency of pyridoxine, produced the fatty acid and bile acid changes characteristic of human cholelithiasis but did not increase the total and esterified cholesterol in the liver or decrease the lecithin in bile; these changes could be induced by adding lard to the EFA-rich, pyridoxine-poor diet. Under these conditions, cholesterol gallstones formed in a high percentage of mice and hamsters. When the results of seven different diets were correlated, a significant negative correlation was observed between the incidence of cholesterol stones and the ratio of EFA to saturated fatty acids plus oleic acid, suggesting that saturated and short-chain fatty acids enhanced and EFA diminished the propensity to stone formation. Hikasa postulated that a diet rich in saturated fat and protein (especially sulfur-containing amino acids) may bring about a relative deficiency of pyridoxine and permit cholesterol gallstone formation.

The experimental data from this laboratory were published in several papers. The changes in biliary constituents in rats fed EFA- and pyridoxine-deficient diets were described in detail by Yoshinaga (78). Maruyama (79) showed that the administration of EFA (soya lecithin) and pyridoxine increased the bile acid/cholesterol ratio in human hepatic bile. The fatty acid changes in patients with gallstones were described by Hirano (80). He also studied rats on EFA- and/or pyridoxine-deficient diets and found a significant negative correlation between the amount of cholesterol in the liver and the fraction esterified with EFA. The administration of ACTH to rats further decreased the content of cholesteryl arachidonate and bile acids. The starving of rats previously fed an EFA-deficient diet, however, resulted in an increase in liver cholesteryl arachidonate, presumably mobilized from storage depots, and in total esterified cholesterol. At the same time, biliary cholesterol increased while bile acids decreased (data not provided). Eguchi (81) found that arachidonic acid constituted 11% of the liver fatty acids in healthy men, 13% in normal hamsters, 8% in patients with gallstones, and 6% in hamsters on the lithogenic diet. The EFA fraction was lower in animals on a sucrose diet than in animals on a starch diet. In general, these workers felt that their results lent further support to the hypothesis that cholesterol esterified with EFA is the preferred substrate for bile acid formation.

Shioda (82) investigated the effect of dietary constituents on gallstone formation in golden hamsters. Like Dam, he found that hamsters on a low-fat diet frequently formed cholesterol gallstones. The addition of unsaturated fats (codliver oil, sesame oil) prevented cholesterol stones but allowed the development of amorphous pigment stones. Cholesterol stones correlated well with increased cholesterol and decreased bile acids and phospholipids in hepatic bile that was consequently supersaturated with respect to cholester-

ol (7). Lactose and starch were effective in preventing gallstones. Occasional animals showed "degeneration of liver cells, proliferation of interstitial tissues, infiltration of lymphocytes and leukocytes, and stagnation of bile." Tanimura (83) showed that sucrose, fructose, and sesame oil were partially effective and starch and codliver oil fully effective in preventing stone formation. Again, the described changes in biliary cholesterol, bile acids, and phospholipids correlated well with stone formation and with the dietary supply of EFA. The starch diet, however, produced the same EFA pattern as did glucose but prevented stone formation. Amorphous pigment stones that formed on a pyridoxine-deficient diet containing sucrose and fat were analyzed and found to contain abundant phosphorus and calcium, moderate amounts of bile salts, and very small amounts of biliverdine. Pyridoxal phosphate completely prevented cholesterol gallstones in the lard- and sesame oil–glucose diet groups (adequate linoleic acid content) but not in the coconut oil- and butter–glucose diet groups (inadequate linoleic acid content) Kotake (referred to in reference 77) has found that diets high in saturated fats and oleic acid inhibit the conversion of pyridoxine to pyridoxal phosphate, which might in turn inhibit the conversion of the decreased amounts of linoleic acid in these diets to polyunsaturated derivatives.

Hashimoto (84) found that vitamin K_1 (in contrast to vitamin K_2—see reference 85) completely prevented cholesterol gallstones in hamsters on a glucose, fat-free diet. Progesterone did not influence stone formation, but estrogen and thiouracil were partially protective. Cortisone was most effective in preventing cholesterol stones and favored amorphous pigment stone formation. Cholesterol (1%) added to the starch, 20% butterfat diet did not significantly increase cholesterol stone formation but markedly enhanced amorphous pigment stone formation; the addition of cholic acid (0.2%) largely counteracted this effect (see Section IVB).

Raw starch (β-starch) protected against gallstones, but when predigested with enzymes (83) or cooked (84) the starch (α-starch) became absorbable and the incidence of cholesterol stones was as great as in animals on the glucose, fat-free diet. The addition of neomycin to the α-starch, butterfat, 1% cholesterol diet protected completely against cholesterol stones but resulted in a 100% incidence of amorphous pigment stones (84).

Hikasa *et al.* (76) interpreted the results of these studies as indicating that cholesterol stone formation in hamsters is related to a relative deficiency in the supply of linoleic acid or its conversion to arachidonic acid and other unsaturated derivatives by pathways dependent on pyridoxal phosphate. Nonabsorbable carbohydrates were thought to favor growth of elements of the bacterial flora that produce vitamin B_6 and thus prevent stone formation. Diets high in saturated and short-chain fats, and low in linoleic acid, may also inhibit conversion of pyrisoxine to pyrisoxal phosphate and so favor stone

formation. Hikasa *et al.* (77) have suggested that the rate of conversion of cholesterol to bile acids may depend on the extent of cholesterol esterification with these unsaturated derivatives of linoleic acid, so that their availability may influence the relative amounts of bile acids and cholesterol in bile and hence the propensity of the bile to sustain cholesterol stone formation. Although this particular hypothesis requires further investigation and confirmation, various changes in biliary composition induced by diets and vitamins in studies by this group are entirely sufficient to account for the observed incidence of cholesterol gallstones when plotted on triangular coordinates according to Small (7).

B. Mice

During investigations on the effect of gold thioglucose on mice, Tepperman *et al.* (86) noted a high incidence of gallstones (averaging 94% cholesterol) in mice fed a diet containing 15% casein, 51% glucose, 31% lard, 1% cholesterol, and 0.5% cholic acid. Serum cholesterol levels were elevated, but omitting either cholic acid or cholesterol from the diet resulted in similar serum cholesterol levels without stone formation. The biliary cholesterol levels in stone-forming mice were markedly higher (550 mg %) than in mice on a chow diet (91 mg %), and the livers were grossly fatty; three of ten had primary hepatomas. Histological examination of the gallbladder wall showed some thickening and leukocytic infiltration, but no stones were observed forming on the mucosal surface. Pedreira and Tepperman (87) analyzed hepatic bile and found similar cholesterol concentrations in experimental and control animals; bile salts and phospholipids were not determined. Bile flow was greater in females than in males, and this could be reversed by treating the animals with hormones (estrone or testosterone) of the opposite sex. Caldwell *et al.* (88,89) analyzed the gallbladder bile in greater detail. The data indicated that the initial response to the lithogenic diet is an increase in all biliary constituents, but the cholesterol-holding capacity of bile is not exceeded. In time, however, the rise in cholesterol becomes disproportionate to the rise in bile salts and lecithin, and the concentration of bile salts actually returns toward normal. Histological examination again showed inflammation of the gallbladder within 2 weeks, and it was suggested that resorption of bile salts had occurred (see references 12 and 13). The influence of the gallbladder was studied further by Caldwell and Levitsky (90). Contrary to the data of Pedreira and Tepperman, hepatic bile flow was greater in males than females. More importantly, hepatic bile consistently maintained a bile acid/cholesterol ratio above the point at which cholesterol precipitates (see reference 7). In contrast, gallbladder bile was characterized by a drop in the bile acid/cholesterol ratio which coincided

temporally with the development of histological changes in the gallbladder. The hypothesis that abnormal resorption of bile acids by the gallbladder leads to an abnormal, lithogenic bile was further supported by observations that cholecystectomized animals did not form stones. Hamsters fed this lithogenic diet (91) had similar though less marked changes in biliary constituents, and no stones were observed; this is in keeping with the inherently greater cholesterol-holding capacity of hamster bile.

Bergman *et al.* (92) showed that feeding mice either starch or sucrose instead of glucose had no effect on this form of gallstone formation. Similarly, substituting deoxycholate for cholate or adding hyodeoxycholate (which protects hamsters against cholesterol gallstone formation) had no ameliorating effect (93). Omitting cholic acid from the diet permitted dissolution of the gallstones in some animals, but the addition of 3% cholestyramine to the diet did not affect stone dissolution (94). Besancon *et al.* (95–97) also noted that dehydrocholate served as effectively as cholate in the lithogenic diet and described the gallbladder as containing calculi, mucus, and desquamated epithelial cells. An eosinophilic inflammatory infiltrate of the gallbladder was found (see Section IVB2). Surprisingly, dehydrocholate without cholesterol also produced stones in a high percentage of animals. With dehydrocholate alone, fatty changes in the liver were not marked, whereas with cholesterol included, fatty infiltration was prominent. The stones were not prevented by adding 0.5% cholestyramine to the diet (97). Frey *et al.* have produced stones in germ-free mice (98) and have been unable to culture bacteria from the gallbladder and bile of conventional animals with stones (99), showing definitively that the gallbladder inflammation and gallstone formation are not related to biliary tract infection. Since neither cholate nor dehydrocholate has important cytotoxic properties (100), and since toxic secondary bile salts would not be expected in germ-free animals, it may be the increased bile flow that leads in some unknown way to inflammation and enhanced bile salt reabsorption in the gallbladder.

C. Rabbits

Borgman (101) noted that rabbits fed a diet containing 30% casein and 20% semipurified oleic acid developed gallstones. Increasing the casein to 40% gave greater numbers of stones, containing 12–25% cholesterol; linoleic acid and semipurified triolein did not affect the incidence of stones (102). When the effects of different proteins were investigated (103), casein resulted in large numbers, lactalbumin and torula yeast protein in moderate numbers, and egg albumin in small numbers of stones. Linoleic acid and oleic acid (20%) were comparable, but lowering the fatty acid content to 10% decreased stone formation. When 40% casein and 15% fat were used, olive oil

(oleic rich) resulted in large numbers of stones; palm oil, cocoa butter, safflower oil (linoleic rich), and combinations of semipurified palmitic acid and palm oil and semipurified oleic acid and cocoa butter resulted in moderate numbers of stones; and boiled linseed oil (linoleic rich) resulted in a small number of stones (104). Cornstarch partially inhibited stone formation, and removing olive oil from the diet or using a commercial diet permitted considerable stone dissolution (105). Furthermore, the breed of rabbit was found to influence the amount of gallstone formation (106).

Complete analyses of these stones have not been published. However, in view of the low cholesterol content reported in the stones, it is doubtful that these stones can be considered to be primarily cholesterol stones. The major constituents have not been identified, but rabbits form stones of various types readily, as shown by work on cholestanol-induced cholelithiasis and the early work on stasis and infection as etiological factors.

In experiments similar to those of Borgman, Bergman *et al.* (92) fed young rabbits diets containing 20% casein, 20% oleic acid, and 34% sucrose or starch. The nine animals fed sucrose all had gallstones, whereas only one of nine fed starch had gallstones. This diet was similar to that used by Borgman, but Bergman *et al.* were unable to identify more than minute amounts of cholesterol by X-ray diffraction, infrared absorption, or chromatographic techniques. They felt that the infrared spectra resembled that of the cholestanol-induced stones, subsequently shown (107) to contain allodeoxycholate, but the composition requires further investigation.

D. Guinea Pigs

Jones and Peric-Golia (108) have described a biliary precipitate composed of cholesterol and some pigment in guinea pigs 1–2 days after the intravenous injection of capsular polysaccharide from *Klebsiella pneumoniae*. No further work has been done with this experimental model.

Schoenfield and Sjövall (109) produced gallstones containing 50% cholesterol and 2% bile acids in guinea pigs fed a pelleted diet containing 15% protein, 3.6% fat, 42% carbohydrate, and 1% cholestyramine. The animals that formed stones had lower values for bile salts and lower bile acid/cholesterol ratios in hepatic bile than did control animals; the changes were attributed to depletion of the bile salt pool by cholestyramine. All of the animals on the pelleted diet, with or without cholestyramine, lost weight and had greatly increased cholesterol levels in hepatic bile. Animals on an unpelleted diet, which gained weight normally, did not form stones when fed cholestyramine. It is probable that the increased excretion of cholesterol in bile associated with starvation (110) acted in conjunction with diminished bile salt excretion due to cholestyramine to produce cholesterol precipitation.

Phospholipid excretion was not determined, and inflammation of the gallbladder did not occur.

E. Dogs

Cholesterol-containing stones, which may or may not be true cholesterol stones, since the cholesterol content has not been quantitated, have been produced by Englert (111) in dogs fed a diet high in carbohydrate (76%), low in protein (10%) and animal fat (5%), and containing cholesterol (1%) but not bile salts. The gallstones varied morphologically from microspheroliths and "structures with crystalline borders" to amorphous pigment conglomerations (112). Stones from four dogs were analyzed qualitatively, and all contained protein, cholesterol, and bilirubin. Carbonate was present in three, calcium in one, phosphorus in one, lecithin in three, free cholate in two, taurocholate in one, and taurochenodeoxycholate in two (112). Serum cholesterol was elevated (431 $\pm$ 56 mg % versus 243 $\pm$ 106 mg %, $p < 0.05$), as was biliary cholesterol (362 $\pm$ 127 mg % versus 93 $\pm$ 29 mg %, $p < 0.01$), in animals on the experimental diet. Serum taurine levels were reduced (0.28 mg % versus 2.34 mg %), and the proportion of taurine-conjugated bile salts in bile was decreased; unconjugated bile salts were also detected despite negative cultures.

Within a week of dietary treatment, Harman *et al.* (113) have observed the formation of apical cellular vesicles in the gallbladder mucosa, PAS-staining material on the surface of the mucosa and in the crypts, and the occurrence of 1–2 mm surface gel particles staining intensely with PAS, mucicarmine, and alcian blue (see also references 5 and 8). Bile hexosamine levels were also increased (169 $\pm$ 40 μg % versus 113 $\pm$ 22 μg %). These findings suggest that increased mucin formation and excretion are early results of the lithogenic diet. A similar conclusion has been from studies on mucin secretion in dihydrocholesterol-induced cholelithiasis in rabbits (114–116) (see Section IVB). It is not known whether this increased mucin secretion is common to all forms of cholelithiasis, what the mechanism is, or whether it is the same in both of these experimental models. In rabbits, it apparently depends on a factor excreted in bile, since it can be prevented by cystic duct ligation (116), but the factor or factors responsible have not been identified.

It is of interest that the diet used in dogs resembles that used by Dam and Christensen (46) to produce gallstones in hamsters in being high in carbohydrate and low in protein and fat. The low protein resulted in an increased glycine/taurine ratio of biliary bile salts in both dogs (113) and hamsters (61). Furthermore, addition of 1% cholesterol to the hamster diet decreased the incidence of cholesterol stones and increased the incidence of

amorphous pigment stones (68). These results tend to suggest that the gallstones produced in dogs may not be true cholesterol stones but may be more analogous to the amorphous pigment stones of hamsters. Further studies on the composition of these stones are necessary.

F. Miscellaneous

Patton *et al.* (117) have reported cholesterol deposits in ground squirrels and prairie dogs fed 50% egg yolk, 50% monkey ration diets for 12–15 months. Further details are not available.

IV. CALCIUM STONES

A. Carbonate and Bilirubinate Stones

Calcium has been recognized as a constituent of gallstones since the earliest chemical studies (see reference 118). It occurs as calcium carbonate (either calcite or aragonite), calcium phosphate (apatite), calcium bilirubinate, or the salts of bile acids and fatty acids. In normal hepatic bile, the calcium concentration is about 21 mg %. It is about 45 mg % in normal gallbladder bile and about twice that in the gallbladder bile of patients with cholesterol stones (119). The concentration of calcium is said to vary with the concentration of bile salts (3). Apparently, calcium in bile is bound in part to bile salt micelles, and under normal conditions this complex remains stable and soluble (120). However, under appropriate conditions of *p*H, calcium concentration, and anion concentration, calcium salts may precipitate.

Rous *et al.* (121–123) made some of the important early observations on the experimental production of calcium stones. In the course of experiments involving the collection of sterile bile from dogs, calcium precipitates were noted on the walls of the cannulas. Pigmented precipitates (calcium bilirubinate) generally occurred in the proximal portions of the cannulas and often served as nidi for the concentric layering of pigmented and nonpigmented (calcium carbonate) deposits. More distally, the stones consisted predominantly of calcium carbonate, although they usually formed around a small pigmented nidus. The calcium carbonate deposition was almost always associated with organic debris, whether deposited as relatively pure carbonate stones or around pre-existing pigment stones.

Hepatic injury resulted in the appearance of organic biliary debris, consisting of hepatic parenchymal cells, ductular cells, red cells, and leukocytes, and injections of hemoglobin caused a proteinaceous thickening of the bile. None of this debris, however, was ever observed acting as a nidus for calcium carbonate deposition. Calcium carbonate deposition seemed to be

associated with organic debris both in the dog experiments and in instances of human carbonate stones; it was therefore postulated that alterations occurred with time in the physicochemical state of the debris that favored deposition of calcium carbonate.

The *p*H of liver bile has been found to range from 7.07 to 8.55, ordinarily being around 8.20 (119,123). The *p*H of gallbladder bile was found to be alkaline when obtained shortly after a meal but declined with fasting to 5.18–6.00 (123). When liver bile that contained calcium carbonate precipitates was neutralized or made slightly acid, the deposits dissolved, either partly or completely. Furthermore, when Drury *et al.* (123) altered the *p*H of human bile samples *in vitro,* they noted that neutral or alkaline specimens formed moderate or large amounts of cholesterol precipitates, whereas acidification prevented these deposits. They therefore proposed that the lower *p*H of gallbladder bile was sufficient to prevent the precipitation of calcium carbonate and mentioned that the failure to acidify gallbladder bile in inflamed gallbladders might predispose to calcium carbonate and cholesterol concretions.

Phemister *et al.* (124) produced calcium carbonate stones in dogs and rabbits following cystic duct ligation. Chemical analyses showed the stones to contain calcium carbonate, 70.7%; calcium phosphate, 1.8%; and organic matter, 17.5%. They observed that the concentration of calcium and carbonate ions in the supernatant fluid exceeded the solubility product tenfold.

Bisgard and Baker (125) noted the formation of pigment stones in goats following obstruction of the common duct below the level of the pancreatic duct, thus permitting the reflux of pancreatic juice. Aronson (24) followed up these observations by actually making an anastamosis between the pancreatic duct and the gallbladder in dogs. In the one dog with an anastamosis that remained patent for 2 years, calcium (radio-opaque) stones developed; cholesterol and pigment were minor constituents (less than 1%) of the stones. Although these methods involved diverting alkaline pancreatic secretions into the gallbladder, with a presumed increase in *p*H, other factors may certainly have contributed to stone formation.

Hamanaka (126) produced gallstones in rabbits by altering calcium metabolism with daily injections of large amounts of vitamin D (0.5% irradiated ergosterol in olive oil). Stones formed in 29% of the controls receiving olive oil, but the frequency was much greater (64%) in the presence of hypervitaminosis D. The incidence of gallstones appeared to be related to the concentration of calcium in the serum and, particularly, gallbladder bile, as well as to the serum cholesterol level. The composition of the stones was not investigated, and it is not clear whether these represent calcium carbonate or cholesterol stones.

Okey (127) produced gallstones "rich in calcium phosphate, although

they contained some cholesterol" in guinea pigs on a 1% cholesterol diet supplemented with riboflavin. Both cholesterol and riboflavin were necessary for stone formation, which was preceded by an abrupt fall in the red cell count. The mechanism of this type of lithiasis is totally obscure. Riboflavin-containing reductases, such as glutathione reductase, might increase mitochondrial NADPH levels and hence inhibit the mitochondrial oxidation of the cholesterol side chain during blie acid synthesis. There is, however, no direct evidence that excessive amounts of riboflavin can inhibit the conversion of cholesterol to bile acids.

Simendinger (25) had observed clinically that patients with cholecystitis were frequently hypothyroid and therefore studied the influence of the thyroid gland on gallbladder function in dogs. Thyroidectomy led to poor or absent radiographic visualization of the gallbladder, and this could be corrected by thyroid administration. The hypothyroid dogs had normal bile salt/cholesterol ratios, but the gallbladders were distended with thick viscous bile containing large amounts of brown precipitate and a supernatant layer of clear yellow bile. The precipitates were composed of "pigment and polymerized pigment soaps," with traces of calcium and cholesterol.

Maki (128) has reported a number of important experiments from his laboratory dealing with the formation of calcium bilirubinate stones. Sato had produced a solid calcium bilirubinate stone by incubating an ascaris worm in the gallbladder of a dog for 10 months. Since bilirubin is excreted in bile as the glucuronide, hydrolysis of the conjugate presumably occurred in this model. Sato then demonstrated that *in vitro* incubation of bile from patients with calcium bilirubinate stones, but not from control patients, resulted in the further precipitation of calcium bilirubinate. These samples were almost invariably infected with *E. coli* and demonstrated β-glucuronidase activity. Precipitation of pigment was accompanied by a decrease in the bilirubin content and an increase in free glucuronic acid. The addition of β-glucuronidase to normal bile produced similar precipitates. Matsushiro identified glucuro-1,4-lactone in human bile and showed it to be a strong inhibitor of biliary β-glucuronidase; it prevented the precipitation of calcium bilirubinate as described above, either when fed *in vivo* or when added to bile *in vitro*. Maki and Susuki then showed that small precipitates of calcium bilirubinate could be made to coalesce by the addition of electrolytes, high molecular weight particles such as "Konanfloc," and motion. Coagulation was enhanced by lowering the *p*H or adding calcium or aluminum chloride.

B. Cholanate Stones

1. Cholestanol-Induced Gallstones

In 1954, Cook *et al.* (129) noted the occurrence of gallstones in rabbits

during cholestanol (dihydrocholesterol) balance studies. Similar observations were made by Mosbach and Bevans (130), who found that feeding 0.25–1.0 g cholestanol daily for 2 weeks or longer consistently resulted in the production of gallstones in male chinchilla-type rabbits. The stones were small and white, with small quantities of pigment, and frequently coalesced to form a cast of the gallbladder (131). Inflammatory lesions of the gallbladder and bile ducts frequently preceded stone formation, and in the early stages "portions of the concretions and bile clung tenaciously to the mucosal surface." Removing cholestanol from the diet led to regression of the cholecystitis and cholelithiasis in rabbits within 14–19 weeks (132).

These results were confirmed by Caira *et al.* (85,133), who found gallstones in New Zealand rabbits of both sexes fed 0.5 g cholestanol for 9 days or more. They observed that the newly formed stones were gelatinous in consistency and were accompanied by marked mucosal inflammatory changes (edema, round cell infiltration, and fibrosis). Choledocholithiasis developed readily in cholecystectomized animals, indicating that the liver bile itself was abnormal and that a gallbladder was not essential for stone formation. Similar concretions formed in two of 20 guinea pigs fed 0.25 g cholestanol daily, but none formed in rats fed 0.125 g daily.

Mosbach and Bevans (134) showed that cholestanol-induced cholecystitis and cholelithiasis could be inhibited by the simultaneous administration of dehydrocholic acid and that the extent of inhibition depended on the relative concentrations of the two steroids. Similar observations were made by Ricci *et al.* (135). Deoxycholic and cholic acids were also effective inhibitors (136), but hyodeoxycholic acid did not suppress gallstone formation and appeared to increase biliary tract inflammation. Several non–bile acid choleretics were without inhibitory effects (136). Lindelöf and van der Linden (32) found that intravenous injections of cholecystokinin every 8 hr did not suppress and may actually have enhanced gallstone formation. The inhibition of cholelithiasis by dehydrocholic, deoxycholic, and cholic acids was not accompanied by a decrease in cholestanol absorption but did result in increased tissue cholestanol levels, suggesting a decrease in the conversion of this sterol to bile acids (134,136). Conversely, methyl testosterone apparently inhibited stone formation by interfering with cholestanol absorption, since tissue and serum levels of cholestanol were reduced (137). Olive oil has been shown to facilitate stone formation (138), perhaps by enhancing cholestanol absorption (137).

Early studies on the composition of cholestanol-induced gallstones established that they did not contain appreciable amounts of sterol (cholesterol or cholestanol) or pigment but consisted largely of glycine-conjugated bile salts (130,133,139,140). Mosbach and Bevans (139), utilizing cholestanol-^{14}C, demonstrated that approximately half of the bile acids in the stones were

derived from cholestanol and were similar to but not identical with glycodeoxycholate and glycocholate. They suggested that these compounds might be 5α-cholanic (allocholanic) acids, in keeping with current concepts of the metabolism of cholestanol, and that these metabolites might be in part responsible for the biological effects of cholestanol administration. Hofmann and Mosbach (107) isolated the major component of the gallstones and conclusively identified it as glycoallodeoxycholate, with calcium and sodium ions associated in roughly equal quantities.

Studies on the physicochemical properties of synthetic glycoallodeoxycholate (107) revealed that at low concentrations (0.02 M) the sodium salt formed a clear micellar solution, capable of solubilizing azobenzene but that at higher concentrations it formed a liquid crystalline, viscoelastic gel. Dilute solutions (0.02 M) in the presence of excess sodium ions (0.15 M) also formed a birefringent, liquid crystalline phase. The calcium salt of glycoallodeoxycholate (5α), like that of glycodeoxycholate (5β), was highly insoluble in water; in the presence of sodium ions, however, the latter dissolved to form a clear micellar solution, whereas the calcium glycoallodeoxycholate remained insoluble.

These results led to the following hypothesis concerning cholestanol-induced gallstone formation: cholestanol, absorbed from the intestinal tract, is metabolized to glycoallocholate in the liver. Following its excretion in bile, it is dehydroxylated by intestinal bacteria to form (glyco)allodeoxycholate, reabsorbed, and re-excreted in the bile. Calcium ions can then precipitate glycoallodeoxycholate, whereas the normal isomer (glycodeoxycholate) and the parent compound (glycoallocholate) remain in solution (107). This hypothesis has been supported by experiments showing that the administration of neomycin decreases the concentration of glycoallodeoxycholate in bile and prevents gallstone formation (141,142). The decrease in glycoallodeoxycholate was accompanied by a reciprocal increase in glycoallocholate and was proportional to the dose of neomycin, leading to the conclusion that the primary effect of neomycin was to inhibit intestinal bacteria responsible for the dehydroxylation of glycoallocholate. Dehydroxylation of glycocholate to glycodeoxycholate was not similarly impaired, however, suggesting either a difference in the type or the neomycin sensitivity of the bacteria dehydroxylating 5α and 5β bile acids or, more likely, a greater affinity of the enzyme involved for 5β bile acids. Alternatively, the effect of neomycin could be unrelated to its antibiotic properties and related instead to its ability to sequester bile acids in the intestinal lumen (143–146), with selective sequestration of secondary and in particular allo bile acids. In either case, reduction in the concentration of glycoallodeoxycholate can protect against stone formation. When the concentration of glycoallodeoxycholate was greater than 24% of total bile salts, all animals had stones; when the concentration

was less than 19%, stones did not occur (142). Precipitation of glycoallodeoxycholate when its concentration reaches approximately 20% of the total bile salts appears to be a fundamental step in the etiology of cholestanol-induced gallstones.

Allo (5α) bile acids have also been implicated in the pathogenesis of the inflammatory lesions in the gallbladder wall, since these changes often precede the formation of gallstones (131,139). However, 5α-steroids are in general less cytotoxic than 5β-steroids (147), and inflammation is not an invariable concomitant of cholestanol feeding, even in the presence of gallstones (32,114,115,140,141,148). Hence the etiology of the lesions is still unclear, and their role in gallstone formation is uncertain. Although inflammation has been shown to facilitate absorption of conjugated bile salts (13), there were no data to indicate whether 5α and 5β bile salts might be absorbed preferentially and hence whether this absorption might be expected to enhance or inhibit this form of experimental cholelithiasis.

While the initial step in cholestanol-induced gallstones probably consists of the precipitation of small bile salt crystals and microliths, attention has been directed recently to the mechanism by which these small stones grow and coalesce. In a comprehensive review of the subject, Freston (114) has called attention to the importance of mucus secretion in stone formation. Bevans and Mosbach (131) had noted small crystals embedded in mucus that was adherent to the gallbladder wall; Fortner (69) and Amberg (148) had made similar observations. Freston (114) extended these studies in rabbits fed 0.75% cholestanol and described the way small spherules became matted together, forming larger stones. Light and electron microscopic studies indicated that small vesicles developed in and between gallbladder epithelial cells in response to the cholestanol diet. The vesicles appeared to enlarge by merging with adjacent vesicles and could be shown by light microscopy to contain PAS-positive material. The large vesicles, which were not associated with inflammatory changes, preceded stone formation; they readily identified after 3 days of treatment, whereas no stones developed before 7 days. Synthesis of mucus could be demonstrated in the gallbladder wall, and its secretion into gallbladder bile was increased in respone to the lithogenic diet. The hexosamine content of both the gallbladder wall and the bile was increased with vesicle formation and, eventually, with gallstone formation, suggesting that the vesicles were indeed mucus-containing secretory vesicles. The secretion of mucus appeared to be dependent on a factor in bile, since it did not occur when the cystic duct was ligated prior to cholestanol feeding. Mucus was incorporated in the gallstones, as evidenced by their hexosamine content. The results strongly suggest that abnormal mucus secretion plays an important role in trapping small biliary concretions and promoting their maturation into fully developed gallstones. It is not clear whether this effect

is simply one of mechanical retention or whether the mucus may actually facilitate the crystallization and growth of stones, as suggested by Kleeberg (9,10) and Womack (5).

2. Lithocholate-Induced Gallstones

Holsti (149) was the first to show that the oral administration of lithocholic acid to rabbits results in extensive bile duct proliferation. This was confirmed by Stolk (150) in *Iguana,* and by Leveille *et al.* (151) and Eyssen and De Somer (152) in chicks. The latter noted that the gallbladders "were filled with a viscous yellow-green fluid contrasting sharply with the clear dark-green bile of the control animals." Attempts to produce liver changes in rats by feeding lithocholic acid then led to the serendipitous production of gallstones in the common duct by three groups independently.

Eyssen and VandePutte, in unpublished experiments, reported (International Conference on Biochemistry of Lipids, Stockholm, August 5–7, 1963) that albino male rats fed a low protein diet containing 0.2% lithocholate developed typical lithocholate-induced liver lesions by 2 weeks, which became more pronounced by 4–8 weeks. The cardinal features were a striking proliferation of intrahepatic bile ducts and ductular cells; an inflammatory portal infiltrate consisting of polymorphonuclear leukocytes, round cells, and connective tissue; and a pronounced mucosal hyperplasis and enlargement of the extrahepatic bile ducts. Mucinous material and cellular debris were found in the dilated ducts, and focal areas of hepatic necrosis were observed. By 8 weeks, small (0.5–3.0 mm) yellow-green gallstones began to appear, being a consistent finding after 3–5 months. Rats fed a "normal" purified diet (25% protein) containing 0.2% lithocholic acid did not show any significant changes.

Palmer (153,154) reported that Sprague-Dawley rats of both sexes consistently developed gallstones after 4 months on an 8% protein diet containing 1% lithocholic acid. There was considerable variation in the morphology of the stones; some were small spherules, some large and faceted, and some compacted to form a solid cast of the dilated common duct. The color varied from pale yellow-green to brown or greenish black, presumably depending on the extent of bacterial degradation of biliary pigments. The stones contained only trace amounts of pigment and cholesterol and consisted primarily of the calcium salts of free and glycine-conjugated lithocholic acid and its 6β-hydroxy derivative. The extreme variations in the ratio of free/conjugated bile salts suggested that the free bile salts were also predominantly the result of bacterial deconjugation.

Stone formation appeared to depend on a deficiency of sulfur-containing amino acids. Increasing the protein content of the diet to 27% or adding 1% taurine to the diet increased urinary taurine excretion, increased con-

jugation of bile salts with taurine, and prevented gallstone formation. Histologically, the liver lesions were similar to those reported previsouly in rabbits and other species. The hepatocytes were generally intact and showed only mild hyperplasia of the Golgi complex and an increase in the smooth endoplasmic reticulum; the bile canaliculi were not distended. In the bile ducts, degenerating epithelial cells were observed sloughing off into the lumen, and eosinophilic material (cellular debris?) was often seen surrounding basophilic crystalline material (bile salts?) to form microcalculi. The histological changes were not diminished by the taurine- or protein-supplemented diets which prevented stone formation.

Zaki *et al.* (155) reported similar findings in Holtzmann male rats fed 75 mg lithocholic acid in 10 g of a low (12%) protein diet daily. At 9 weeks, the common ducts contained semisolid concretions, which matured to solid, sometimes faceted stones at 15 weeks. When lithocholic acid was removed from the diet, the semisolid concretions disappeared within 5 weeks. In contrast, the solid stones persisted for as long as 15 weeks after discontinuing lithocholic acid. The main bile acids in hydrolyzed gallstone extracts were 6β-hydroxylithocholic acid and lithocholic acid, with smaller amounts of β-muricholic and chenodeoxycholic acids. Analysis of serum bile acids in these rats showed highly variable increases with 3α,6β-dihydroxy-5β-cholanic acid predominating. Histological changes were similar to those described above, except that midzonal and peripheral areas of hydropic degeneration were more prominent. Removing lithocholic acid from the diet also caused regression of the liver lesions, as described by Holsti (149), in rabbits, with complete return to normal in 5 weeks. Rats fed the same amount of lithocholate in laboratory chow did not develop gallstones, and the liver histology was normal.

The histological changes induced by lithocholic acid do not appear to be immutably related to the production of gallstones, although ductural cell desquamation and degeneration, or mucus secretion by common duct epithelium, may facilitate the retention and maturation of small gallstones. On the contrary, the liver changes seem to be the result of an independent pharmacological property of lithocholic acid. Stone formation can be abolished by adding protein or taurine to the diet, by simultaneously administering cholic acid or cholesterol, or by feeding lithocholic acid esterified with sulfuric acid; in all cases, bile duct proliferation remains a prominent feature (156). The lesions resemble in part those produced by bile duct ligation (157) or vitamin A deficient diets (157–159) (see Section V). Biliary stasis, however, could not have been a factor during simultaneous feeding of cholic acid and cholesterol, and vitamin A supplementation did not inhibit either stone formation or bile duct proliferation (154).

The definitive step in lithocholate-induced gallstone formation thus

appears to be precipitation of calcium salts of lithocholic acid and various derivatives. Free and glycine-conjugated dihydroxy bile acids (e.g., 3α,6β-dihydroxy-5β-cholanic acid) readily precipitate from aqueous solutions as the calcium salts, but the exact solubilities of these salts have not been studied. In contrast, even the sodium and potassium salts of monohydroxy bile acids are relatively insoluble. Small and Admirand have studied several lithocholates in some detail (160). Contrary to expectations, conjugation of lithocholate with glycine or taurine actually decreases its solubility (taurine to a greater degree than glycine), so that the solubility of these compounds is inversely related to their polarity. The only physiological derivatives of lithocholate with appreciable solubilities are the recently described sulfate esters (161), which are probably not quantitatively important metabolites in rats (156).

The associated counter-ion is also important in determining bile salt solubility (160). For each lithocholate (free or conjugated with glycine or taurine), the potassium salt is more soluble than the sodium salt, and the solubility of each decreases with increasing counter-ion concentration. The solubility of each lithocholate is enhanced in the presence of micelle-forming bile salts, presumably through the formation of mixed micelles, and in such solutions the same effects of different counter-ions and their concentration can still be demonstrated. Small and Admirand have postulated that lithocholates absorbed from the intestine may be solubilized in the portal blood by proteins and in the hepatocyte as the potassium salt; their transfer to the canalicular fluid then might result in precipitation of the less soluble sodium salt, with consequent cholestasis. [Cholestasis has been demonstrated by Javitt (162,163) during infusions of lithocholates.] However, the relative proportions of cations in lithocholate-induced gallstones, expressed as meq/g dry weight, are calcium, 1.6; sodium, 0.32; potassium, 0.02 (154)—suggesting that precipitated sodium lithocholates, if present, may be redissolved in mixed micellar solutions and then precipitated as the calcium salt.

The relative absence of taurolithocholate in these stones is difficult to explain, since it is less soluble than either lithocholate or glycolithocholate. It may be that the calcium salts have anomalous solubility properties, but it is possible that binding of bile salts to protein (or to cellular debris), which varies inversely with the polarity of the bile salt (164), might be of greater importance in stone formation than pure solubility factors. Such binding might explain the relatively greater incorporation of the less polar free and glycine-conjugated bile salts and greater exclusion of the more polar, though less soluble, taurine conjugates. Furthermore, such binding might greatly facilitate the retention and growth of stones, as suggested by morphological studies (see above) which appear to show cellular debris surrounding aggre-

gates of precipitated bile salts in the larger bile ducts (154). Similar considerations might apply to cholestanol-induced gallstones, since the protein-binding of 5α-steroids is greater than that of 5β-steroids (165,166).

3. Amorphous Pigment Stones

In the course of studies on cholesterol gallstone formation in hamsters on fat free, 74.3% sucrose diets (see Section IIIA), Dam and Christensen (58,65) noted that certain dietary manipulations reduced the cholesterol content of the stones and resulted in the appearance of amorphous pigment stones containing 46% ash, 20% glycine-conjugated bile acid (presumably mainly dihydroxy but also some trihydroxy), and 12% calcium (61,66). Adding 2% fat to the diet (lard, soybean oil, and codliver oil, in order of increasing unsaturation) decreased the total number of animals with stones from 79.5% to 61, 18, and 20.5%, respectively. At the same time, the number of animals with amorphous pigment stones increased from 0% to 2.5, 10.3, and 20.5% (58,65). Similarly, decreasing the amount of sucrose or glucose in the diet eliminated cholesterol gallstones but not amorphous pigment stones (60). The stones were twice as common in females as in males, though the incidence of cholesterol gallstones was similar in both sexes (60,65), and older animals formed more amorphous stones (61). Gonadectomy, treatment with desiccated thyroid, or treatment of females with progesterone decreased the incidence of cholesterol stones and increased the incidence of amorphous stones; testosterone treatment of males and estradiol treatment of females did not affect gallstone formation (66). Cholesterol feeding increased the incidence of pigment stones, though only in females, as did margarine with a high linoleic content; both together increased the incidence in females from 24% to 85% (68). The glycine/taurine ratio of bile salts in bile appeared to be greater in animals with amorphous stones (61).

These results raise the question of whether these amorphous stones containing calcium bile salts could be related to the pathogenesis of the cholesterol stones. Glycine-conjugated bile salts, particularly of the less soluble compounds such as chenodeoxycholate and lithocholate, have been found in greater proportions in the centers of human gallstones (167), and it would be of interest to know whether this is the case in the cholesterol stones that frequently coexisted in these animals.

The increased glycine/taurine ratio in bile from hamsters with amorphous pigment stones is not unexpected, considering the composition of the stones, but the reason for this change is unclear. The increase ratio results from a relative deficiency of taurine and could be produced by a decreased protein intake, which supplies the sulfur-containing amino acids used for taurine synthesis, or by an excessive fecal loss of taurine conjugates, as in

patients with ileal bypass (168) or in lithocholate-induced cholelithiasis (see Section IVB2). In the last example, the increased glycine/taurine ratio is intimately related to the pathogenesis of the gallstone formation. More complete information on bile acid metabolism and the nature of the bile salts and other constituents in these amorphous pigment stones could be of considerable importance.

V. VITAMIN A DEFICIENCY

Fujimaka (158), in the course of experiments on vitamin-deficient diets, noted that rats kept for 22–37 weeks on a vitamin A deficient diet developed urinary calculi and, later, gallstones in the common duct. When calcium and phosphorus in the diet were also restricted, gallstones formed in 6–8 weeks. The gallstones were said to contain calcium salts, cholesterol, and pigment; the methods used for these determinations were not described.

Usuki (169) raised rabbits on a diet deficient in fat-soluble vitamins and found a 9% incidence of gallstones after 6–8 weeks. Qualitative analysis revealed that these small sandlike stones contained calcium, bilirubin, and traces of cholesterol.

Erspamer (170) quotes several other investigators as having noted gallstones produced by vitamin A deficient diets in rats (de Langer, 1929), guinea pigs (Emiliani and Bazzochi, 1933), and dogs (Galli and Bazzochi, 1934). Erspamer made detailed observations on gallstones produced by a vitamin A deficient diet in guinea pigs. Stones developed as early as 5–11 days and appeared to consist of soft masses of degenerated and desquamated epithelial cells and mucus, colored by small amounts of pigment. The stones were soft, did not stain for lipid, and did not exhibit birefringence under polarized light. Cholesterol (Liebermann–Burchardt method) was not detected. Histologically, the gallbladders and intra- and extrahepatic bile ducts showed desquamating cells, whether or not stones were present. The gallbladder wall was thickened and inflamed, and vascular thromboses were observed. Keratinization of the gallbladder epithelium was not observed, and apocrine secretion was prominent in the gallbladder and ducts. Fatty droplets (probably triglycerides) were common in the basal areas of biliary tract epithelial cells and liver cells.

Fortner (69) showed that vitamin A deficiency, and, to a lesser extent, vitamin D deficiency, increased the incidence of cholesterol gallstone formation in the hamster, using the glucose, fat-free diet described by Dam and Christensen (see Section IIIA).

Gillman *et al.* (157), in a study of factors affecting intrahepatic bile ducts,

found that vitamin A deficiency in rats resulted in the production of marked intrahepatic bile duct proliferation, extrahepatic bile duct dilation, and gallstones that were "generally soft and crumble easily under pressure. Many of the calculi consist of organic material together with a variable amount of calcium phosphate heavily impregnated with bile pigment." They noted that bile was invariably present in the intestine, so that complete obstruction of the common duct did not occur. Furthermore, the degree of intrahepatic bile duct proliferation, which was often extensive, did not correlate with the extent of common duct dilation, indicating that they hyperplasia was not a consequence of obstruction.

Thiourea (0.5% in the diet) produced mild duct proliferation, but the ducts in the portal tracts developed cystic changes and a hyperplastic lining of epithelial cells, which desquamated into the lumen and degenerated to form debris. There was no enlargement of the extrahepatic ducts, and no calculi were observed. However, when 0.5% thiourea was added to the Coward's (vitamin A deficient) diet, the intrahepatic bile duct hyperplasia seen with Coward's diet alone was almost completely suppressed, and enlargement of the common duct did not occur. Thyroidectomized rats fed Coward's diet also had less extrahepatic duct dilation. In contrast, parathormone, 10 units daily, administered after 39 days of Coward's diet, appeared to increase the extent of bile duct proliferation and the incidence of calculi (two of five). Cholic acid (0.5%) added to Coward's diet resulted in a marked enlargement of the common duct, but bile duct proliferation was not striking and calculi were not mentioned.

In 1956, Hamre (159) reported a detailed study of the liver and biliary tract changes associated with vitamin A deficiency in rats. The animals were fed the diet for approximately 5 weeks. At autopsy, 86% of the rats showed pathological changes. The proximal two-thirds of the common ducts as dilated, and the bile contained masses of epithelial cells. Calculus-like bodies, either hard and chalky white or fragile and dull, were found in the dilated common ducts and consisted predominantly of masses of compacted epithelial cells. Crystals of inorganic salts, cholesterol, bile salts, and others were identified by microscopic examination of crushed stones. All calculi contained both acid-soluble and ether-soluble material.

The epithelium of the common duct was stratified (five to ten layers of cells), with cells becoming rounded or cuboidal. As the process increased in severity, polyploid masses of cells could be seen projecting into the lumen, where they often could be found lying free. Numerous glandlike evaginations occurred in the wall of the common duct and were lined by columnar cells, except for areas in contact with the lumen at the neck and mouth of the evagination, where stratification sometimes was seen. The thickening of the

epithelium in the intraduodenal portion of the duct was sufficient to reduce the cross-sectional area of the lumen considerably. The connective tissue of the duct was not greatly increased, but there were large numbers of polymorphonuclear leukocytes and eosinophils.

Similar changes were seen in the larger hepatic ducts, which were dilated and contained masses of desquamated cells. The medium and large duct epithelium was usually columnar but occasionally stratified. The portal fields were only slightly enlarged but contained greatly dilated lymphatics. Inflammation was not prominent, and bile duct proliferation was not typical. In some animals, however, numerous masses of multiplying ducts filled the portal fields and extended to adjacent portal fields, accompanied by some inflammation and degeneration of peripheral hepatocytes. Changes of complete biliary obstruction, i.e., well-developed cirrhosis, did not occur.

Vitamin A deficiency characteristically results in hypoplasia of mucus-secreting tissues and "keratinizing metaplasia" of epithelial surfaces (171). The inhibition of mucus secretion appears to be related to a defect in mucopolysaccharide synthesis which can be attributed to a diminished incorporation of sulfate into 3′-phosphoadenosine-5′-phosphosulfate (active sulfate) (172). The impaired synthesis of active sulfate can be demonstrated in cell-free colon preparations from vitamin A deficient rats and can be restored to normals by a metabolite of vitamin A.

The keratinization of epithelial structures has long been recognized as a feature of vitamin A deficiency, but the biochemical and ultrastructural details of this lesion are poorly understood. Keratinization implies accumulation of keratin, an insoluble protein rich in cystine sulfhydryl linkages. The incorporation of methionine-^{35}S into the skin of vitamin A deficient rats is markedly increased (173), and the incorporation of inorganic $^{35}SO_4$, presumably into mucopolysaccharides, is decreased. There is also evidence for an increased conversion of cystine into inorganic sulfate (174), and the total cystine content of skin is depressed (175).

The defect in mucopolysaccharide synthesis together with the increased incorporation of methionine sulfur and the decreased cystine content may reflect an increased epithelial cell turnover, as suggested by the hyperplastic mucosa and the masses of epithelial cells in the biliary tract, but the picture is not yet complete. In any case, it is clear that desquamated cells appear to be the most important factor in this form of cholelithiasis. The contribution of cellular debris to other types of gallstone formation has been discussed in previous sections.

Another consequence of vitamin A deficiency which may influence gallstone formation is the reduced conversion of squalene to cholesterol, with subsequent diversion of mevalonate to ubiquinone synthesis (176). This effect,

which could in part explain the increased oxygen consumption seen in vitamin A deficiency (177), would be expected to substantially reduce the formation of bile salts from cholesterol, with a consequent reduction in the bile salt pool. This would in turn reduce the flow bile of with obvious effects on the kinetics of gallstone formation. There is, however, no direct evidence bearing on this possibility.

There are some remarkable similarities between cholelithiasis induced by vitamin A deficiency and that by lithocholate administration. In both, bile duct and ductular cell proliferation are striking, and the common duct extremely hyperplastic. Desquamating cells are prominent, and cellular debris is important in the structure of the calculi. Both conditions are characterized by an unusual infiltration of polymorphonuclear and eosinophilic leukocytes. In addition, cholestasis, which may be present in vitamin A deficiency, is a well-recognized pharmacological effect of lithocholate administration (162, 163). Vitamin A deficiency does not result from lithocholate administration, and the pathological changes produced by lithocholate cannot be reversed by vitamin A (154). Similarly, preliminary data indicate that there is also no major change in the pattern of bile acid metabolism in vitamin A deficiency (156). Further work will be necessary to determine whether any of the changes found in these conditions have a common pathogenesis.

VI. CONCLUSIONS

It is clear that research in experimental cholelithiasis is at a point where the various factors influencing gallstone formation can be separated and investigated. As pointed out by Small (7), they fall into two main categories: (a) factors resulting in the formation of an abnormal (lithogenic) hepatic bile and (b) factors resulting in the alterations of normal bile in the extrahepatic biliary system. It seems safe to predict that most of the factors regulating cholesterol solubility have or are being delineated (see reference 178), and a number of investigations are now being directed toward the physiological processes that govern the excretion of cholesterol, phospholipids, and bile salts in bile. Just as important, however, are the factors governing the absorption of substances (particularly bile salts) from the gallbladder. The studies of Ostrow (12,13) have emphasized the importance of inflammatory changes in the gallbladder wall in permitting diffusion of ionized bile salts and of alterations in the chemical nature of bile salts, e.g., bacterial deconjugation, in permitting nonionic diffusion of these compounds as un-ionized acids across the normal gallbladder mucosa. These two lines of investigation should make it possible to accurately identify bile that is potentially lithogenic with

respect to cholesterol stone formation, whether by virtue of the production of abnormal hepatic bile or by subsequent alterations in the composition of normal bile. In addition, the investigations may be expected to result in practical methods for altering the composition of bile in a direction calculated to prevent cholesterol lithiasis or cause the dissolution of pre-existing stones.

Much less is known about the formation of pigment stones. Good qualitative analyses are lacking, and animal models have not, as yet, yielded a great deal of useful information. The role of biliary cations, particularly calcium, has not been studied sufficiently in relation to the known anions, such as bilirubin, bile acids, and fatty acids, to provide much understanding about the formation of these insoluble salts and their association to form stones. The work on β-glucuronidase activity (128) is exciting and appears to provide a promising approach to the etiology of pigment stones. If animal models are to be helpful in these investigations, as they should be, more comprehensive qualitative and quantitative analyses of experimental pigment stones will be necessary to define clearly the particular biliary constituents involved in the early stages of stone formation.

The role of mucus in the pathogenesis of gallstones is under active investigation. The results of these studies should clarify how gallstones are organized and the function of the organic matrix. A better understanding of how mucus affects midus retention, together with more details on factors affecting bile flow and gallbladder contractility, should help to evaluate the role of stasis in gallstone formation and may lead to clinically useful methods of therapy. Several of the experimental models described above may prove helpful in these further investigations.

The studies reported here on experimental cholelithiasis have reemphasized the fact that bile salts deserve particular attention in considering the pathogenesis of gallstones. They appear to constitute the most important variable determining the solubility of cholesterol in bile, and factors influencing their concentration in bile are certainly germane to the problem of cholelithiasis. These include various aspects of bile acid metabolism which affect the excretion of bile salts in bile and their reabsorption from the biliary tract. In addition, studies on experimentally induced cholelithiasis have demonstrated that certain bile salts are quite insoluble and may participate, with other organic anions, in forming mixed stones, pigment stones, and the centers, or nidi, of cholesterol stones, Finally, bile salts possess pharmacological properties that may affect mucus production and bile flow. These properties, like other pharmacological properties of bile salts, are probably related to specific structural configurations, so that more information about the specific effects of individual bile salts will be necessary to evaluate their importance in the pathogenesis of cholelithiasis.

REFERENCES

1. Editorial, *New Engl. J. Med.* **277,** 932 (1967).
2. F. J. Ingelfinger, *Gastroenterology* **55,** 102 (1968).
3. A. J. H. Rains, "Gallstones, Causes and Treatment," C. C Thomas, Springfield, Ill. (1964).
4. I. S. Russell, *Brit. J. Surg.* **55,** 161 (1968).
5. N. A. Womack, R. Zeppa, and G. L. Irvin, III, *Ann. Surg.* **157,** 670 (1963).
6. I. A. D. Bouchier and J. W. Freston, *Lancet* **1,** 340 (1968).
7. D. M. Small, *New Engl. J. Med.* **279,** 588 (1968).
8. O. Hultén, *Acta Chir. Scand.* **134,** 125 (1968).
9. J. Kleeberg, *Gastroenterologia* **80,** 313 (1953).
10. J. Kleeberg, *Gastroenterologia* **85,** 271 (1956).
11. F. Nakayama and H. Miyake, *J. Lab. Clin. Med.* **67,** 78 (1966).
12. J. D. Ostrow, *J. Clin. Invest.* **46,** 2035 (1967).
13 J. D. Ostrow, *J. Lab. Clin. Med.* **72,** 999 (1968); **74,** 482 (1969).
14. P. Fitz-James and A. C. Burton, *Can. J. Res.* **27,** 309 (1949).
15. C. A. Hospers, *Arch. Pathol.* **14,** 66 (1932).
16. H. Miyake, *Mitt. Grenzgeb. Med. Chir.* **6,** 479 (1900).
17. T. Aoyama, *Deutsch. Z. Chir.* **132,** 234 (1914).
18. H. Iwanaga, *Zentralbl. Allg. Pathol. Anat.* **33,** 191 (1922).
19. S. Hansen, *Acta Chir. Scand.* **62,** 483 (1927).
20. L. R. Whitaker, *J. A. M. A.* **88,** 1542 (1927).
21. K. Westphal and F. Gleichmann, *Z. Klin. Med.* **115,** 329 (1931).
22. J. H. Cascao de Anciaes, *Compt. Rend. Soc. Biol.* **113,** 740 (1933).
23. H. G. Aronson, *Arch. Surg.* **41,** 960 (1940).
24. H. G. Aronson, *Arch. Pathol.* **34,** 843 (1942).
25. E. A. Simendinger, *Surg. Gynec. Obstet.* **79,** 10 (1944).
26. K. Imamoglu, J. F. Perry, Jr., and O. H. Wangensteen, *Surgery* **42,** 623 (1957).
27. K. Imamoglu, E. G. Yonehiro, J. F. Perry, Jr., and O. H. Wangensteen, *Surg. Forum* **8,** 225 (1957).
28. D. M. Nicoloff, W. O. Griffen, Jr., S. L. Wangensteen, K. Imamoglu, and O. H. Wangensteen, *Fed. Proc.* **20,** 248 (1961).
29. K. Imamoglu, J. F. Perry, Jr., H. D. Root, N. W. Crisp, C. B. Jenson, and O. H. Wangensteen, *Surg. Forum* **9,** 521 (1958).
30. K. Imamoglu, S. L. Wangensteen, H. D. Root, P. A. Salmon, W. O. Griffen, Jr., and O. H. Wangensteen, *Surg. Forum* **10,** 246 (1960).
31. G. Ricci, U. Bani, G. Bani, F. Occhionero, F. Soccorsi, and G. C. Urbinati, *Epatologia* **10,** 453 (1964).
32. G. Lindelöf and W. van der Linden, *Acta Chir. Scand.* **130,** 494 (1965).
33. W. Taylor, ed., "The Biliary System," p. 455, F. A. Davis Co., Philadelphia (1965).
34. S. Bergström, H. Danielsson, and B. Samuelsson, *in* "Lipid Metabolism" (K. Bloch, ed.), p. 323, Wiley, New York (1960).
35. O. Strand, *Proc. Soc. Exptl. Biol. Med.* **109,** 668 (1962).
36. E. Thureborn, *Acta Chir. Scand. Suppl.* **126,** 303 (1962).
37. R. Abourre, S. G. Gordon, J. G. Mann, and F. Kern, Jr., *J. Lab. Clin. Med.* **72,** 849 (1968).
38. Z. R. Vlahcevic, I. Buhac, C. C. Bell, Jr., J. T. Farrar, and L. Swell, *Gastroenterology* **56,** 1204 (1969).

39. M. J. Kreek, R. E. Peterson, M. H. Sleisinger, and G. H. Jeffries, *J. Clin. Invest.* **46,** 1080 (1967).
40. N. B. Javitt and M. Harkavy, *Gastroenterology* **56,** 400 (1969).
41. M. J. Kreek, R. E. Peterson, M. H. Sleisinger, and G. H. Jeffries, *Proc. Soc. Exptl. Biol. Med.* **113,** 646 (1969).
42. R. H. Palmer, T. F. Gallagher, Jr., M. N. Mueller, and A. Kappas, *in* "Metabolic Effects of Gonadal Hormones and Contraceptive Steroids," Plenum Press, New York (1969).
43. E. L. Forker, *Gastroenterology* **56,** 427 (1969).
44. J. Tepperman, *Gastroenterology* **48,** 261 (1965).
45. H. Dam, *in* "Proceedings of the Sixth International Congress of Nutrition in Edinburgh" (August 9–15, 1963), p. 6, E. S. Livingston Co., Edinburgh (1964).
46. H. Dam and F. Christensen, *Acta Pathol. Microbiol. Scand.* **30,** 236 (1952).
47. F. Christensen, H. Dam, and I. Prange, *Acta Physiol. Scand.* **27,** 315 (1953).
48. H. Dam, I. Prange, and F. Christensen, *Z. Ernährungswiss.* **6,** 97 (1965).
49. H. Muroya, *Arch. Jap. Chir.* **37,** 76 (1968).
50. H. Muroya, R. Suzue, and Y. Hikasa, *Arch. Biochem. Biophys.* **124,** 12 (1968).
51. F. Christensen and H. Dam, *Acta Physiol. Scand.* **31,** 75 (1954).
52. H. Dam and F. Christensen, *Z. Ernährungswiss.* **2,** 154 (1962).
53. A. Snog-Kjaer, I. Prange, F. Christensen, and H. Dam, *Z. Ernährungswiss.* **4,** 14 (1963).
54. H. K. Hanel, F. Christensen, and H. Dam, *Acta Pathol. Microbiol. Scand.* **35,** 237 (1954).
55. H. K. Hanel, F. Christensen, and H. Dam, *Acta Pathol. Microbiol. Scand.* **35,** 423 (1954).
56. B. Jensen and H. Dam, *Biochim. Biophys. Acta* **125,** 367 (1966).
57. F. Christensen, H. Dam, and G. Kristensen, *Acta Physiol. Scand.* **36,** 329 (1956).
58. H. Dam and F. Christensen, *Z. Ernährungswiss.* **2,** 36 (1961).
59. I. F. Prange, F. Christensen, and H. Dam, Abstracts of Communications, Fifth International Congress of Biochemistry (Moscow, 1961), p. 297 (quoted in reference 61).
60. H. Dam and F. Christensen, *Z. Ernährungswiss.* **2,** 223 (1961).
61. I. Prange, F. Christensen, and H. Dam, *Z. Ernährungswiss.* **3,** 59 (1962).
62. I. Prange, F. Christensen, and H. Dam, *Z. Ernährungswiss.* **4,** 193 (1964).
63. I. Prange, F. Christensen, and H. Dam, *Z. Ernährungswiss.* **7,** 59 (1966).
64. F. Christensen, I. Prange, and H. Dam, *Z. Ernährungswiss.* **3,** 117 (1962).
65. F. Christensen, I. Prange, and H. Dam, *Z. Ernährungswiss.* **4,** 186 (1964).
66. H. Dam and F. Christensen, *Z. Ernährungswiss.* **5,** 149 (1965).
67. H. Dam, I. Kruse, H. E. Kellehauge, O. E. Hartkopp, and M. Krogh Jensen, *Scand. J. Clin. Lab. Invest.* **18,** 385 (1966).
68. H. Dam, I. Prange, and E. Sondergaard, *Z. Ernährungswiss.* **9,** 43 (1968).
69. J. G. Fortner, *Surgery* **36,** 932 (1954).
70. W. van der Linden, F. Christensen, and H. Dam, *Acta Chir. Scand.* **118,** 113 (1959).
71. J. Drews, *Deutsch. Arch. Klin. Med.* **208,** 593 (1963).
72. F. Bergman and W. van der Linden, *Acat Chir. Scand.* **129,** 547 (1964).
73. F. Bergman and W. van der Linden, *Acta Chir. Scand.* **131,** 319 (1966).
74. F. Bergman and W. van der Linden, *Acta Chir. Scand.* **132,** 724 (1966).
75. Y. Hikasa, I. Maruyama, M. Yoshinaga, M. Hirano, T. Eguchi, R. Shioda, H. Tanimura, and K. Hashimoto, *Arch. Jap. Chir.* **33,** 601 (1964).
76. Y. Hikasa, T. Kuyama, I. Maruyama, M. Yoshinaga, M. Hirano, T. Eguchi, R. Shioda, H. Tanimura, K. Hashimoto, M. Muroya, and M. Togo, *Arch. Jap. Chir.*

34, 1430 (1965).
77. Y. Hikasa, S. Matsuda, M. Nagase, M. Yoshinaga, T. Tobe, I. Maruyama, R. Shioda, H. Tanimura, R. Muraoka, H. Muroya, and M. Togo, *Arch. Jap. Chir.* **38,** 107 (1969).
78. M. Yoshinaga, *Arch. Jap. Chir.* **34,** 1 (1965).
79. I. Maruyama, *Arch. Jap. Chir.* **34,** 19 (1965).
80. M. Hirano, *Arch. Jap. Chir.* **34,** 939 (1965).
81. T. Eguchi, *Arch. Jap. Chir.* **34,** 1181 (1965).
82. R. Shioda, *Arch. Jap. Chir.* **34,** 571 (1965).
83. H. Tanimura, *Arch. Jap. Chir.* **34,** 1160 (1965).
84. K. Hashimoto, *Arch, Jap. Chir.* **35,** 981 (1966).
85. E. G. Caira, D. R. Webster, and S. C. Skoryna, *in* "Proceedings of the World Congress of Gastroenterology, Washington, D. C.," p. 294, Williams & Wilkins, Baltimore (1958).
86. J. Tepperman, F. T. Caldwell, and H. M. Tepperman, *Am. J. Physiol.* **206,** 628 (1964).
87. F. Pedreira and J. Tepperman, *Am. J. Physiol.* **206,** 635 (1964).
88. F. T. Caldwell, Jr., K. Levitsky, and B. Rosenberg, *J. A. M. A.* **188,** 437 (1964).
89. F. T. Caldwell, Jr., K. Levitsky, and B. Rosenberg, *Am. J. Physiol.* **209,** 437 (1965).
90. F. T. Caldwell, Jr., and K. Levitsky, *Ann. Surg.* **166,** 753 (1967).
91. F. T. Caldwell, K. Levitsky, and E. B. Sheman, *Fed. Proc.* **27,** 513 (1968).
92. F. Bergman, H. Bogren, G. Lindelöf, and W. van der Linden. *Acta Chir. Scand.* **132,** 715 (1966).
93. F. Bergman and W. van der Linden, *Acta Chir. Scand.* **133,** 479 (1967).
94. F. Bergman and W. van der Linden, *Acta Chir. Scand.* **134,** 287 (1968).
95. F. Besancon, C. Marche, M. Souchard, and C. Debray, *Compt. Rend. Soc. Biol.* **159,** 2333 (1965).
96. F. Besancon, C. Marche, M. Souchard, and C. Debray, *Rein et Foie* **10,** 229 (1967).
97. F. Besancon, C. Marche, M. Souchard, and C. Debray, *Gastroenterology* **52,** 1071 (1967).
98. C. Frey, C. Thorpe, and G. Abrams, *Am. J. Surg.* **115,** 75 (1968).
99. C. F. Frey and F. Freter, *Am. J. Surg.* **116,** 868 (1968).
100. R. H. Palmer, P. B. Glickman, and A. Kappas, *J. Clin. Invest.* **41,** 1573 (1962).
101. R. F. Borgman, *J. Food Sci.* **29,** 20 (1964).
102. R. F. Borgman, *Am. J. Vet. Res.* **26,** 1167 (1965).
103. R. F. Borgman and F. H. Haselden, *Arch. Pathol.* **82,** 72 (1966).
104. R. F. Borgman and F. H. Haselden, *Am. J. Vet. Res.* **28,** 1161 (1967).
105. R. F. Borgman and F. H. Haselden, *Am. J. Vet. Res.* **29,** 1287 (1968).
106. R. F. Borgman and F. H. Haselden, *Arch. Pathol.* **85,** 598 (1968).
107. A. F. Hofmann and E. H. Mosbach, *J. Biol. Chem.* **239,** 2813 (1964).
108. R. S. Jones and L. Peric-Golia, *Proc. Soc. Exptl. Biol. Med.* **109,** 991 (1962).
109. L. J. Schoenfield and J. Sjövall, *Am. J. Physiol.* **211,** 1069 (1966).
110. P. D. McMaster, *J. Exptl. Med.* **40,** 25 (1924).
111. E. Englert, Jr., *Clin. Res.* **15,** 128 (1967).
112. E. Englert, Jr., *Clin. Res.* **15,** 231 (1967).
113. C. G. Harman, J. W. Freston, and E. Englert, Jr., *Gastroenterology* **56,** 1165 (1969).
114. J. W. Freston, thesis submitted for the degree of Doctor of Philosophy (Faculty o Medicine) in the University of London (1967).
115. J. W. Freston and I. A. D. Bouchier, *Clin. Res.* **16,** 151 (1968).
116. A. F. Hayward, J. W. Freston, and I. A. D. Bouchier, *Gut* **9,** 550 (1968).
117. D. E. Patton, K. Plotner, C. E. Cox, and C. B. Taylor, *Fed. Proc.* **20,** 248 (1961).

118. W. von Meier and H. Moenke, *Zentralbl. Allg. Pathol.* **103,** 339 (1962).
119. W. Burnett, *in* "The Biliary System" (W. Taylor, ed.), p. 601, F. A. Davis Co., Philadelphia (1965).
120. A. F. Hofmann, *Gastroenterology* **48,** 484 (1965).
121. P. Rous, P. D. McMaster, and D. R. Drury, *J. Exptl. Med.* **39,** 77 (1924).
122. P. Rous, D. R. Drury, and P. D. McMaster, *J. Exptl. Med.* **39,** 97 (1924).
123. D. R. Drury, P. D. McMaster, and P. Rous, *J. Exptl. Med.* **39,** 403 (1924).
124. D. B. Phemister, L. Day, and A. B. Hastings, *Ann, Surg.* **96,** 595 (1932).
125. J. D. Bisgard and C. B. Baker, *Ann. Surg.* **112,** 1006 (1934).
126. S. Hamanaka, *Jap. J. Gastroenterol.* **8,** 103 (1936).
127. R. Okey, *Proc. Exptl. Biol. Med.* **51,** 349 (1942).
128. T. Maki, *Ann. Surg.* **164,** 90 (1966).
129. R. P. Cook, A. Kleiman, and L. F. Fieser, *Arch. Biochem. Biophys.* **52,** 439 (1954).
130. E. H. Mosbach and M. Bevans, *Arch. Biochem. Biophys.* **63,** 258 (1956).
131. M. Bevans and E. H. Mosbach, *Arch. Pathol.* **62,** 112 (1956).
132. E. H. Mosbach, M. Bevans, R. Kaplan, and E. Halpern, *Arch. Pathol.* **66,** 72 (1958).
133. E. G. Caira, S. C. Skoryna, A. C. Ritchie, and D. R. Webster, *Surg. Forum* **8,** 222 (1957).
134. E. H. Mosbach and M. Bevans, *Arch. Pathol.* **64,** 162 (1957).
135. G. Ricci, F. Occhionero, and F. Soccorsi, *Fegato (Rome)* **10,** 75 (1964).
136 E. H. Mosbach, M. Bevans, R. Kaplan, E. Halpern, and D. Harris, *Arch. Pathol.* **67,** 197 (1959).
137. E. H. Mosbach and M. Bevans, *Arch. Pathol.* **75,** 558 (1963).
138. W. van der Linden, *Acta Chir. Scand.* **129,** 553 (1965).
139. E. H. Mosbach and M. Bevans, *Am. J. Pathol.* **37,** 631 (1960).
140. S. Bergström, C.-M. Fajers, and W. van der Linden, *Acta Chir. Scand.* **126,** 329 (1963).
141. E. H. Mosbach, V. Bokkenheuser, D. P. Chattopadhyay, M. Schmidt, R. L. Hirsch, and A. F. Hofmann, *Nature* **208,** 1226 (1965).
142. A. F. Hofmann, V. Bokkenheuser, R. L. Hirsch, and E. H. Mosbach, *J. Lipid Res.* **9,** 244 (1968).
143. R. C. Powell, W. T. Nunes, R. S. Harding, and J. B. Vacca, *Am. J. Clin. Nutr.* **11,** 156 (1962).
144. H. Eyssen, E. Evrard, H. Vanderhaege, and P. De Somer, *Overdruk Tijdschr. Gastroenterol.* **8,** 121 (1965).
145. W. G. Hardison and I. H. Rosenberg, *Gastroenterology* **54,** 1243 (1968).
146. W. W. Faloon, A. Rubulis, and M. Rupert, *Clin. Res.* **17,** 158. (1969)
147. A. Kappas and R. H. Palmer, *Pharmacol. Rev.* **15,** 123 (1963).
148. J. R. Amberg, *Radiology* **88,** 584 (1967).
149. P. Holsti, *Nature* **186,** 250 (1960).
150. A. Stolk, *Experientia* **16,** 507 (1960).
151. G. A. Leveille, H. E. Sauberlich, and R. D. Hunt, *Poultry Sci.* **41,** 1991 (1962).
152. H. Eyssen and P. De Somer, *Poultry Sci.* **42,** 1020 (1963).
153. R. H. Palmer, *Science* **148,** 1339 (1965).
154. R. H. Palmer and Z. Hruban, *J. Clin. Invest.* **45,** 1225 (1966).
155. G. F. Zaki, J. B. Carey, Jr., F. W. Hoffbauer, and C. Revokolo, *J. Lab. Clin. Med.* **69,** 737 (1967).
156. R. H. Palmer, unpublished observations.
157. J. Gillman, C. Gilbert, and I. Spence, *Cancer* **7,** 1109 (1954).
158. Y. Fujimaki, *Jap. Med. World* **6,** 29 (1926).

159. C. J. Hamre, *Am. J. Med. Sci.* **220,** 183 (1950).
160. D. M. Small and W. H. Admirand, *Nature* **221,** 265 (1969).
161. R. H. Palmer, *Proc. Natl. Acad. Sci.* **58,** 1047 (1967).
162. N. B. Javitt, *Nature* **210,** 1262 (1966).
163. N. B. Javitt and S. Emerman, *J. Clin. Invest.* **47,** 1002 (1968).
164. D. Rudman and F. E. Kendall, *J. Clin. Invest.* **36,** 538 (1957).
165. K. Eik-Nes, J. A. Schellman, R. Lemmry, and L. T. Samuels, *J. Biol. Chem.* **206,** 411 (1954).
166. A. A. Sandberg and W. R. Slaunwhite, Jr., *Rec. Progr. Hormone Res.* **13,** 209 (1957).
167. L. J. Schoenfield, J. Sjövall, and K. Sjövall, *J. Lab. Clin. Med.* **68,** 186 (1966).
168. R. Abourre, S. G. Gordon, J. G. Mann, and F. Kern, Jr., *J. Lab. Clin. Med.* **72,** 849 (1968).
169. K. Usuki, *Jap. J. Gastroenterol.* **1,** 18 (1929).
170. V. Erspamer, *Virchows Arch. Pathol. Anat. Physiol.* **302,** 766 (1938).
171. J. L. Gaylor, *N.Y. State J. Med.* **64,** 905 (1964).
172. G. Wolf, *Am. J. Clin. Nutr.* **9,** 36 (1961).
173. T. Koyanagi and T. Tatebayashi, *Tohoku J. Exptl. Med.* **81,** 138 (1963).
174. J. W. Smudski and H. M. Myers, *J. Dental Res.* **42,** 959 (1963).
175. T. Koyanagi and S. Odagiri, *Nature* **186,** 809 (1960).
176. O. Wiss, U. Gloor, and F. Weber, *Am. J. Clin. Nutt.* **9,** 27 (1961).
177. H. F. DeLuca, M. R. Manalt, N. Madsen, and E. B. Obson, *Nutrition* **81,** 383 (1963).
178. A. F. Hofmann and D. M. Small, *Ann. Rev. Med.* **18,** 333 (1967).

Chapter 7

CLINICAL IMPLICATIONS OF BILE ACID METABOLISM IN MAN*

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I. INTRODUCTION

Cholesterol molecules once formed in the body or absorbed from the diet can be eliminated from the human organism primarily by the gastrointestinal route as such or after conversion to bile acids. The latter are synthesized exclusively in the liver via a series of reactions which are initiated by 7α-hydroxylation of cholesterol (1). Bile acids subsequently formed are called "primary bile acids," in contrast to "secondary bile acids," which are formed by intestinal microorganisms from the primary ones during the enterohepatic circulation.

Primary bile acids, the major components of which in man are cholic and chenodeoxycholic acids, are mainly excreted as glycine and taurine conjugates via the bile into the intestine, where they facilitate micellar solubilization and absorption of lipids. Most of the bile salt conjugates are reabsorbed in the ileum and re-excreted, completing the enterohepatic circulation of bile acids (2). A small portion escapes during each circulation into the colon and finally into feces, the daily fecal excretion being in a normal man about 250 mg/day. During intestinal passage, bacteria convert the primary bile acids into a great number of secondary products, deoxycholic acid

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being the major component of cholic acid and lithocholic acid that of chenodeoxycholic acid. Since secondary bile acids (especially deoxycholic acid) are absorbed to a small extent, bile usually contains deoxycholate conjugates as a third major component.

Bile acids have two major functions in man: (a) they form a catabolic pathway of cholesterol metabolism, and (b) they play an essential role in intestinal absorption of fat, cholesterol, and fat-soluble vitamins. These functions may be so vital that a genetic mutant with absence of bile acids, if at all developed, is obviously incapable of life, and therefore this type of inborn error of metabolism is not yet known clinically. A slightly decreased bile acid production, i.e., reduced cholesterol catabolism, as a primary phenomenon can lead to hypercholesterolemia without fat malabsorption, as has been suggested to be the case in familial hypercholesterolemia. A relative defect in bile salt production may lead to gallstone formation. A more severe defect in bile acid synthesis and biliary excretion found secondarily in liver disease causes fat malabsorption. This may be associated with hypercholesterolemia according to whether the bile salt deficiency is due to decreased function of parenchymal cells, as in liver cirrhosis, or whether the biliary excretory function is predominantly disturbed, as in biliary cirrhosis or extrahepatic biliary occlusion. Finally, an augmented cholesterol production in obesity is partially balanced by increased cholesterol catabolism via bile acids, while interruption of the enterohepatic circulation by ileal dysfunction or cholestyramine leads to intestinal bile salt deficiency despite an up to twentyfold increase in bile salt synthesis, to fat malabsorption, and to a fall in serum cholesterol.

The biochemistry of bile acid synthesis, production of secondary bile acids, and enterohepatic circulation have been extensively studied, particularly by Bergström's group, and have been thoroughly reviewed in both earlier (3–5) and more recent studies (1,2). In the late 1960s and in the beginning of the 1970s, more and more attention has been paid to the clinical significance of bile acids, especially in the fields of gastroenterology (6–10), hypercholesterolemia, and atherosclerosis (11). This chapter therefore concentrates on recent findings in bile acid metabolism in man, the clinical implications in particular being emphasized.

II. SYNTHESIS OF PRIMARY BILE ACIDS

The synthesis of cholic acid from cholesterol in rodents proceeds via 7α-OH-cholesterol, leading to a trihydroxy derivative which finally loses a side chain, resulting in the formation of cholic acid (1). The synthesis appears to proceed similarly in the human liver (12). Trihydroxycoprostanic acid, which is a precursor of cholic acid and which is formed from cholesterol

in man, has been isolated in small amounts from human bile and belongs thus to the primary bile acids (13–15). Cholic acid appears to be produced also from 26-OH-cholesterol in man (16).

It seems quite apparent that 7α-OH-cholesterol serves as a primary intermediate in human chenodeoxycholic acid synthesis (1). 5β-cholestane-3α,7α-diol and, further, dihydroxycoprostanic acid have been found in human bile (17). The latter is known to be converted finally to chenodeoxycholate (18). Thus dihydroxycoprostanic acid belongs to the primary human bile acids.

Bile acids are conjugated in the liver with glycine or taurine. The conjugation, carried out by bile acid acyl transferase(s), is located in the lysosomal fraction, catalyzed by the microsomal fraction, and inhibited by bilirubin and particularly by conjugated bile salts (19). The last factors apparently explain the reduced conjugation of bile acids in biliary occlusion. Taurine deficiency develops easily in man (see Section VIIC2d), and oral administration of taurine decreases the glycine/taurine ratio (20). Bile acids are conjugated also with other substances in man. Thus, glyco- and taurolithocholic acid sulfates (21) and ornithine conjugates (22) have been found in human bile.

III. ENTEROHEPATIC CIRCULATION OF BILE ACIDS

Though human bile contains a small amount of unconjugated bile acids, most of them are in the form of taurine and glycine conjugates (23). The ratio of taurine conjugates/glycine conjugates is approximately 1:3, and the cholic/deoxy/cheno ratio is about 1.1:0.6:1.0. The individual bile acids are conjugated in the same proportions with taurine and glycine. The concentration of bile acids in the gallbladder bile is usually above 30 mM and in the upper intestinal contents of fasting subjects about 10 mM. After a test meal, the lower normal limit of the bile acid concentration in the duodenal or jejunal contents is about 4 mM (24–28). The first $\frac{1}{2}$ hr sample after the meal administered following an overnight fast usually exhibits a higher concentration than the later ones, because the bile salt pool is accumulated and concentrated during the night into the gallbladder, its sudden emptying increasing markedly the initial intestinal bile acid concentration. Successive meals usually reduce slightly the intestinal concentration, because concentrated bile has not yet been formed in the gallbladder, but even then the levels are usually maintained above 4 mM in normal subjects (28). This is clearly above the critical micellar concentration needed for micelle formation (29) but appears to be necessary in order to facilitate micellar solubilization of lipids effectively enough during fat absorption.

Bile acids are reabsorbed along the whole small intestine, preferentially, however, by the distal ileum. Absorption seems to take place by different

mechanisms, *viz.* passive ionic, nonionic and micellar diffusion, and active transport (30).

Taurine conjugates are not absorbed in the upper intestine of human subjects (31,32), the major transport taking place in the lower ileum by both an active mechanism and passive ionic diffusion. Glycine conjugates, particularly those of dihydroxy bile acids, on the other hand, are absorbed also in the jejunum by passive ionic diffusion (33). Negligible amounts of free bile acids are normally found in the upper small intestine (23), while deconjugation is known to occur in the lumen of the terminal ileum. Absorption of free bile acids appears to take place by both ionic and nonionic diffusion, the transport for dihydroxy bile acids being particularly rapid even in the upper intestine (33).

Reabsorption of bile acids is very effective, so that only a small percentage escapes into the cecum. The rest returns via the portal circulation as conjugates [or as unconjugated bile salts if free bile acids were present in the gut lumen (34)] into the liver, thus completing the enterohepatic circulation. Before resecretion, free bile salts are conjugated in the liver with taurine and glycine.

It has been calculated that the human bile salt pool, amounting to about 2–4 g (35–38), circulates two to three times during each meal or five to ten times daily (7). Thus, theoretically, 10–40 g of bile acids is secreted by the liver into the intestine daily. Almost the same amount is transported by the portal blood back to the liver, yet the serum bile acid concentration is normally low, indicating that the normal liver effectively extracts bile salts from the portal blood. In view of this relatively high bile acid load to the liver, it is understandable that disturbances in liver function can lead to an augmented bile acid concentration in the systemic circulation, though under these conditions bile acid secretion into the intestine is also reduced.

Most of the bile acids which escape from the terminal ileum into the colon undergo under normal conditions a transformation to secondary bile acids via the action of colonic bacteria (1,4). The amount of primary bile acids in feces is thus negligible, if any, the mixture of fecal bile acids consisting of compounds with a wide range of polarity (38–41). However, in contrast to neutral sterols, bile acids are not degraded to any appreciable extent into undetectable metabolites during the intestinal passage (42).

After the deconjugation of bile salt conjugates in the lower ileum and colon by a great number of different bacteria, the removal of the 7α-hydroxyl group takes place very effectively by bacterial enzymes (1,4). The latter originate from anaerobes, gram-positive rods. Oxidation of 3α-OH and 12α-OH to keto groups and subsequent reduction of 3-beta group to 3β-OH are the other major transformations taking place in the human colon. Thus the major product of cholic acid is deoxycholic acid and that of chenodeoxy-

cholic acid is lithocholic acid (or its epimer), two secondary components comprising normally the bulk of fecal bile acids in man (38–40,43).

Colonic reabsorption of secondary bile acids seems to be clearly established. The presence of deoxycholic acid as a normal biliary constituent indicates that it has been absorbed from the colon. Furthermore, the human bile contains a variety of other bacterial transformation products such as lithocholic acid and other cholanic acids, some of which may have been further metabolized by the liver (44–47). In contrast to the case in some other mammalian species, human liver is not able to convert deoxycholic acid back to cholic acid. Colonic perfusion with different labeled bile acids has clearly shown that colonic absorption takes place in man (48). Administration of labeled cholic acid into the lumen of the large bowel during operation for cholecystectomy is followed by the appearance of labeled cholic acid and deoxycholic acid in the T-tube bile, the recovery from the T-tube being about 60% of the dose (49). This clearly shows that cholic acid is converted to deoxycholic acid in the human colon and that both of them are absorbed from the large bowel. Colonic reabsorption has been calculated to amount to 200 mg/day (49). The colonic absorption of secondary bile salts could be even higher if the physical state of some bile acids were not unfavorable for absorption. Lithocholic acid, for example, is a very nonpolar compound and precipitates in the colonic content; in addition, it and other secondary bile acids as well are partially associated with fecal debris and bacteria (41). As a result of poor absorption, the amount of secondary bile acids, other than deoxycholic acid, is usually low in human bile. After a continuous biliary drainage, secondary bile acids disappear from the bile in a few days (49–51).

As already mentioned, some bile acids escape from the portal blood to the general circulation. Serum bile acid concentrations range normally from 30 to 230 μg/100 ml (52). Most of them are in conjugated form, the proportions of the individual bile acids being about the same as in the bile, *viz.* cholic, chenodeoxycholic, and deoxycholic acids are the major constituents.

IV. QUANTITATION OF BILE ACID METABOLISM

The methods available for the quantitation of bile acid metabolism in man have been reviewed recently by Hofmann *et al.* (53). The measurement can be performed by the following methods: (a) fecal determination of bile acids, (b) fecal excretion of administered labeled bile acids, (c) isotope dilution, and (d) measurement of bile salt pool.

A. Determination of Fecal Bile Acids

Since urinary excretion (4) and output through the skin (54) of bile

acids are nomally negligible and since virtually no degradation of bile acids takes place during intestinal transit (42,55), bile acids are eliminated from the body by the fecal route. Therefore, determination of total fecal bile acids gives exact figures (mg/day) for the individual's capacity to synthesize bile acids and to catabolize cholesterol via this pathway. Because of heterogeneity of the fecal bile salt mixture, presence of other disturbing components in feces, and irregularities of human bowel movements, a reliable quantitation of total fecal bile acids is a very difficult and time-consuming procedure. Furthermore, end products of cholic and chenodeoxycholic acids are not usually measured separately. The following methods are available for quantitation of total fecal bile acids: (a) direct chemical measurement, chemical balance (55–58), and (b) measurement of radioactivity in the fecal bile acid fraction after administration of labeled cholesterol, isotopic balance (43,59–61). Both of the methods require a large pool of feces to be collected over several days, and even then the use of an unabsorbable marker, which allows a correction to be made for fecal flow, is preferable. The isotope method necessitates that radioactive cholesterol be given several weeks earlier so as to reach an isotopic steady state when the specific activity of fecal bile acids equals that of their precursor, serum cholesterol. The results obtained with the isotopic method are in good agreement with those found chemically, provided that the isotopic steady state is achieved (55,61).

In contrast to many earlier studies using less specific procedures (*cf.* 55), the chemical methods, which apparently give the most reliable results, have shown that the daily bile acid synthesis is normally relatively low in man, being about 250 mg/day (range from about 100 to 400 mg/day), i.e., about one-third of total cholesterol catabolism. Dietary factors, and especially body size and obesity, affect the values sensitively; impaired liver function and hypercholesterolemia decrease, and malabsorption, especially ileal dysfunction, increases markedly the fecal bile acid elimination (11,62,63). Determination of the fecal bile salt excretion is a sensitive method for detection of ileal dysfunction (64).

B. Fecal Excretion of Administered Isotopic Bile Acids

Measurement of fecal excretion of isotopic bile acids (65) gives only the half-life of the labeled bile acid used. The isotope is injected intravenously, and the daily fecal excretion of radioactivity is measured. According to this procedure, the fractional excretion rate of cholic acid in man is normally about 12–13% per day (66,67). Disadvantages of the method are that the absolute values are not obtained, the cholic and chenodeoxycholic acid excretions must be measured separately or a double label method must be used, and the fecal flow should be regular, though an unabsorbable fecal marker can be used. The method appears to be suitable for screening of ileal dysfunction.

C. Isotope Dilution

In the isotope dilution method, described originally by Lindstedt (35), a tracer dose of a labeled bile acid is given orally or intravenously and the disappearance is followed by determining the specific activity of that bile acid in duodenal contents serially for up to 7 days. The specific activity–time curve is exponential. Thus the pool size and turnover (equals synthesis) of that bile salt can be determined. Cholic and chenodeoxycholic acids appear to have slightly different turnover data in some individuals (38,67). Therefore, the metabolism of the two should be measured separately if exact figures are wanted, by using both labeled cholic and chenodeoxycholic acids. A gross estimate of the one can be obtained, however, from the ratio of these two bile acids in the bile if the pool and turnover of the other are measured (35,37).

The studies with this method (35,37,38) have revealed that the cholic acid pool is 0.5–2.3 g and that of chenodeoxycholate and deoxycholate about the same, so that the total bile acid pool is 1.9–5.0 g. Turnover data have indicated that the fractional turnover rate is about 20% per day, and the daily synthesis of cholic and chenodeoxycholic acids is normally 190–690 mg and 290–390 mg, respectively, totaling on an average 700 mg. This figure is markedly higher than that obtained with the chemical methods, though no comparable studies with the two methods in the same subjects have been performed. The reason for the difference is not apparent, but it should be borne in mind that though the primary bile salts of the enterohepatic circulation apparently form a single rapidly equilibrating pool, colonic bile acids form another relatively slowly equilibrating pool (68). It is apparently for this reason that the fractional turnover of bile salts is slightly lower when measured with the isotope excretion method (66,67) than with the isotope dilution method.

The isotope dilution method is relatively easy to perform technically. The procedure has been widely used for measurement of cholesterol catabolism in different conditions (69–74) and of bile salt kinetics in gastrointestinal disorders (75–78). Markedly augmented loss of bile acids in ileopathy makes the procedure less reliable because the administered isotope may disappear totally into feces during the first day, sometimes even during the first enterohepatic circulation of the bile acid pool after administration of the label. Under these conditions, no quantitative figures are obtained, the method being suitable for screening of this disorder.

D. Measurement of Bile Acid Pool

After an overnight fast, the bile acid pool is almost totally in the gallbladder. If under these conditions a tracer amount of an isotopic bile acid

is injected intravenously, it is rapidly secreted into the gallbladder and mixed with the endogenous bile acids (77,78). The dilution can be easily measured from a single duodenal bile salt sample taken about 3 hr after the injection. The pool size calculated from the dilution is of the same magnitude as that obtained by Lindstedt's serial dilution technique (77). The value of this method is limited to the detection of a reduced size of the fasting pool. Erroneous results are apparently obtained if liver function is impaired, because under these conditions intravenously administered bile acids are eliminated slowly from the circulation (79–81).

The jejunal bile salt concentration obtained after a test meal following an overnight fast is some kind of indication of the effective bile acid pool (52). As already stated (24–28), this concentration is normally more than 4 mM even after successive meals (28). In bile salt deficiency (7) and in cases of impaired gallbladder contraction (82–84), the concentration is frequently but not consistently decreased. The value of this method may be increased if the intestinal bile salt pool, instead of the concentration, is determined, because differences in the dilution of the jejunal contents are mostly eliminated. Thus, though the fecal fat and bile salt excretion is not correlated with the jejunal bile salt concentration after a test meal in patients with ileal resection, they show a highly significant negative correlation with the bile salts (average intestinal bile salt pool was normally about 2.5 g) found in the intestinal pool in which the β-sitosterol administered with the test meal was dispersed (64). This suggests that the fasting bile salt pool in the gallbladder and subsequently the effective intestinal pool during fat digestion are reduced proportionally to the fecal bile acid loss. Determination of the gallbladder pool in this way becomes apparently still more accurate if the internal marker is instilled into the upper duodenum by constant infusion and the amounts of biliary constituents are related to this marker (85). The biliary secretion rate of bile acids can be measured by this method.

V. PHYSIOLOGICAL FUNCTIONS OF BILE ACIDS

A. Effects on Biliary Secretion

Bile salts appear to have a central role in the biliary secretion of water and solutes (103,104). Thus about half of biliary water secretion is bile acid dependent and the other half bile acid independent (104,105). In addition, cholesterol and lecithin secretion is also, at least in part, bile acid dependent. Feeding of bile acids to patients with interrupted enterohepatic circulation has a normalizing effect on the biliary secretion of bile acids, cholesterol and lecithin, so that despite an augmented cholesterol output its solubilization is improved (106,107). When the variation in the biliary bile acid secretion

rate is made wider by interruption of enterohepatic circulation and by duodenal bile acid feeding in patients with T-tube, a positive correlation can be seen between the secretion rate of bile acids and those of cholesterol and particularly lecithin (105). An interrupted enterohepatic circulation appears to inhibit, and its restoration to stimulate, hepatic lecithin synthesis (108). At a low rate of bile acid secretion, cholesterol no longer remains in micellar form and is under these conditions secreted independently of bile acids (105). The negative correlation between the fecal bile salt output and the intestinal cholesterol pool after a fat meal in patients with ileal resection was suggested to be due to depletion of hepatic cholesterol for bile acid synthesis but may also be caused by a reduced rate of biliary bile salt secretion (64).

Jejunocolic bypass, which interrupts enterohepatic circulation and which has been used in the treatment of intractable obesity, is occasionally associated with the development of fatty liver (109,110). It can be speculated that the disturbance in bile acid metabolism might be associated with a disturbed release of lipids not only into the bile but also in the form of lipoproteins into the circulation, thus contributing to the development of fatty liver.

B. Catabolism of Cholesterol via Bile Acids

Elimination of cholesterol from the human body takes place primarily by the fecal route as bile acids and neutral sterols, *viz.* cholesterol, coprostanol, and coprostanone. About one-third of cholesterol is normally catabolized by way of bile acids (11). As will be shown later, the amount of the latter depends on the body size, so that the weight correlates with the fecal bile acids, the average daily output of 250 mg corresponding to about 4 mg/kg. The factors regulating hepatic bile acid production under normal conditions are, however, unknown in many respects.

It seems evident that (1) if bile acid elimination is inhibited or impaired as a primary phenomenon, e.g., in biliary obstruction and hypercholesterolemia, a decreased catabolism of cholesterol leads to hypercholesterolemia and reduced cholesterol synthesis; (2) if bile acid elimination is primarily augmented, e.g., after an external bile fistula, ileal bypass, ileal resection, cholestyramine treatment, or perhaps a diet rich in fibrous material, conversion of cholesterol to bile acids is enhanced, leading almost always, despite stimulated cholesterol synthesis, to a fall in serum cholesterol; (3) if endogenous cholesterol production is primarily increased, e.g., by obesity and excess of calories, bile acid synthesis and elimination are augmented, preventing together with increased neutral sterol elimination in some but not all cases the increase of serum cholesterol. This suggests that removal, not production, of cholesterol is the primary factor which determines serum cholesterol level.

There is no direct evidence so far that the actual concentration of serum cholesterol would determine bile acid production and elimination in man. For instance, increase of serum cholesterol by dietary cholesterol is not associated with compensatory increase in bile acid production (63,71,86,87). This does not exclude the possibility that an increase of some lipoprotein subfraction would stimulate bile acid synthesis. Thus determinations of bile acid synthesis by the isotope dilution method have shown markedly high values in triglyceridemic subjects (69), though according to sterol balance data this association is mostly determined by the degree of obesity of these patients (11,63). It is also interesting to note that though the serum cholesterol level and bile acid production are not normally correlated with each other, bile acid synthesis and the serum cholesterol pool are closely correlated in normocholesterolemic nonobese and obese subjects and in hypercholesterolemic individuals (88).

1. Impaired Bile Acid Production

An isolated defect in bile acid production has been found so far only in familial hypercholesterolemia (62), though even in this entity cholesterol catabolism as a whole may be decreased. Essential hypercholesterolemics (11) and hypothyroid patients (11,89) also tend to have a low bile salt elimination, though the excretion of cholesterol as such appears to decrease, too, particularly in the latter condition. In the circumstances in which bile salt elimination is decreased as a result of decreased hepatic function, elimination of cholesterol as such is also reduced (11). Under these conditions, serum cholesterol apparently increases only when the amount of elimination is decreased more than the feedback mechanism(s) are able to suppress synthesis, i.e., when the production exceeds elimination.

2. Augmented Bile Salt Elimination in Interrupted Enterohepatic Circulation

Bile acid elimination can be stimulated easily in man experimentally with cholestyramine (90). This resin interrupts enterohepatic circulation by binding bile acids so that their fecal elimination increases in normal subjects to almost tenfold. Since this increase persists as long as cholestyramine is given, the loss must be balanced by increased bile acid synthesis from cholesterol, which in turn leads to a fall in the serum cholesterol level. Excessive decrease of the serum cholesterol level is inhibited by augmented cholesterol synthesis and to a small extent by mobilization of tissue cholesterol.

An understanding of the mechanisms by which the compensatory increase in bile acid and cholesterol synthesis takes place following interrupted enterohepatic circulation of bile acids would be important not only

theoretically but also practically. Cholestyramine and ileal bypass appear to be the most effective therapeutic measures in reducing the serum cholesterol levels of hypercholesterolemic patients. The magnitude of this reduction seems to depend on the compensatory increase in cholesterol synthesis. If the latter could be prevented, though only partially, it would be possible to titrate serum cholesterol to any desirable level, e.g., during cholestyramine treatment.

Biliary diversion in the rat stimulates bile acid production and intestinal and hepatic cholesterologenesis to maximum within 24 hr, primarily by increasing the activity of cholesterol 7α-hydroxylase and hydroxymethylglutaryl–coenzyme A reductase, respectively (*cf.* 1,91,92). The activity of these enzymes is repressed via a double feedback mechanism within 6–12 hr after administration of bile acids, possibly by direct action of these compounds on the metabolism of the enzyme protein. An alternative explanation is that bile acids, though inhibiting directly an augmented cholesterologenesis in the intestinal mucosa, enhance the intestinal absorption of cholesterol, the increased return of cholesterol as chylomicrons to the liver suppressing hepatic cholesterol production (92).

A continuous biliary diversion in man augments bile acid secretion severalfold (49,50,93). Interruption of enterohepatic circulation of bile acids by cholestyramine, ileal bypass, or ileal resection has actually been shown to augment cholesterol synthesis also in human subjects (11,64,94,95). Furthermore, oral administration of cholic acid reduces bile acid and cholesterol synthesis in man (94), the production of cholic acid having been inhibited by oral chenodeoxycholic acid treatment in Indian women with lithogenic bile (96). Administration of taurocholate to normal subjects did not change consistently the cholesterol synthesis as measured by the acetate–mevalonate test (97). In patients treated with cholestyramine, in whom the acetate–mevalonate test showed a severalfold increase in cholesterol synthesis (11), the simultaneous administration of cholic acid (given 1–2 hr before each cholestyramine dose so as to facilitate absorption of cholic acid in the absence of resin) reduced the cholesterol synthesis significantly (88). The latter was not, however, normalized, and because serum cholesterol tended to increase the cholesterol synthesis may have exceeded the elimination. It is not known actually whether in man the supplementation of bile salt loss with exogenous bile acids is able to suppress both hepatic and intestinal cholesterologenesis. Some evidence has been presented that cholesterol production by intestinal mucosa is stimulated in man by cholestyramine-induced interruption of enterohepatic circulation (98).

The interval before the feedback mechanism activates bile acid synthesis after interruption of the enterohepatic circulation is not known in man, nor has it been established how soon the augmented synthesis is

normalized after restoration of the normal bile acid circulation. Bile drainage following cholecystectomy shows that secretion is markedly augmented after the third day, indicating that activation is slow or that operative "stress" slows it down (49,50,93). Stimulation of cholesterol synthesis appears to be a faster phenomenon. Thus cholesterol synthesis measured with the acetate–mevalonate test is significantly stimulated after 1 day on cholestyramine (Fig. 1), the maximal stimulation being seen in 3–4 days (88). One day after interruption of the treatment, there is a twofold reduction in cholesterol synthesis. In a hypercholesterolemic patient, the acetate–mevalonate test indicated that synthesis was increased severalfold 1 day after the ileal bypass operation, the maximum stimulation being reached on the third day. These experiments of course did not tell anything about the temporal relationship between bile acid and cholesterol synthesis. Stimulation and suppression of cholesterol synthesis seem to be so fast in man that they may even precede those of bile acid, as has been suggested to be the case in the rat (99).

The maximal capacity of human subjects to catabolize cholesterol via bile acids is not known, because complete external biliary fistula, ileal bypass,

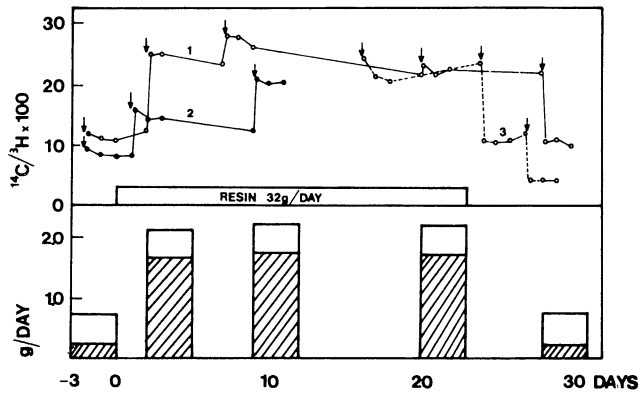

Fig. 1. Effect of cholestyramine (resin) on the ratio $^{14}C/^{3}H \times 100$ in serum cholesterol after intravenous administrations (injections indicated by arrows) of an acetate-^{14}C–mevalonate-^{3}H mixture (upper panel), and fecal bile acids (lower panel, shaded area of bars) and neutral steroids of cholesterol origin (lower panel, unshaded area of bars). 1, Slightly obese triglyceridemic (type IV) patient whose fecal steroid excretion is illustrated in the lower panel. 2, Obese patient with familial hypercholesterolemia (type II). 3, Nonobese patient with familial hypercholesterolemia (type II), had been on cholestyramine for 2 months before the first injection of the isotope mixture. Low-cholesterol solid food diet.

or ileal resection alone or associated with cholestyramine treatment—the clinical conditions employed in these kinds of studies—usually results in malabsorption and malnutrition, which as such limit bile acid production. Patients with biliary fistula have been reported to produce 0.8–5.1 g of bile acids daily (100), though occasionally a value as high as 8.4 g has been recorded (49). Fecal bile acid excretion in patients with ileal bypass or resection may increase up to 4–5 g/day (64,88,101), and if the patient is treated additionally with cholestyramine the value may increase up to 100 mg/kg of body weight (102). It is not known exactly which of the two factors in bile acid synthesis, i.e., depletion of the substrate, cholesterol (via limited increase of cholesterol synthesis), or limited enzyme activity (primarily cholesterol 7α-hydroxylase) of the bile acid synthesis pathway, is the limiting factor of the overall bile acid production. In hypercholesterolemia, at least, the lack of cholesterol seems not to be responsible for the relatively low bile acid production (11,62,63,90), though very little is known about the magnitude of the intrahepatic cholesterol pool which feeds cholesterol to bile acid synthesis.

3. Augmented Bile Acid Production Subsequent to Increased Cholesterol Synthesis

Increased body size (without obesity) is associated with augmented cholesterol production and, apparently owing to larger liver size, with increased catabolism of cholesterol via bile acids. If the body size is further increased by obesity, cholesterol production, probably in the liver, is also increased. This is balanced by enhanced bile acid synthesis and excretion and augmented cholesterol elimination as fecal neutral sterols so that the serum cholesterol level is not consistently increased (11,63,88). This finding suggests that bile acid production is increased as an obligatory consequence of the augmented cholesterol production.

This raises a question of whether the mechanism of stimulated bile acid production during interrupted enterohepatic circulation of bile acids is different from that found during the enhanced cholesterol production in obesity. It is reasonable to assume that in obesity the biliary secretion of both bile acids and cholesterol is augmented and that subsequent intestinal reabsorption from the expanded intraluminal pool is increased in absolute figures (probably decreased relatively). Thus the fluxes both of bile acids via the portal blood and of cholesterol via the lymphatics back to the liver are augmented. Despite these two fluxes, from which the former at least is supposed to inhibit bile salt production (and cholesterol synthesis as well), the hepatic synthesis of bile acids is actually increased, suggesting that it is an increased cholesterol synthesis which stimulates bile acid production.

In interrupted enterohepatic circulation, the biliary secretion of both bile acids and cholesterol is decreased and their reabsorption reduced, so

that both the portal and the lymphatic fluxes of bile acids and cholesterol, respectively, to the liver are diminished. Initially, one or both of these reduced fluxes may stimulate bile acid production and secondarily cholesterol synthesis. Alternatively, however, the latter may at first be augmented, resulting, as in obesity, secondarily in enhanced bile acid synthesis. However, it is of a different magnitude than in obesity. The reason for this can be speculated to lie in the reduced bile acid secretion, which reduces biliary cholesterol secretion so that more cholesterol might remain in the liver. If biliary cholesterol secretion and bile acid synthesis have, even if partially, a common cholesterol pool, the reduced cholesterol secretion could rapidly expand this pool unless the bile acid synthesis were markedly increased. To explain the initial serum cholesterol reduction, the increase in bile acid synthesis should exceed the increase in cholesterol synthesis. In obesity, the biliary secretion of bile acids apparently is slightly increased so that a considerable portion of augmented cholesterol synthesis can be balanced by augmented biliary cholesterol secretion. Under these conditions, serum cholesterol may increase occasionally.

An interrupted enterohepatic circulation of cholesterol itself is seen in malabsorption (119), very little extra bile salts being lost. Under these conditions, the bile acid fluxes to and from the liver are probably normal, the return of cholesterol being markedly reduced and the biliary secretion normal or increased. Thus (a) cholesterol synthesis is increased, (b) bile acid synthesis is normal or slightly elevated, and (c) serum cholesterol is low due to augmented catabolism of cholesterol via fecal neutral sterol excretion. The relationship between bile acid and cholesterol metabolism under different conditions in which cholesterol synthesis is altered is illustrated in Table I.

C. Role in Lipid Absorption

It is a general clinical observation that in conditions in which the intestinal bile salt concentration is low, as in ileopathy, biliary obstruction, or liver cirrhosis, fat absorption is only moderately reduced. Interruption of the enterohepatic circulation of bile salts by cholestyramine increases slightly the fecal fat on a low-resin intake, 12 g three times a day increasing the fat output above 20 g/day (111). Eight grams of cholestyramine four times daily on a normal fat intake has, however, virtually no detectable effect on fat absorption (88). Steatorrhea, as compared to that found in gluten enteropathy, is distinctly less in states with bile salt deficiency, provided that the intestinal mucosa is unaffected. This means that, although the bile salt induced micellar solubilization of hydrolysis products of triglycerides, free

TABLE I. Probable Sequences in Synthesis of Cholesterol and Bile Acids and in Fluxes of These Compounds Under Different Conditions in Man[a]

Condition	Hepatic synthesis		Intestinal	Biliary secretion		Flux to liver		Fecal output		Serum	Pertinent
	BA	Cholesterol	cholesterol synthesis	BA	Cholesterol	BA	Cholesterol	BA	NS	cholesterol	references
Obesity	+	↑ ↑	=+?	+	++	+(=)	(+)=	+	++	+=	(11,63,88,136)
IEHC of BA	++	++	+?	−	−	↓	−	↑ ↑	=	−−	(7,11,64,92,105)
IEHC of cholesterol	=(+)	+	=−?	=(+)	=(+)	=(−)	−	(=)+	↑ ↑	−−	(87,88,119)
Cholesterol feeding	=	−	=	=	+	=	↑	=	+	+=	(11,63,71,86,87)
BA feeding	−	=−?	−(=)	+	+=?	↑	=(+)	+	=?	=+	(88,94,96,97,150)

[a] Assumed primary event is illustrated by arrow (↑, increased; ↓, decreased); +, increased secondarily; −, decreased secondarily; =, unchanged; NS, neutral sterols of cholesterol origin; IEHC of BA, interrupted enterohepatic circulation of bile acids, e.g., during cholestyramine treatment or in ileopathy; IEHC of cholesterol, sequences judged from fecal data and intestinal concentration of patients with gluten enteropathy. (119), and on neomycin (88) and β-nitosterol (87) treatment. Nonobese and obese patients with type IV hyperlipoproteinemia can exhibit the same pattern as normolipidemic obese subjects (88).

fatty acids, and monoglycerides, is generally known to be essential for fatty acid and monoglyceride absorption in the upper intestine (6,10,112–114), a certain portion of fat is absorbed without micellar solubilization in the absence of bile acids. This absorption has been assumed to take place along the small intestine by pancreatic lipase splitting triglycerides to fatty acids, which are then absorbed by diffusion (6,115).

Cholesterol and fat-soluble vitamins require bile acid induced micellar solubilization for absorption, which takes place in the upper small intestine (*cf.* 32,116–118). Accordingly, in bile salt deficiency states, cholesterol absorption should be markedly impaired and fecal neutral sterol excretion increased. The fact that fecal neutral steroid excretion on a low-cholesterol diet is actually normal, as after ileal resection, ileal bypass, and cholestyramine treatment, or even decreased, as in cirrhosis of the liver or biliary occlusion (11), is due to a markedly reduced biliary secretion of cholesterol. In gluten enteropathy, in which no excessive bile salt loss usually exists, fecal neutral sterol excretion is markedly augmented (119). However, in occasional cases in which fecal bile salt elimination is markedly enhanced, the fecal neutral steroid excretion is quite normal, probably owing to decreased biliary cholesterol secretion as a consequence of low biliary bile salt secretion. Detailed information on the role of bile salts in both intraluminal and mucosal phases of fat and sterol absorption is presented in many recent reviews (6,10,113,114,117).

D. Role in Water Absorption

Because bile acids are known to be cathartic, they have been used therapeutically as laxatives (120). Free bile acids especially inhibit water and electrolyte absorption in both the large and the small intestine (121), indicating that though bile acids have lipid transport functions they also have antitransport functions. Preventing excessive colonic water absorption, they can be regarded as physiological laxatives. Perfusion of the colon has shown that dihydroxy bile acids, depending on concentration, can actually stimulate colonic sodium and water secretion (48,122). Thus excessive amounts of unconjugated bile salts in the colon can cause diarrhea, called "cholerheic enteropathy" (7). Patients with ileopathy usually have bile salt induced diarrhea, which can be treated successfully in most cases by binding excessive colonic bile acids with orally administered cholestyramine (123–127) or even lignin (128). The mass of ileostomy discharge correlates with the bile salt output and can be increased by augmenting the latter with orally administered bile acids (129), indicating that bile salts exert their antitransport action in the human small intestine, too.

VI. BILE SALT METABOLISM IN PHYSIOLOGICAL CONDITIONS

A. Age

That the human fetus is already able to convert cholesterol to bile acids during the second half of gestation is indicated by the presence of bile salts in the gallbladder (130,131), cholic acid being the major component (132, 133). However, deoxycholate has been found in cord blood and in newborn serum, indicating that maternal bile acids cross the placenta to the fetus (134). Deoxycholate appears to be absent in the gallbladder bile of fetuses and the duodenal contents of newborns, suggesting that the transfer of bile acids from the maternal side actually is negligible, that the fetus is not able to secrete it into the bile, or that the fetal liver converts deoxycholate to cholate or some other product. The gallbladder bile of the newborn appears to be less concentrated than that of adults (131). The relatively small amount of bile acids in the meconium suggests that the fetus secretes bile acids via the bile and that intestinal reabsorption is very effective (135). In view of the capacity of bile acids to stimulate water and electrolyte secretion, the low bile salt concentration in the meconium is understandable. The human fetus and newborn resemble many adult animal species in that most of the bile acids are conjugated with taurine (130,132,135). During the first year of life, the glycine/taurine ratio reaches almost the adult level. A small amount of cholic acid may be conjugated with ornithine in newborns (130).

Because the newborn lacks microbial 7α-dehydroxylation, deoxycholic acid disappears rapidly from the serum during the first days after birth (134) and appears in the bile and blood of infants at about the age of 1 year. Concentrations of newborn serum bile acids are within the range for adults, a small amount of unconjugated bile salts being detected. During the first days of life, the unconjugated derivatives also seem to disappear from the serum (a sign of negligible bacterial action) and concentrations of serum bile acids are subnormal; normal values are reached after 1 year. The intestinal bile salt concentration and the micelle formation after a fat meal already appear to be similar to those of adults during the first year of life (88).

As far as the rate of bile acid synthesis is concerned, no exact figures are available for normal children, because fecal bile acid determinations have not been performed and the use of isotopes may not be desirable at this age. In view of a relatively high caloric expenditure and rapid growth of the tissues, one can imagine that cholesterol synthesis and, accordingly, bile acid production may be augmented. The few fecal bile acid analyses made have indicated that synthesis per kilogram of body weight may be higher in children than in adults (88).

B. Body Size

Fecal bile acid excretion bears a close correlation with body weight in normocholesterolemic nonobese human subjects ($r = 0.85$ in 13 subjects). This indicates that the body size should always be taken into account when bile acid synthesis is studied in different groups of patients (Table II). When the values are expressed per square meter of body surface, the correlation is still significant ($r = 0.72$), but when expressed per kilogram of body weight the correlation no longer reaches a significant level ($r = 0.52$). This applies only to adults. As already mentioned, children may synthesize relatively more bile acids than adults. Regression between body weight and bile acid excretion, which is linear on the adult weight area, seems to be curvilinear when extended to the weight scale of children (88).

Daily cholic acid production appeared to bear some relationship to body weight in a study in which the effect of butter and corn oil on the cholesterol metabolism was investigated. Thus the correlation coefficient between the average cholic acid production rate of each control subject and the average weight is 0.75 (73).

C. Sex

Normocholesterolemic women (without obesity) excrete and synthesize distinctly smaller amounts of bile acids than males (63) (Table II). The sex difference, however, almost disappears when the values are expressed per kilogram of body weight, though even then the bile acid production tends to be higher in males (4.20 ± 0.27 mg/kg) than in females (3.27 ± 0.31 mg/kg). Thus the possibility of an actual sex difference in bile acid metabolism is not excluded. Furthermore, women tend to eliminate slightly less cholesterol in the form of bile acids (28% of balance) than men (33%). Table II shows bile acid production in both sexes as measured by fecal analysis in various conditions with abnormalities in lipid metabolism.

D. Obesity

Obesity as such without hyperglyceridemia and hypercholesterolemia is the most potent physiological factor in stimulation of cholesterol synthesis in man (11,37,63,138). Though this increased synthesis is mainly balanced by elimination of cholesterol as fecal neutral sterols, bile acid synthesis is also increased (see Table II and Section VB3). This was not seen, however, constantly in every patient, though in a group of 13 overweight subjects the average fecal bile salt excretion (438 ± 64 mg/day) was significantly higher than in normocholesterolemic nonobese controls (238 ± 25 mg/day). Since

the ideal weights of the two groups were the same, obesity as such was inducing an extra production of 200 mg/day of bile acids, which in this particular series corresponded to 3.6 mg of bile salts/day/kg in contrast to 3.8 mg/day/kg of body weight in the controls (Table II). In the controls, 31% of synthesized cholesterol was catabolized by way of bile acids, this figure being in obese subjects only insignificantly less, *viz.* 26%. However, obese women seem to catabolize significantly less cholesterol via bile acids (21% of total synthesis) than nonobese women (28%). This finding will be discussed in Section VII with respect to gallstone development.

Since it is quite apparent that augmented bile acid synthesis is a primary phenomenon which leads to increased fecal bile acid excretion in obesity, the bile acid pool should also be large. Unfortunately, measurement of pool size has not yet been performed in relation to body weight or obesity. Enhanced bile acid production in obesity seems not to be an irreversible phenomenon. Thus weight reduction in a few overweight patients brought cholesterol synthesis and also bile acid production down to almost normal limits (136; see Fig. 2).

It is quite clear from the literature that all studies dealing with bile salt metabolism in man, no matter what the methodology, neglect totally both

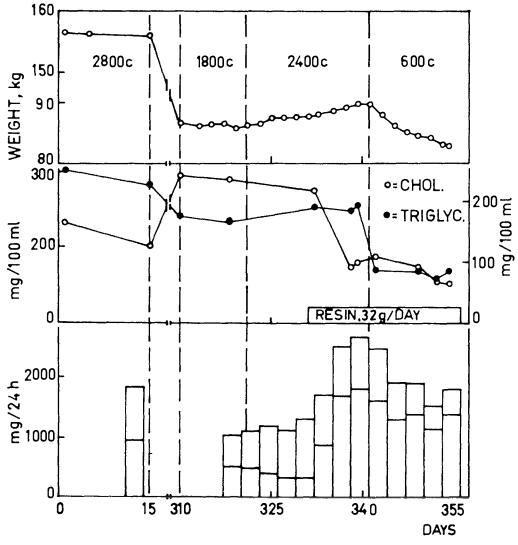

Fig. 2. Response of blood lipids and fecal steroids to weight reduction, high-calorie diet, and cholestyramine (resin) in a patient with excessive initial overweight. The lower parts of bars indicate fecal bile acids and the upper parts fecal neutral steroids of cholesterol origin. Low-cholesterol solid food diet.

TABLE II. Bile Acid Production as Measured by Fecal Excretion in Different Clinical Conditions with Abnormalities in Lipid Metabolism[a]

Group		Relative weight	Serum lipids (mg/100 ml)		Fecal bile acids			
			Cholesterol	Triglyceride	mg/day	mg/m²/day	mg/kg/day	Percent of balance
Controls								
Total	(13)	0.96 ± 0.02	203 ± 11	87 ± 8	238 ± 25[b]	136 ± 10	3.80 ± 0.23	31 ± 2
Males	(7)	1.01 ± 0.02	197 ± 15	97 ± 11	303 ± 25	160 ± 10	4.20 ± 0.27	33 ± 4
Females	(6)	0.90 ± 0.02[c]	212 ± 18	73 ± 7	163 ± 16[c]	107 ± 11[c]	3.27 ± 0.31[c]	28 ± 2
Obesity								
Total	(13)	1.73 ± 0.10[b]	207 ± 9	124 ± 9[b]	392 ± 58[b]	177 ± 22[b]	3.60 ± 0.40	26 ± 3
Males	(6)	1.59 ± 0.13[b]	196 ± 11	123 ± 15	488 ± 110	211 ± 40	4.23 ± 0.65	30 ± 6
Females	(7)	1.84 ± 0.15[b]	217 ± 13	127 ± 8[b]	310 ± 36[b]	148 ± 19	3.14 ± 0.37	22 ± 1[b]
Familial hypercholesterolemia (type II)								
Total	(69)	1.22 ± 0.02[b]	462 ± 14[b]	132 ± 6[b]	176 ± 9[b]	103 ± 5[b]	2.70 ± 0.10[b]	24 ± 1[b]
Males	(32)	1.12 ± 0.03[b]	462 ± 16[b]	142 ± 10[b]	210 ± 13[b]	114 ± 7[b]	2.91 ± 0.18[b]	24 ± 1[b]
Females	(37)	1.12 ± 0.03[b]	463 ± 23[b]	124 ± 7[b]	145 ± 11[c]	93 ± 6[c]	2.49 ± 0.16[b]	23 ± 1[b]
Obese	(15)	1.34 ± 0.04[b]	462 ± 11[b]	138 ± 17[b]	201 ± 22	112 ± 10	2.70 ± 0.20[b]	24 ± 2[b]
Males	(4)	1.39 ± 0.10[b]	437 ± 21[b]	219 ± 30[b]	280 ± 17	130 ± 4[b]	2.90 ± 0.13[b]	26 ± 4
Females	(11)	1.33 ± 0.03[b]	471 ± 12[b]	108 ± 10[b,c]	172 ± 24[c]	105 ± 14	2.55 ± 0.33	24 ± 3
Nonobese	(54)	1.06 ± 0.01[b]	462 ± 18[b]	131 ± 7[b]	168 ± 10[b]	100 ± 5[b]	2.70 ± 0.10[b]	24 ± 1[b]
Males	(28)	1.08 ± 0.02[b]	465 ± 18[b]	131 ± 10[b]	200 ± 13[b]	112 ± 8[b]	2.91 ± 0.21[b]	24 ± 1[b]
Females	(26)	1.04 ± 0.02[b]	459 ± 32[b]	130 ± 9[b]	134 ± 11[c]	87 ± 6[c]	2.47 ± 0.18[b]	23 ± 2

TABLE II (Continued)

Group		Relative weight	Serum lipids (mg/100 ml)		Fecal bile acids			
			Cholesterol	Triglyceride	mg/day	mg/m²/day	mg/kg/day	Percent of balance
Essential hypercholesterolemia (type II)								
Total	(15)	1.14 ± 0.04[b]	327 ± 13[b]	143 ± 14[b]	215 ± 25	125 ± 14	3.20 ± 0.40	29 ± 4
Males	(10)	1.15 ± 0.04[b]	344 ± 11[b]	155 ± 17[b]	219 ± 37	120 ± 20	3.02 ± 0.40[b]	28 ± 4
Females	(5)	1.14 ± 0.08	295 ± 27[b]	119 ± 20[b]	208 ± 24	134 ± 18	3.64 ± 0.60	30 ± 7
Obese	(5)	1.30 ± 0.06[b]	356 ± 10[b]	166 ± 29	246 ± 76	133 ± 41	3.20 ± 1.00	23 ± 6
Nonobese	(10)	1.06 ± 0.03[b]	313 ± 17[b]	132 ± 14[b]	200 ± 12	121 ± 10	3.30 ± 0.30	31 ± 4
Males	(6)	1.06 ± 0.05	341 ± 19[b]	144 ± 16[b]	201 ± 10	114 ± 9[b]	2.98 ± 0.23[b]	32 ± 4
Females	(4)	1.07 ± 0.06[b]	271 ± 16[b]	113 ± 25	200 ± 29	131 ± 22	3.65 ± 0.78	31 ± 9
Hyperglyceridemia (type IV)								
Total	(14)	1.28 ± 0.05[b]	379 ± 57[b]	627 ± 146[b]	369 ± 80	187 ± 35	4.37 ± 0.77	29 ± 3
Males	(11)	1.26 ± 0.06[b]	317 ± 20[b]	437 ± 63[b]	370 ± 103	181 ± 45	4.19 ± 0.98	30 ± 4
Females	(3)	1.36 ± 0.13[b]	575 ± 226	1255 ± 471[b]	369 ± 49[b]	210 ± 29[b]	5.03 ± 0.63[b]	28 ± 5
Hyperthyroidism								
Total	(12)	1.02 ± 0.03	157 ± 11[b]	91 ± 18	366 ± 106	227 ± 66	6.39 ± 1.90	35 ± 5
Males	(1)	1.15	158	177	557	292	7.20	43
Females	(11)	1.01 ± 0.03[b]	157 ± 12[b]	88 ± 8	349 ± 115	221 ± 71	6.32 ± 2.08	34 ± 5
Hypothyroidism								
Total	(15)	1.27 ± 0.05[b]	502 ± 50[b]	290 ± 43[b]	187 ± 28	103 ± 14	2.53 ± 0.35[b]	38 ± 4
Males	(6)	1.14 ± 0.04[b]	317 ± 42[b]	223 ± 30[b]	202 ± 20[b]	108 ± 11[b]	2.72 ± 0.32[b]	39 ± 3
Females	(9)	1.35 ± 0.07[b,c]	589 ± 65[b,c]	341 ± 69[b]	177 ± 45	99 ± 23	2.41 ± 0.55	37 ± 6

[a] Figures in parentheses indicate the number of subjects in each group. In essential hypercholesterolemia (type II), familial history is unclear, tendon xanthomata are absent, and serum cholesterol is usually only moderately increased.
[b] Difference from the controls.
[c] Sex difference.

the body size and obesity. This is quite apparently the reason, in addition to heterogeneity of the material as far as hyperlipidemias are concerned, for the relatively large variation in the values reported.

E. Diet

It is apparent that both the quantity and quality of the diet affect bile acid production. Both have been insufficiently studied in man, though more attention has been paid to the effects of different types of fats on fecal bile acid elimination.

1. Quantity of Diet

As already discussed, obesity is associated via augmented cholesterol synthesis with an increased bile acid production. Since obesity as such is a result of excessive consumption of calories, it is logical to infer that overeating stimulates cholesterol synthesis and secondarily bile acid production. An increased number and quantity of daily meals may, however, change under these conditions the metabolism of both cholesterol and bile acids in complicated ways which are not yet completely understood, by augmenting the number of enterohepatic circulations of bile salts. Increased intestinal contents and fecal mass may also interfere with reabsorption of bile acids.

Figure 2 illustrates, however, that in a patient whose weight had been reduced by total fast and low-calorie diet a markedly decreased fecal bile acid excretion was further reduced when the diet contained an excessive amount of calories. Total cholesterol elimination increased correspondingly. Cholestyramine promptly enhanced the bile acid excretion by a factor of 5, the production being again slightly decreased, despite continuous cholestyramine treatment, when the patient was put on a low-calorie diet. It appears to be a general observation that a total fast or a low-calorie diet decreases the bile acid production both in basal conditions and when the production is already stimulated by, e.g., cholestyramine or ileal bypass (11, 88,139,140). It should be borne in mind, however, that patients with extensive intestinal resections and severe malnutrition can excrete relatively large amounts (2.5–3.6 g/day) of bile acids into the feces (64). Reduced bile acid excretion found during fasting can be increased by cholestyramine (88,139,140), which is also a finding indicating that negative caloric balance is not an absolute inhibitor of bile acid production.

It can be expected that total fast or a low-calorie diet, which effectively inhibit cholesterol synthesis in man (11,88,139,141), would dramatically reduce the serum cholesterol level during interrupted enterohepatic circulation of bile acids. Occasionally, this has turned out to be true (139) (see Fig. 2), but frequently a moderate or even only a negligible further fall is seen (11,88)

in serum cholesterol, indicating that catabolism of cholesterol via bile acids decreases proportionally to the changes in cholesterol synthesis and mobilization from lost tissues. On the other hand, compensatory increase in cholesterol synthesis during fasting can prevent any excessive reduction of serum cholesterol by cholestyramine.

Reduced fecal bile acid excretion during fasting may be secondary to decreased cholesterol synthesis, reduced flow of bile acids into the gut lumen in the absence of stimulus for gall bladder contraction, or diminished trapping of intestinal bile salts by markedly decreased intestinal contents and food residues of cellulose and mucillaneous components. Absence or marked reduction of biliary secretion of bile acids should lead to the accumulation of the bile salt pool in the gallbladder and to stopped or diminished bile acid flux back to the liver via the portal blood. This indicates interrupted enterohepatic circulation and should lead, in contrast to what fecal excretion shows, to an augmented bile acid synthesis (see Section VB3). Thus some bile acids are apparently secreted via the bile, reabsorption being in the absence of dietary substances augmented so that fecal elimination is decreased and flux of bile acids back to the liver inhibits (probably in association with low cholesterol synthesis) hepatic bile acid production. The size of the bile acid pool during total fast is not known.

2. Quality of Diet

Table III lists the effects of unsaturated fats on fecal bile acids or bile acid turnover in the studies in which specific methods have been used for the measurement of bile acid metabolism. It can be seen that the well-known reduction of serum cholesterol by unsaturated fats is in most studies associated with a small average increase in bile acid excretion. Though the increase may be statistically significant, this material, including 72 patients, comprises several subjects in whom the change from saturated to unsaturated fat provoked no alteration or even decreased the bile acid excretion and turnover. Therefore, though the material is rather heterogeneous in view of hyperlipidemia and obesity, augmented bile acid elimination does not appear to be any constant and specific response to an unsaturated fat induced fall in serum cholesterol. This increase, when recorded, may be primarily due to augmented bile acid synthesis in the liver or more likely to inhibited intestinal reabsorption of bile acids. The changes observed are usually so small that they hardly result in any alteration in the pool size of bile acids. Two investigations in which turnover and pool size of cholic acid were measured did not reveal any consistent change in the pool during fat exchange.

Very little is known about the effects of other dietary factors on bile acid metabolism in man. As already discussed, an increase in the dietary cholesterol content did not consistently enhance bile acid synthesis. It has

TABLE III. Effects of Unsaturated Fats on Bile Acid Metabolism in Man

Number of cases	Type of hyperlipidemia	Method of analysis	Dietary fat	Bile acid production, mg/day	Change, mg/day Range	Change, mg/day Mean	References
7	Six normals and	Cholic acid	Coconut oil	110–260			
7	one type II	turnover	Corn oil	130–410	−20–+150	+71	(72)
9	Normal	Cholic acid	Butter	80–450			
9		turnover	Corn oil	140–480	−105–+110	+19	(73)
4	Hypercholesterolemia	Cholic acid	Butter	105–370			
4		turnover	Corn oil	110–265	−105–+5	−30	(73)
6	One normo-, others	Isotopic	Saturated	240–2200			
6	hypercholesterolemic	balance	Unsaturated	100–2320	−790–+120	−186	(142)
5	Normal	Isotopic	Butter	392–533			
5		balance	Safflower oil	455–673	−7–+179	+91	(137)
1	Normal	Chemical	Butter	57		−23	(143)
1		balance	Corn oil	34	—		
3	Hypercholesteremia	Titration of	Saturated;				
3		isolated fecal bile acids	sterols−	101 ± 50			(144)
			Unsaturated; sterols−	134 ± 54	—	+33	

2	Normal	Titration of isolated fecal bile acids	Saturated; sterols+	247 ± 70			
2			Unsaturated; sterols+	271 ± 48	—	+24	(144)
11	Nine type II, one type IV, and one type V	Chemical balance	Butter	81–899			
11			Corn or safflower oil	98–891	−48–+203	+52	(145)
6	Normal	Chemical balance	Cocoa butter	149–467			
6			Corn oil	337–649	−18–+290	+127	(146)
5	Normal	Isotopic balance	Palmitate–oleate	about 380			
5			Trilinolein	about 780	—	+460[a]	(147)
1	Normal	Isotopic balance	Coconut oil	125			
1			Safflower oil	418	—	+293	(148)

[a] Obtained from one patient.

been generally felt that increased amounts of fibrous material, cellulose, and mucillaneous substances make the intestinal contents and stools more bulky, thus increasing bile acid elimination (89,140). Vegetarians in particular would be interesting in this respect. Lignin, one of the major components of plant material, has been reported to bind bile acids (128), and a mucinous material, Metamucil®, increases the fecal bile salt elimination (149).

VII. BILE SALT METABOLISM IN CLINICAL CONDITIONS

A. Hyperlipidemia

Hyperlipidemia is known to be one of the most potent factor associated with the premature development of atheromatous arterial disease. Thus an increased serum cholesterol level is frequently found in patients with ischemic heart disease and myocardial infarction, and hypercholesterolemic patients have a high incidence of coronary artery disease. Therefore, because cholesterol is partially eliminated from the body as bile acids (see Section VB), it would be important to know the role of bile acid metabolism in the development of different types of hypercholesterolemia.

1. Hypercholesterolemia (Type II)

It has been reported that fecal elimination of bile acids correlates negatively (62,90) with the serum cholesterol level in patients with familial hypercholesterolemia, suggesting that in this condition conversion of cholesterol to bile acids is defective and that this defect contributes to the development of hypercholesterolemia. A low average fecal output of bile acids has been repeatedly detected in this condition (11,62,63,90). The detailed relationship of fecal bile acids to obesity and sex based on 69 subjects with familial hypercholesterolemia is presented in Table II.

The negative correlation between fecal bile acids and serum cholesterol was of low degree for the total series ($r = -0.24$) and for nonobese patients ($r = -0.27$), the correlation being positive with the serum cholesterol pool ($r = 0.28$). Fecal bile acids also correlated positively with body weight ($r = 0.52$), relative body weight ($r = 0.31$), and body surface ($r = 0.53$).

The average fecal bile acid elimination is seen to be subnormal, particularly when expressed per kilogram of body weight. The obese subgroup of 15 patients had a normal bile acid elimination, the output in the obese males being significantly higher than in the lean ones. Thus obesity associated with familial hypercholesterolemia slightly stimulates bile salt production, though apparently to a lesser degree than in normolipidemic or triglyceridemic subjects. The sex difference appears to be similar in familial hypercholesterolemia as in normal subjects.

Table II also shows, in agreement with earlier results (62), that subnormal amounts (24%) of body cholesterol are catabolized by way of bile acids in familial hypercholesterolemia. Thus the elimination defect concerns primarily bile acids, the excretion of neutral sterols being less affected so that the sterol balance and hence the overall cholesterol synthesis tend to be decreased in familial hypercholesterolemia.

Another group of hypercholesterolemic (type II) patients, indicated in Table II by the term "essential hypercholesterolemia," was also studied. These patients differed from familial hypercholesterolemia patients in that the family history was less clear, serum cholesterol was less elevated, and xanthomata were not present. Hypercholesterolemia may be primarily caused by environmental, primarily dietary, factors. Bile acid production in this group is less significantly reduced than in the familial group, and the relative catabolism of cholesterol by way of bile acids is within normal limits. Sodhi (151) observed in this type of hypercholesterolemia a markedly low fecal bile acid excretion.

In agreement with the findings presented above, the turnover of cholic acid has been reported to be low in patients with hypercholesterolemia (73, 152). Production of cholic and chenodeoxycholic acids has also been shown to be markedly lower in hypercholesterolemic than in triglyceridemic patients, the former subjects exhibiting a smaller cholic but not chenodeoxycholic acid pool than the latter ones (69).

Though the basal bile acid production in hypercholesterolemic patients is decreased, these subjects are nevertheless able to augment their bile acid synthesis as a response to interrupted enterohepatic circulation of bile acids. Thus cholestyramine treatment (11,63,90,94,153,154) or an ileal bypass operation (11,63,127,155,156) constantly and markedly augments fecal bile acid elimination. Average increments in patients with familial hypercholesterolemia have been 1 and 2 g/day, respectively. However, in normocholesterolemic patients the increment by cholestyramine appears to be significantly higher than that in familial hypercholesterolemia (90) (Fig. 3), again indicating defective bile acid production. The increment of bile acids correlates with basal bile acid elimination, while the correlation with the decrement of serum cholesterol remains insignificant (90), owing to a variable compensatory increase in cholesterol synthesis to balance the enhanced elimination.

The reason for the low bile acid formation in familial hypercholesterolemia is not known. It could be due to impaired availability of cholesterol to the pool which is utilized for bile acid synthesis, a partial deficiency or inhibition of the enzyme system producing bile acids, deficient hepatic secretion of bile acids, or augmented intestinal reabsorption. An interesting observation is that the bile of hypercholesterolemic patients may contain relatively little or no deoxycholic acid (23,73,157), a finding recorded also in patients

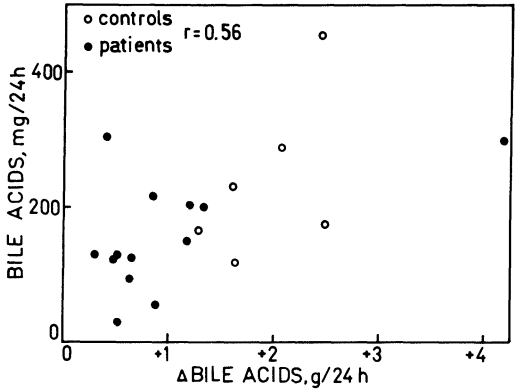

Fig. 3. Correlation between basal fecal bile acid excretion and cholestyramine-induced increment of bile acid output in normocholesterolemic control subjects (○) and patients with familial hypercholesterolemia (●). Cholestyramine dose 32 g/day and low-cholesterol solid food diet.

with liver cirrhosis (23). This is explainable by a reduced entry of bile acids from the small intestine into the colon when little deoxycholic acid is formed and absorbed and little bile acid excreted into the feces. A prerequisite for this explanation is that the intestinal and overall bile acid pool is small or the intestinal absorption is effective. The cholic acid pool is insignificantly smaller than normal in hypercholesterolemic patients, while the deoxycholic acid pool apparently is only a half of normal (73).

The causal relationship between low bile acid production and high serum cholesterol in familial hypercholesterolemia remains unknown. It can be postulated that they are not associated initially, particularly because augmented conversion of cholesterol to bile acids is not able to normalize the serum cholesterol level. On the other hand, it can be speculated that cholestyramine treatment, for instance, stimulates cholesterol production by hepatocytes so that subnormal amounts of newly synthesized cholesterol are utilized for bile acid synthesis, a relatively large amount being released as lipoproteins into the bloodstream so that blood cholesterol still remains high.

2. Triglyceridemia (Type IV)

Earlier fecal bile salt analyses (11,63,138) and those presented in Table III indicate that frequently, but not constantly, bile acid elimination is augmented in patients with triglyceridemia. In some patients with both obesity and triglyceridemia, the values were normal, in others markedly

elevated, and sometimes triglyceridemia in a relatively lean patient was associated with an augmented bile acid excretion. For these reasons, the bile acid production in triglyceridemic patients was not correlated significantly with either body weight or serum triglycerides.

High fecal bile acid values are seen in the few type IV and type V patients of Ahren's group (61,145,158), and Sodhi (151) found a clearly elevated excretion in triglyceridemia as compared to that in hypercholesterolemia. Turnover studies with cholic and chenodeoxycholic acids also indicated that triglyceridemic patients synthesized large quantities of bile acids, the values being higher than in hypercholesterolemia, though a relatively large variation was observed (69).

Administration of cholestyramine to triglyceridemic patients usually has no effect on serum cholesterol. Yet a marked increase is seen in fecal bile acid elimination, the increment showing, in contrast to the results in Fig. 3, no distinct positive correlation with initial bile acid values (88). Furthermore, a cholestyramine-induced increase in fecal bile acid excretion was almost constantly associated with a decrease in neutral sterol elimination despite an apparent reduction of cholesterol absorption. Thus, under these conditions, biliary secretion of cholesterol was probably markedly decreased, particularly because cholesterol production in the intestinal mucosa was obviously enhanced by the reduction of intraluminal bile salt concentration (92,98).

B. Endocrinological Factors

1. Thyroid Hormones

It has been generally accepted, mainly on the basis of animal experiments (159,160), that the production of bile acids is increased in hyperthyroid and decreased in hypothyroid patients. Treatment of the latter subjects with thyroid hormones was actually shown to increase the turnover of cholic acid (161). The determination of fecal bile acids indicated, however, that bile acid elimination was inconsistently decreased in hypothyroid patients and that thyroid hormone therapy increased the bile acid excretion insignificantly (89,162).

Table II shows the fecal bile salt output and the contribution of bile acids to the cholesterol catabolism in both hyper- and hypothyroid patients. The fecal bile acid excretion tended to be supernormal in hyperthyroidism, but the variation was wide because two of the patients had a mild diarrhea associated with a markedly elevated bile acid output (557 and 1480 mg/day, respectively). If these two patients are excluded, the remaining ten women had slightly elevated values, though even then the output per kilogram of body

weight remained within the normal limits. Hypothyroid patients, especially males, produced subnormal amounts of bile acids, particularly per kilogram of body weight. As compared with hyperthyroidism, the bile acid output of hypothyroid women was significantly reduced but only if the body weight (35% overweight) was taken into consideration. Accordingly, it can be concluded that the average bile acid synthesis is slightly augmented or normal in hyperthyroidism, provided that it is not complicated by diarrhea, and slightly depressed or normal in hypothyroidism.

In hypothyroidism, the fecal steroids tended to contain relatively more bile acids (38%) than normally (31%), while in hyperthyroidism quite normal values were obtained. This indicates that the hypercholesterolemia found consistently in hypothyroidism is caused by a defect in the elimination of cholesterol itself, the catabolism of cholesterol via bile acids being less significantly affected.

Slight changes in bile acid metabolism in thyroid imbalance may be attributable to a direct and specific action of thyroid hormone or may be consequences of alterations in the gastrointestinal functions. In experimental animals, thyroxine quite apparently specifically inhibits 12 α-hydroxylation, leading to augmented chenodeoxycholic acid formation (1,160). A slight relative increase of chenodeoxycholate in the bile acid pattern of hyperthyroid patients (161) suggests that the same is true in man, too, though the finding is not a consistent one (163). However, the indirect actions of thyroid hormones may be even more important. A relatively low food intake and slow intestinal motility may allow an effective reabsorption of bile salts in hypothyroidism, leading to reduced fecal bile salt elimination. Bile salt synthesis may in addition be reduced by a good flux of bile salts back to the liver and especially by decreased cholesterol synthesis. In hyperthyroidism, hyperphagia and enhanced intestinal motility may result in the opposite changes, though a negative caloric balance and continuous weight loss may limit not only cholesterol but also bile acid production so that the overall cholesterol synthesis may not be markedly increased. The diarrhea found frequently in patients with hyperthyroidism is mainly caused by augmented intestinal motility (164). Two of the patients in Table II had a marked diarrhea and were the only patients who had an increased fecal bile acid elimination. The diarrhea was easily controlled with cholestyramine, indicating that it was due to "cholerheic diarrhea"; i.e., an augmented small intestinal motility caused increased flux of bile salts into the colon, leading to diarrhea (see Section VD).

The few experiments performed with cholestyramine have clearly shown that both hyper- and hypothyroid patients are able to augment their bile acid production (88). Bile acid production tended to be even higher in hypothyroidism than in hyperthyroidism, suggesting that the presence of thyroid hormones may not be important for bile acid production.

2. Other Hormones

For the role of sex hormones, see Section VIC.

Children with growth hormone deficiency produce only trihydroxy bile acids and have a low intestinal bile salt concentration (165). Growth hormone treatment restores these abnormalities and leads to improved intestinal absorption. A patient with acromegaly exhibited, after hypophysectomy with an adequate thyroid and adrenal hormone substitution, a markedly augmented bile acid synthesis (593 mg/day) which, however, was normal when related to the body weight (88).

The significance of other hormones in bile acid metabolism is not known. Most of the obese and triglyceridemic patients presented in Table II had latent diabetes and, as obese patients in general, apparently a marked hyperinsulinemia. Since hyperinsulinemia markedly stimulates hepatic lipogenesis in man (166), it is logical to infer that insulin causes in these patients an augmented cholesterol production and an indirectly increased bile acid synthesis. The metabolism of bile acids in insulin-dependent diabetes and in hyperlipidemia of ketotic diabetes is not known.

Intestinal bile salt deficiency associated with impaired gallbladder contraction and slightly augmented fecal bile salt excretion during hypocalcemic steatorrhea of patients with primary hypoparathyroidism (84) will be discussed later (see Section VIIE2a).

C. Liver Diseases

1. Gallstone Formation

It has been shown that the ratio of cholesterol to bile acids plus lecithin is in general high in patients with cholesterol gallstones and low in normal subjects, indicating that bile acids and lecithin are the primary cholesterol-solubilizing agents in the human bile (167). More recent studies have shown that when biliary cholesterol, bile acids, and lecithin are plotted on triangular cordinates the normal bile is separated from abnormal or lithogenic bile. Normal bile is less than saturated with cholesterol, while abnormal bile with cholesterol gallstones is saturated or even supersaturated with cholesterol, so that crystallization occurs (168). The precipitation of cholesterol can in an unknown way lead finally to formation of cholesterol stones (*cf.* 168–171).

Analyses of hepatic bile and gallbladder bile have shown that in patients with cholesterol gallstones the hepatic bile is supersaturated and the gallbladder bile saturated or supersaturated with cholesterol (172,173). This indicates that the liver itself forms lithogenic bile and that it is not formed in the gallbladder. Accordingly, gallstone disease, as far as cholesterol stones are concerned, is ultimately a liver disease which might be due to an abnormally low production and secretion of bile acids and lecithin or to exces-

sive production and secretion of cholesterol in relation to the two detergents. A markedly reduced cholic acid pool and turnover with a normal half-life in patients with gallstone disease suggest that bile acid synthesis is abnormally low (174).

As discussed earlier (see Section VA), a reduced bile salt secretion decreases cholesterol and lecithin secretion so that the bile becomes supersaturated with cholesterol, a condition which can be corrected by oral bile salt feeding (105–108,175,176). It is thus apparent that any condition which interrupts the enterohepatic circulation of bile salts, inhibits the hepatic bile salt synthesis and secretion, or stimulates the biliary cholesterol elimination could lead via lithogenic bile to gallstone formation. As a matter of fact, there seems to be an augmented incidence of gallstones in patients suffering from ileal dysfunction (177,178), a condition with interrupted enterohepatic circulation of bile salts. The centers of "normal" gallstones contain proportionally less deoxycholic acid and taurine conjugates of bile acids than the bile or the remainder of the gallstone, suggesting that at the time of gallstone formation the enterohepatic circulation of bile acids may have been actually disturbed (179).

In patients with cirrhosis of the liver, the incidence of gallstones is also higher than normal (180), a finding which can be related to an abnormally low bile acid production by damaged parenchymal cells and a reduced capacity to secrete bile (see Section VIIC2). However, secretion of cholesterol appears also to be markedly reduced (11,181,182), though its relationship to bile acids and phosphatides is not known in this disease. Furthermore, insufficient gallbladder emptying, which has been demonstrated to impair bile and bile acid secretion in patients with cirrhosis (183), may lead to retention of bile in the gallbladder, an important factor in gallstone formation.

Obesity is frequently associated with gallstone disease (*cf.* 184), and cholelithiasis has been related to an increased intake of calories (176). Increased caloric intake has been demonstrated to augment the biliary cholesterol concentration in a way which is independent of the bile salt concentration, so that precipitation of cholesterol may occur (176). Because obesity very potently stimulates cholesterol production (see Sections VB3, VID, and Table II), it could be assumed that the biliary elimination of excess cholesterol takes place proportionally more as cholesterol itself, a relatively smaller amount being catabolized by way of bile acids. This would result in the production of lithogenic bile, precipitation of biliary cholesterol, and finally a proportionally high prevalence of cholesterol gallstones. It is interesting to note that in experimental animals on a gallstone-producing diet the cholesterol synthesis is usually markedly elevated (185,186). Table II shows that obese patients, particularly women, catabolize a relatively small amount of their excessive cholesterol production via bile acids, suggesting that cholesterol catabolism

may be "lithogenic" in most of the obese women. Three of the seven obese women in Table II later actually underwent cholecystectomy for cholesterol gallstones. In contrast to what was found on the basis of the cholic acid turnover (174), all these patients had a clearly increased bile acid production as measured by the chemical balance technique. On the other hand, though the relative amount of fecal bile acids was in general low in familial hypercholesterolemia, the gallstone prevalence was not as high as in obesity (88).

Lithogenic bile has been found in the absence of gallstones in some apparently normal subjects (96,172). It has been suggested that these individuals are going to have gallstones and that lithogenic bile precedes, perhaps by long periods of time, gallstone formation. As already mentioned, oral bile acid feeding normalizes lithogenic bile in patients with interrupted enterohepatic circulation of bile acids (105–108,175,176). A similar type of normalization is obtained not only in patients with gallstones (187) but also in those with lithogenic bile without gallstones (96). It thus seems quite apparent that in most cases the origin of lithogenic bile and gallstone disease itself depends on an insufficient relative production of bile acids.

2. Cirrhosis

Liver cirrhosis is known to markedly change bile acid metabolism. On the other hand, the role of bile acids as an etiological factor in liver cirrhosis has also been widely discussed (188), particularly because lithocholic acid feeding results in the development of experimental cirrhosis (189,190). Major changes of bile acid metabolism in liver diseases are presented in Table IV.

Fecal bile acid elimination is markedly reduced in patients with liver cirrhosis (11,182,191), even if icterus and signs of biliary obstruction are absent (11,182). Since, in addition, the urinary excretion of bile acids is quantitatively, if not proportionally (81), negligible, it can be concluded that the overall bile acid synthesis is markedly depressed in liver cirrhosis (182). The administration of cholestyramine, which is associated with reduction of both serum bile acids (188,192,193) and urinary bile acids (88,182), augmented the fecal bile acid excretion to a very small extent only (182). This indicates that the ability of the parenchymal cells to increase their bile acid production is clearly decreased compared to that of normal subjects. Though the fecal neutral sterol excretion was also increased by cholestyramine, only a relatively small decrease in serum cholesterol was found, suggesting that the patients still were able to increase their cholesterol production (182).

Hepatic bile acid production is also changed qualitatively. The conjugation of bile acids with glycine is significantly decreased in liver biopsies from cirrhotic patients (194). However, the ratio of glycine- to taurine-

TABLE IV. Most Commonly Detected Changes of Bile Acid Metabolism in Some Gastroenterological Disorders[a]

Disease	Fecal BA	Urinary BA	Synthesis of BA	BA pool	Micellar BA			Serum bile acid pattern					Steatorrhea	References
					Free	Conjugated	Total	Concentration	G/T	C/CD	DC	Free BA		
Cirrhosis	−	+	−	−	+≈	−	−	+	−≈	−	−	+	None to moderate	(11,23,26,28,50,52, 80,81,88,134,181,182, 188,191–193,195–199, 200–209,213–220,222)
Hepatitis	−	+	−	−		−	−	+	−≈	−≈+	−	≈	None to moderate	
Obstructive jaundice	−	+	−	−		−	−	+	≈	+	−	≈	Moderate	
Pancreatic insufficiency	+(≈)		+(≈)		≈	≈	≈						Moderate to gross	(26,28,88,229)
Gluten enteropathy	+(≈)		+(≈)	≈	+(≈)	≈(−)	≈(−)						Gross	(11,26,28,88,119,181,203, 204,239)
Stagnant-loop syndrome					+	−	≈	+	+		≈+	+	Moderate	(208,237–239,2243,244, 254–256,259)
Ileal dysfunction	+		+	−	+≈	−	−	≈(+)	+	−≈	−	≈(+)	Moderate	(11,26,28,63,64,66,67, 75–78,94,101,127,155, 156,208,244,255,256, 260–267)

[a] BA, bile acids; G, glycine conjugate; T, taurine conjugate; C, cholic acid; CD, chenodeoxycholic acid; DC, deoxycholic acid; −, decreased; +, increased; ≈, unchanged.

conjugated bile acids in the bile of these patients is within normal limits (23), though in serum the average of the ratio appears to be decreased (193).

It seems to be a general observation that the proportion of chenodeoxycholic acid is increased in liver cirrhosis. Thus the ratio cholic acid/chenodeoxycholic acid has been found to be decreased in the bile (23), serum (52,134,193,195–198), and urine (88,199) of cirrhotic subjects. Since the ratios of cholic acid, chenodeoxycholic acid, and deoxycholic acid appear to be approximately the same in bile and serum (200,201), and perhaps also in urine, it seems quite obvious that the bile acid pattern in any of these three sources is similar to that produced by the liver. Simultaneous determinations of bile acids from bile, serum, and urine have not been made, however. The relative increase of chenodeoxycholic acid has been interpreted to indicate a hindrance of 12α-hydroxylation in liver injury when the formation of cholic acid is decreased in favor of chenodeoxycholic acid (202). This, on the other hand, changes the pattern of secondary bile acids so that relatively more lithocholic acid is formed in the colon (191,200,202), the amount of deoxycholic acid being reduced (23,52,134,193,195–198), particularly because quantitatively only a small portion of the bile acids escapes daily from the ileum to the colon (23).

Depending on the degree of biliary obstruction and impaired parenchymal cell function, the following major abnormalities can be found in the bile acid metabolism of cirrhotic subjects (see Table IV): (a) reduced bile acid synthesis and biliary secretion, (b) low intraluminal micellar bile acid concentration, (c) reduced fecal bile acid elimination, (d) decreased cholic acid production in relation to chenodeoxycholic synthesis, (e) low deoxycholic and high lithocholic acid production by intestinal bacteria, (f) increased concentrations of conjugated and unconjugated bile acids in serum, (g) decreased clearance of bile acids from serum, (h) slightly decreased glycine conjugation in relation to taurine conjugation, (i) increased urinary bile acid excretion, (j) increased bile acid excretion via the skin.

One of the major consequences of altered bile acid metabolism is impaired absorption of fats and particularly of fat-soluble vitamins. This is caused primarily by impaired micellar solubilization of lipids in the intestinal contents because of insufficient bile acid concentration (25,181,203,204). Cirrhotic patients with signs of biliary obstruction (181) or those with steatorrhea (25) have especially low intestinal bile salt levels. The relative amount of free bile acids is increased in intestinal contents as a probable sign of augmented bacterial deconjugation (181) and may contribute to increased free serum bile acids (52,134,193,198), but apparently it plays no important role in the impaired micellar formation. Decreased hepatic removal (81) of absorbed free bile acids may be another factor resulting in the increased serum bile acid concentration occasionally seen in cirrhotic patients. Insufficient gallbladder emptying (82) may contribute, in addition to a decreas-

ed effective bile acid pool, to a low micellar bile salt concentration in the intestinal contents. It should be borne in mind, however, that though occasionally virtually no micellar lipids (except a small amount of free fatty acids) or micellar bile acids are found in the jejunal contents, only a moderate steatorrhea (181) is detected, probably because fats are absorbed as fatty acids by diffusion through the normal intestinal mucosa. Under these conditions, absorption of cholesterol and fat-soluble vitamins should be grossly disturbed, because their absorption requires micelle formation (see Section VC).

Another clinical symptom which can be associated with abnormal bile acid metabolism is pruritus, particularly in patients with signs of biliary obstruction and biliary cirrhosis (204). Though the association between the serum bile acid concentration and pruritus is not distinct, patients who have markedly elevated serum bile acid levels are very likely to suffer from pruritus (193,205–207). It is interesting to note that though the bulk of skin bile acids in pruritic patients are in free form (54), the serum free bile acids are not correlated with pruritus in liver diseases (193), and patients with the stagnant-loop syndrome and high levels of serum free bile acids have no pruritus (208). Even though cholestyramine increases the fecal bile acid elimination only to a small extent in patients with liver cirrhosis, it clearly reduces the urinary output of bile acids (182) and the serum bile acid levels (188,192,193,205) so that pruritus, if present, usually disappears (205). Administration of bile acids orally to a patient with alcoholic cirrhosis increased the serum bile acid level, yet no pruritus occurred (195). On the other hand, administration of whole bile to patients with biliary cirrhosis greatly aggravated their pruritus (209).

Lithocholic acid has been associated with the development and progression of human liver cirrhosis (188,202). This acid is found in human serum, particularly in cirrhotic patients (202,210), in whom chenodeoxycholic acid is the predominant bile acid. Serum lithocholic acid is decreased by cholestyramine and neomycin in cirrhosis, and it has been suggested that the treatment of cirrhotic patients with these drugs warrants consideration (202). Usually, however, the correlation between the levels of lithocholic acid and its precursor chenodeoxycholic acid is poor (188,193). Long-term treatment of patients with lithogenic bile with chenodeoxycholic acid led to an almost complete predominance of this bile acid in bile, yet the amount of lithocholic acid was not increased significantly (96). Predominance of chenodeoxycholic acid appears to be related to the parenchymal cell function (195); the poorer it is the more predominant is chenodeoxycholic acid among the bile acids. However, a simultaneous decrease of hepatic secretory function possibly associated with intrahepatic biliary obstruction reduces the quantitative flow of chenodeoxycholate to the colon, so that bacterial formation and

subsequent absorption of lithocholic acid may also be reduced. The significance of the direct hepatic production of lithocholic acid is not known in man, but it may have little import, if any, because this acid is not found consistently in the serum of normal or cirrhotic patients (193). Progressive fatal familial biliary cirrhosis of the liver has been described recently and was postulated to be caused by lithocholic acid (211). The patient with this abnormality exhibits a markedly augmented chenodeoxycholic acid production and very high chenodeoxycholic and lithocholic acid levels in the serum. An inborn error of hepatic chenodeoxycholic acid overproduction was proposed to be the primary factor which leads via augmented intestinal production and absorption of lithocholic acid to liver injury (211).

The liver damage found frequently in association with inflammatory bowel disease, regional ileitis, and ulcerative colitis has also been postulated to be caused by lithocholic acid (212). The serum concentration of lithocholic acid appears to be, however, quite normal or zero in patients with these diseases (188,193) even if liver damage is present (193), indicating that the association, if any, is not a simple one.

3. Acute Hepatitis

Bile acid metabolism in acute hepatitis resembles very much that found in cirrhosis; i.e., the degree of impaired hepatocellular function and of biliary obstruction is the determining factor. Fecal bile acids and hence overall bile acid synthesis have not been measured at any stage of acute hepatitis. However, as can be expected, the decay of labeled cholic acid indicates a low or low normal daily production of bile acids (80) which in association with biliary obstruction decreases intestinal bile salt concentration and micelle formation, resulting in steatorrhea (213). Due to diminished uptake and excretion of bile acids by the liver cells, the removal of bile acids from the circulation is slow (80), so that the serum bile acid concentration is elevated (52,134,193,198,214–216) and the urinary output slightly increased (80,199). Serial analyses have indicated that serum bile acids reflect impaired hepatic function more sensitively than serum bilirubin, transaminases, and bromsulfophthalein excretion do (134,215,216). The proportion of free and secondary bile acids is usually negligible in serum, while, in contrast to liver cirrhosis, serum bile acids may consist predominantly of cholic acid, the conjugation with glycine being reduced. However, the bile acid pattern, as far as both conjugation and hydroxylation are concerned, is variable and depends on the stage of the disease (215).

4. Biliary Obstruction

The most striking initial change in biliary obstruction is a decrease or complete stopping of biliary bile acid secretion, which explains the reduction

or absence of micellar lipids and bile acids in the intestinal contents (26,28, 181), the marked reduction or absence of fecal bile acids (88), and the moderate steatorrhea. Since the urinary bile acid excretion, which may occasionally be the major route of bile acid elimination (217), is only a few tens of milligrams (88,196,199), even in the presence of complete intra- or extrahepatic obstruction, it can be concluded that bile acid synthesis in man, in contrast to that reported in the rat, is markedly reduced in this condition. The serum bile acid concentration is markedly increased and correlates positively with the serum bilirubin level (193,196,198,218–220), pruritus being frequently but not constantly present. Hepatic conjugation of bile salts is reduced relatively more with glycine than with taurine *in vitro* (19,194,221); yet most of the serum and urinary bile acids are in conjugated form (196,199), taurine conjugates predominating frequently (23,50,193) but not consistently (218). In contrast to the situation in parenchymal cell injury, the proportion of cholic acid increases conspicuously in biliary obstruction (23,50,195,196).

In patients with Dubin–Johnson syndrome, in whom fecal bile acid output is not grossly impaired (222) and in whom serum-conjugated bilirubin level is elevated, serum bile acid concentrations are within normal limits. This clearly indicates that secretory mechanisms are not the same for conjugated bile acids and conjugated bilirubin (218).

Serum cholesterol is usually markedly elevated in biliary obstruction, with especially high values in biliary cirrhosis. A positive correlation is found occasionally (196) but not constantly (193) between the serum bile acid and cholesterol levels. An absence of bile acids in the gut lumen and a reduced cholesterol absorption may stimulate, at least initially, both intestinal and hepatic cholesterol production (*cf.* 92,223), and this in association with a block in elimination both as bile acids and as cholesterol itself rapidly raises the serum cholesterol level. However, in biliary obstruction of long duration, e.g., in biliary cirrhosis, sterol balance studies and urinary bile acid measurement indicate that cholesterol synthesis is markedly reduced (88). In parenchymal cell damage of the liver, the serum cholesterol is normal or decreased, probably because the hepatic cholesterol synthesis, due to cell injury, is reduced in proportion to, or proportionally more than, the decreased cholesterol elimination (11,182) and, furthermore, intestinal and hepatic cholesterogenesis may still be under the partial feedback control of bile acids and absorbed cholesterol, respectively.

Bile acids inhibit plasma lecithin cholesterol acyl transferase (LCAT) (*cf.* 224), the activity of which is low in obstructive jaundice and in diseases with impaired liver function (*cf.* 224,225). However, it has been pointed out that the serum bile acid concentrations found in patients with obstructive

jaundice are not high enough to alone reduce LCAT activity (226). On the other hand, the red cell membrane accumulates cholesterol in obstructive jaundice as a consequence of the elevated levels of bile salts, particularly because the relative amount of free cholesterol, apparently due to the low LCAT activity, is increased (227). Thus, though bile acids are known to have hemolytic effects (228), in obstructive jaundice and in other conditions with elevated serum bile acid levels they should counteract hemolysis by contributing to the formation of osmotically hemolysis-resistant red cells (target cells).

D. Pancreatic Diseases

Table IV presents changes in some parameters of bile acid metabolism in different types of clinical conditions causing fat malabsorption.

Chronic pancreatic insufficiency is known to cause a marked fat malabsorption. This is associated with a normal or decreased intestinal bile salt concentration (26,28,229) and, owing to impaired lipolysis of triglycerides, with decreased micellar solubilization of ingested lipid (229). Fecal bile acid elimination is slightly but significantly increased for unknown reasons (88). Despite marked malnutrition, the hepatic bile acid production is increased sufficiently to balance the fecal loss, so that the intestinal bile salt concentration remains quite normal. A slightly increased catabolism of cholesterol via bile acids and more significantly an augmented fecal neutral sterol excretion (88) and a malnutrition-induced reduction of the compensatory increase in cholesterol production for the increased cholesterol catabolism are apparent reasons for the marked hypocholesterolemia found consistently in pancreatic insufficiency.

The pancreatic gastrin-secreting tumor in the Ellison–Zollinger syndrome is known to be associated with a marked steatorrhea and diarrhea. Though fat malabsorption is primarily due to irreversible inhibition of lipase activity by a low duodenal *p*H, the latter brings glycine-conjugated bile salts to nonionic form and to precipitation, thus contributing via impaired micelle formation to steatorrhea (230). Furthermore, precipitated bile salts may enter the colon in enhanced amounts and disturb the colonic water absorption, thus worsening the diarrhea (230).

Bile salts apparently play a certain role in the development of acute pancreatitis. The flux of bile and bile acids into the pancreatic ducts activates phospholipase A, which may cause via lysolecithin formation autodigestion of pancreatic tissue. Bile acids may also act directly on pancreatic cells as toxic agents by producing coagulation necrosis (*cf.* 231).

E. Gastrointestinal Tract

1. Stomach

Regurgitation of bile and bile acids from the duodenum to the stomach has been recently suggested to play a role in the development of gastric ulcer (232,233). Thus the gastric bile salt concentration, an obvious index of regurgitation of duodenal contents into the stomach, is higher in patients with gastric ulcer than in controls (233). Since a small portion of the bile salts is in soluble form, they can act directly as cytotoxic agents on the gastric mucosa or prevent healing of already existing gastric ulcer. In addition, instillation of bile salt conjugates in the pyloric gland area of the stomach causes the release of gastrin and thus secondarily an augmented hydrochloric acid secretion, which is known to be a potential ulcerogenic factor (234). It should be borne in mind, however, that desiccated bile and unconjugated and conjugated bile acids have been fed to patients for different purposes as therapeutic agents for long periods of time, and no convincing evidence has been reported for the development of gastric ulcer.

The bacterial transformation of bile acids, particularly deconjugation, has been shown to be markedly accelerated in patients with gastrectomy, probably because the upper intestinal contents were colonized by a large number of bacteria (235). The deconjugation of doubly labeled glycocholate (labeled in glycine and bile acid portion) amounted to 25–27% 25–26 hr after administration of the isotope in normal subjects (236), the figure in gastrectomized patients being 82–96% (235). Despite the enhanced deconjugation, free bile acids were seldom found in intestinal contents, and in most patients the bacterial formation of deoxycholic acid was very low (235). Deconjugated bile acids have been detected frequently in the intestinal contents of patients with postgastrectomy steatorrhea, and this condition has been related to bacterial overgrowth in the upper small intestine (237–239) (for bacterial overgrowth, see Section VIIE12b).

2. Small Intestine

a. Malabsorption Syndromes. Fecal bile salt elimination appears to be slightly elevated in patients with gluten enteropathy (119), the values being about twice those in normal subjects if expressed per kilogram of body weight (11,88). Bile acids apparently contribute to diarrhea, because the administration of cholestyramine strikingly reduces fecal frequency (88). Since biliary cholesterol secretion is apparently normal or increased in gluten enteropathy (181), reduced cholesterol absorption results in a markedly augmented elimination of cholesterol as neutral sterols (119). Thus the relative catabolism of cholesterol by way of bile acids, in spite of their slightly increased production, appears to be one of the lowest seen in dif-

ferent clinical conditions (11). The reason for the slightly decreased reabsorption of bile acids is not known. The mucosal morphology of the terminal ileum is usually normal, though its functional reserve is lacking (240) and may contribute to a defective reabsorption of bile salts. Hypomotility of the small intestine associated with a larger than normal intestinal fluid volume (241) may also impair bile acid absorption.

Despite the small extra loss of bile acids into the feces, patients with the malabsorption syndrome usually but not always have a normal intestinal bile salt concentration (26,28,181,203,204,229,239) even after repeated test meals (28). The micellar solubilization of lipids by intestinal contents appears to be correspondingly decreased in occasional cases, though for a given bile salt concentration usually relatively little of exogenous lipids and sterols is solubilized, while endogenous cholesterol seems to enter the micellar phase properly (181). Bacterial overgrowth associated with deconjugation of bile salts (239) in the upper small bowel may sometimes be responsible for poor micelle formation and contribute to steatorrhea, but in most cases the absolute concentrations of conjugated bile acids are within normal limits (181). However, compared to the normal bile salt pattern, the intestinal contents of patients with gluten enteropathy have significantly elevated levels of unconjugated bile acids (4% of total in contrast to 0.4% in controls), as if some bacterial deconjugation had actually occurred in almost every subject or the absorption of free bile acids by the damaged mucosa had been reduced. Despite an apparently augmented bacterial action, the deoxycholic acid concentration is decreased, probably owing to reduced bacterial 7α-dehydroxylation of cholic acid or to decreased colonic absorption of bile acids. As in control subjects, the relative amount of intestinal chenodeoxycholic acid is higher in the free than in the conjugated bile salt fraction (181). Because of the rapid absorption of chenodeoxycholic acid in the upper intestine (33), this is a somewhat unexpected finding.

Steatorrhea found during the hypocalcemic stage of primary hypoparathyroidism is associated with a normal intestinal mucosa, slightly increased fecal bile acid loss, low intestinal bile salt concentration, poor micelle formation, and deficient emptying of the gallbladder (84). The last may be an important etiological factor in the abnormal bile acid metabolism and ultimately in steatorrhea, since turnover studies with chenodeoxycholic acid indicate that the pool size is within the normal limits. Therapeutic normalization of serum calcium reduces steatorrhea and normalizes intestinal micellar bile salt and lipid concentrations. Similar abnormality has been detected in patients with gluten enteropathy, though the abnormality, as far as gallbladder function and bile salt metabolism are concerned, appears to be of a lesser degree of severity (83).

b. Stagnant-Loop Syndrome. Malabsorption found in the intestinal

blind-loop syndrome was originally speculated by Dawson and Isselbacher (242) to be caused by bacteria-induced deconjugation of bile acids. The resulting toxic free bile acids could then disturb fat absorption. Subsequent studies showed that deconjugation and dehydroxylation of bile acids occurred in the small intestine and that these bacterial processes appeared to be a major etiological factor in causing steatorrhea in these patients, probably by interfering with intestinal micelle formation (237,238).

Bacterial colonization of the small intestine can take place in many conditions, such as blind intestinal loops, stagnant afferent duodenal loop after partial gastrectomy or gastroenterostomy, intestinal strictures, small intestinal diverticulosis, atonia due to scleroderma, vagotomy, or diabetes, degeneration of the myenteric plexus, gastrojejunocolic fistulas, gluten enteropathy, and extensive intestinal resections (243,244). Under these conditions, a large number of different bacteria occupy the intestinal lumen, and many of them, particularly anaerobic bacteroides, veillonella, clostridia, and bifidobacteria, are able to deconjugate and dehydroxylate bile acids (245–251). Since unconjugated bile salts are relatively ineffective in promoting micelle formation [on the contrary, they may themselves be solubilized by micelles, thus inhibiting the solubilization of digestion products of lipids (252)], the concentration of the remaining conjugated bile salts may become too low for micellar solubilization of hydrolyzed intestinal lipids so that steatorrhea of moderate degree develops (244,253). Treatment with broad-spectrum antibiotics kills bacteria and prevents deconjugation so that micelle formation is improved and steatorrhea reduced (237–239). A similar improvement is obtained by feeding bile salt conjugates orally (238).

The amount of deconjugated bile salts found in intestinal contents is variable (238,239,243,254–256) and depends on anatomical abnormality, the site of sampling of intestinal contents, and ingested meal. Occasionally, a complete deconjugation may have occurred without steatorrhea (243), though in general steatorrhea is related to the loss of conjugated bile salts (239,244). Local deconjugation in duodenum or ileum may not cause steatorrhea (244,255). Precipitation of free bile acids may lead to formation of bile acid enteroliths (257).

Intestinal bacterial overgrowth markedly changes bile salt pattern. Thus, in the stagnant-loop syndrome, glycine conjugates are the predominant bile acids, and the dihydroxy fraction is reduced, a finding suggested to be due to effective absorption of the dihydroxy conjugates from the upper jejunum (254). Augmented absorption of free bile acids in the stagnant-loop syndrome may increase the overall reabsorption of bile acids, because under these conditions absorption may take place along the whole small intestine. However, no information on fecal bile acid excretion in the blind-loop syndrome with normal ileal function appears to be available. Bacterial

deconjugation of bile salts found in patients with gluten enteropathy (181) or resection of terminal ileum (64) is associated with a slight (11,119) or marked (64) fecal loss of bile acids, respectively, indicating that under these conditions, at least, bile salt wastage was not prevented by deconjugation.

Most of the bile acids found in the portal blood are normally in conjugated form in man (198). In patients with the stagnant-loop syndrome, serum bile acids are markedly elevated, but, in contrast to the normal state or liver diseases (except cirrhosis), the bulk of them are in free form (255, 258,259), suggesting that deconjugated bile acids have been absorbed and that the hepatic extraction has not been complete from the portal blood. The pattern of conjugated bile acids in the general circulation resembles that in the small intestinal lumen, so taurine conjugates may be absent. The ratio of trihydroxy/dihydroxy bile acids appears to be low, as if the latter ones had been absorbed at a higher rate than cholic acid. The serum bile acid pool is markedly increased in the stagnant-loop syndrome, fractional turnover being, however, decreased (*cf.* 244,258).

c. Inflammatory Diseases. Crohn's disease may affect bile acid metabolism in at least two ways: strictures formed at any level of the small intestine may be associated with the stagnant-loop syndrome and hydrolysis of the bile salt conjugates (244), and affection of the terminal ileum may inhibit reabsorption of bile acids, resulting in bile salt deficiency and fat malabsorption (66,101,260–262). When the disease presents primarily as terminal ileitis, fecal bile salt loss is a dominating feature, while the stagnant-loop syndrome may be present when the more proximal intestine is affected with strictures. In the latter case, serum free bile acids may be elevated (244). It should be borne in mind that the severe malnutrition which usually belongs to the clinical picture of Crohn's disease may limit bile salt production in patients with interrupted enterohepatic circulation and contribute to the reduction of the serum cholesterol level. Postradiation ileitis also markedly impairs reabsorption of bile salts (76).

d. Resection of Terminal Ileum. Interruption of enterohepatic circulation of bile salts by removal of the terminal ileum results in excessive fecal bile acid loss which is associated with markedly increased bile acid synthesis, proportionally reduced intestinal bile salt, cholesterol, and micellar lipid levels, and moderate steatorrhea with bile salt induced diarrhea. Cholesterol is catabolized primarily by way of bile acids at a markedly enhanced rate, which results in a proportional decrease of the serum cholesterol level despite severalfold increase of cholesterol synthesis (see Fig. 4). Bacterial overgrowth of the upper small intestine associated with deconjugation of bile salts is found in occasional cases, particularly if the ileocaecal valve is not functioning or if an extensive intestinal resection has been performed (64,244,255,256).

Bile salt deficiency as a result of a marked fecal loss of bile acids was

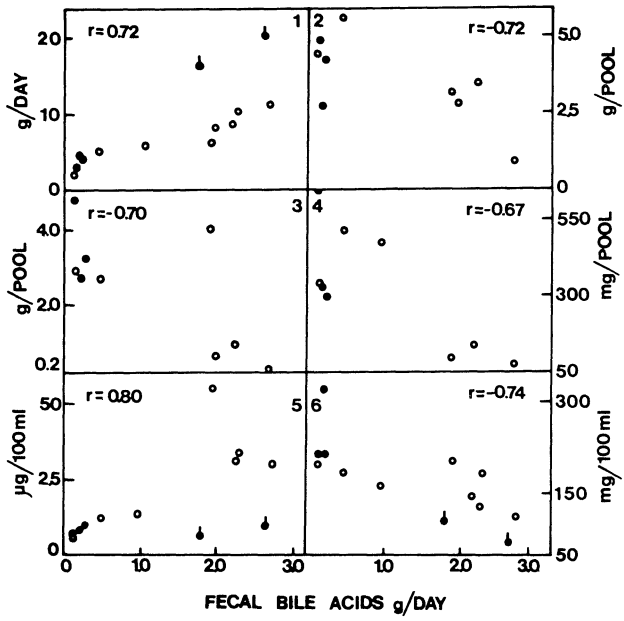

Fig. 4. Correlation of fecal bile acid excretion with the following: 1, fecal fat, 2, 3, and 4, jejunal micellar fat and bile acids, and jejunal total cholesterol, respectively, calculated for amounts found in the intestinal pool in which 136 mg of β-sitosterol administered in the test meal was dispersed. 5, Serum methyl sterol (diunsaturated dimethyl sterol), used as an indicator of augmented cholesterol synthesis (see reference 11). 6, Serum cholesterol. ●, Patients with incomplete ileal resection (25–50 cm of the very terminal ileum intact, 1–2 m of the more proximal gut removed); ○, complete ileal resection; ⬮, extensive intestinal resections with severe malnutrition (the values omitted from calculations in 5).

first described after extensive intestinal resections in a patient who exhibited a low intraluminal bile acid concentration and low micellar solubilization of digested fats in the intestinal contents (263). Resulting steatorrhea became less severe with oral bile acid therapy. Several other cases with ileal resections associated with abnormal bile acid metabolism and impaired micellar solubilization of digested lipids were reported at about the same time or somewhat later (28,67,77,155,264–267). The bile acid pool in these patients is usually markedly reduced, because, depending on the length of intestinal resection, reabsorption of bile acids may be almost completely ceased. On the other hand, retention of a relatively short segment, 25–50 cm, of the very terminal ileum may be enough to prevent fecal bile salt loss, even if the rest of the more proximal ileum has been removed (64). Occasionally, however, removal of the proximal colon and terminal ileum has no effect on fecal bile

salt excretion, suggesting that the colon or, more likely, the more proximal small intestine adapts to reabsorb bile salts.

Reabsorption by the upper small intestine may be improved provided that the proportion of glycine-conjugated or free dihydroxy bile acids is increased, because they are reabsorbed to a considerable extent from the jejunum by passive diffusion (33). It appears to be a general observation that ileal dysfunction actually markedly decreases the proportion of a taurine-conjugated bile acid so that glycine conjugates predominate among the intestinal bile salts (26,77,78,260). The increase in the glycine/taurine ratio is so constant that determination of the ratio has been used for the early diagnosis of the terminal ileopathy (262), even though similar increase is seen in the stagnant-loop syndrome (254). Impaired hepatic conjugation of bile acids with taurine is apparently due to taurine deficiency, because oral administration of this compound reduced the ratio in patients with ileal dysfunction (260,262).

Reduced bile acid pool has been reported to concern primarily cholic acid conjugates, while both relative and absolute amounts of chenodeoxycholate, the glycine conjugate of which is reabsorbed at a relatively high rate from the jejunum (33), may remain unchanged after ileectomy (260). Bacterial transformation of bile acids appears to be also changed. Thus augmented amounts of free bile acids can be frequently found in the upper intestinal contents as an obvious sign of bacterial deconjugation (26,64,244,260). In spite of this possible bacterial overgrowth, deoxycholic acid is usually absent or markedly decreased in the upper intestinal contents, because enterohepatic circulation of deoxycholate is interrupted (26,75,261) and bacterial action on fecal steroids may be decreased (64,101). Occasionally, however, especially in patients with extensive intestinal resections and marked bacterial overgrowth, the formation of secondary bile acids, including deoxycholate and lithocholate, is normal or increased (78,244,268).

It is partly owing to adaptation of the remaining small intestine to reabsorb bile acids that the length of ileal resection is not correlated with fecal bile acid excretion (64,101). In addition, accompanying malnutrition may limit the capacity of the liver to enhance its bile acid synthesis, so that in patients with extensive intestinal resections fecal bile acid excretion may be only moderately increased (64,101). In these cases, serum cholesterol is usually very low and depletion of body cholesterol in the presence of insufficiently enhanced cholesterol synthesis may be one factor for limited bile salt production.

For diarrhea in ileopathy, see Section VD. Ineffectiveness of cholestyramine on diarrhea in patients with extensive ileal resection has been explained by the presence of hydroxy fatty acids and a low level of solubilized bile salts in feces (268).

e. Intestinal Bypass. Partial ileal bypass has been used as a therapeutic measure for hypercholesterolemia (269), more extensive exclusion of the distal small intestine (jejunotransversocolostomy or jejunoileostomy) being performed to create malabsorption and induce weight loss in obese patients (see Section VC). In both conditions, reabsorption of bile acids is discontinued and fecal bile salt excretion grossly increased. No comparative studies have been performed on the capacity of subjects with the two conditions to synthesize bile acids.

Studies with radioactive glycocholate or taurocholate demonstrated a virtual absence of the enterohepatic circulation of bile acids in patients with jejunotransversocolostomy (77). The small amount of absorbed bile acids contained some deconjugated cholate and deoxycholate (which had been reconjugated in the liver), indicating a rapid bacterial action during an apparently fast intestinal passage. Under these conditions, steatorrhea is apparently not solely due to bile salt deficiency induced impairment of micelle formation, but reduced absorptive area may play an important contributory role. No direct measurement of bile acid synthesis by fecal determination has been performed in this condition.

Ileal bypass (see Section VB2) performed for hypercholesterolemic patients (269) increased cholesterol elimination in the study by Moore *et al.* (156) about fivefold as bile acids and threefold as neutral steroids according to the isotopic balance technique. Serum cholesterol reduction was about 40%. In our own series, consisting of patients with familial hypercholesterolemia only, similar results have been obtained by the chemical balance technique except that elimination of cholesterol increased solely as bile acids (11,63,127). The fecal bile salt loss was associated with a 35% fall in the serum cholesterol level, a severalfold increased cholesterol synthesis, impaired micellar solubilization of digested lipids, reduced intestinal bile salt concentrations, and decreased cholesterol level in the intestinal contents. The last suggests that biliary secretion of cholesterol was markedly decreased so that, despite impaired cholesterol reabsorption, fecal neutral steroid excretion remained quite unchanged. An increased fecal bile salt loss associated with stimulated cholesterol production has been reported also by Grundy *et al.* (94).

Since intestinal mucosa, especially in ileum, appears to synthesize cholesterol at a high rate even in man and since this synthesis is increased by the absence of bile acids (223), it is to be expected that in patients with intestinal bypass mucosal cholesterol production is markedly increased in the bypassed portion of the gut and should contribute to serum cholesterol. Ileal or more extensive intestinal resections, on the other hand, prevent this increase in synthesis and should reduce serum cholesterol more effectively than the sole bypass does. However, animal experiments indicate that in monkeys prevention of dietary-induced hypercholesterolemia is less effective by ileal resection

than by ileal bypass (270) and that in rats sterol balance is not significantly more negative after the bypass than after resection (88,267). No detailed study seems to be available for human subjects in this respect.

The markedly augmented escape of glycine bile salt conjugates into the colon in ileal dysfunction appears to have a specific consequence; patients with ileal resection have been reported to have a marked oxaluria, which may lead to subsequent development of oxalate stones in the urinary tract (271). The suggested mechanism is that the glycine conjugates are split by colonic bacteria and the liberated glycine is converted by bacterial enzyme systems, e.g., to CO_2 and glyoxylate. The latter is absorbed and converted by the liver further to oxalate, which is then excreted into the urine. Oral administration of taurine, which normalizes the abnormal glycine/taurine ratio of bile acids in ileopathy (260,262), also abolishes the secondary hyperoxaluria found in patients with ileal resection (272).

3. Colon

Fecal bile acid excretion by patients with ulcerative colitis is usually within normal limits, suggesting that diarrhea associated with this disease is not induced by bile acids (273). Cholestyramine has no beneficial effect on the general condition, diarrhea, or fecal composition, findings which further strengthen the view that bile salts play no role in the diarrhea of ulcerative colitis and that bile salts have hardly any additional damaging effect on the diseased colonic mucosa. Fecal bile acid excretion is increased by cholestyramine significantly less in patients with ulcerative colitis than in control subjects, probably owing to negative caloric balance and possibly associated decrease of liver function (273).

The amount of secondary products of neutral steroids and bile acids in feces of patients with ulcerative colitis appears to be reduced as compared to normal subjects (88). Thus bacterial action on steroids is decreased, probably owing to enhanced colonic motility, and may lead to reduced absorption of primary and especially secondary bile acids from the colon. This may explain the absence or low level of lithocholic acid in serum (188,193) of these patients and does not support the concept that lithocholic acid causes the liver damage (212) found frequently in ulcerative colitis.

Patients with ileostomy, e.g., colectomized for ulcerative colitis, usually have normal bile salt output in ileostomal discharge provided that only a small segment of the terminal ileum has been removed and that the remaining small bowel is unaffected (129,274,275). Furthermore, as compared to normal feces, a relatively large portion of bile acids in dejecta is in conjugated form, and secondary bile acids are found infrequently in the ileal effluent or upper intestinal contents (129,276). These findings suggest that apart from deconjugation most of the bacterial action on bile acids takes place normally in

the colon in man. In addition, though bile salts are absorbed from the colon (48,49), quantitatively colonic absorption may be normally negligible or terminal ileum may be adapted to prevent any extra loss of bile salts after colectomy. It is interesting to note that though a large number of different bacteria colonize the terminal ileum after the colectomy and ileostomy (276, 277), patients apparently have no bacterial overgrowth in the upper small intestine, because only negligible amounts of unconjugated bile salts are found in the jejunal contents (275).

Serum cholesterol, which during active ulcerative colitis is markedly reduced, owing to insufficient synthesis to offset slightly increased cholesterol elimination as neutral steroids and occasionally as bile acids (273), increases rapidly after panproctocolectomy. This may be due to normalization of cholesterol elimination and augmented synthesis during improved nutritional state (275).

VIII. DRUGS AFFECTING BILE ACID EXCRETION

Of the hyperlipidemic drugs, clofibrate appears to decrease fecal elimination of bile acids (278–280). However, this decrease is less than the increase of the neutral steroid output, so the net elimination of cholesterol is increased (280). Thyroid hormones may occasionally, especially if associated with diarrhea, cause a marked increase in bile acid elimination (see Section VIIB), while nicotinic acid only occasionally augments fecal bile salt output (221, 281). Of the more recently developed absorbable hypolipidemic drugs, DH-581 appears to stimulate bile acid excretion at least transiently, probably by inhibiting intestinal bile acid reabsorption (282).

As discussed earlier (see Section VB2), cholestyramine is an unabsorbable bile acid sequestering agent which reduces serum cholesterol by markedly augmenting fecal elimination of bile acids in both normo- and hypercholesterolemic patients (Fig. 3; 11,90,94,153,154,283,284).

Neutral sterol excretion is not changed consistently (11,90), suggesting that biliary cholesterol secretion is decreased because absorption of exogenous and endogenous cholesterol is decreased (285,286). Cholestyramine administered to patients with T-tube has actually been shown to reduce both biliary bile acid and cholesterol secretion, decrement of cholic acid being less significant than that of chenodeoxycholic acid (287,288). Though large cholestyramine doses have been reported to cause fat malabsorption (111), no studies appear to be available dealing with the effect of long-term cholestyramine treatment on the cholesterol and bile acid concentrations and on micelle formation in the intestinal contents during fat absorption.

DEAE-Sephadex increases fecal bile acid elimination in patients with

familial hypercholesterolemia to an extent which at a comparable dose is almost as great as with cholestyramine (11). Colestipol is a newer bile acid sequestering agent (289). Lignin, which is present in, e.g., fiber fraction of vegetables, fruits, and grain, has been shown to bind bile acids *in vitro* (290) and probably also *in vivo* (128). Oral administration of lignin had, however, no significant effect on the turnover of labeled taurochalate, suggesting that its bile acid binding capacity is weak (290a). A hydrophilic colloid (Metamucil®) derived from the blond psyllium seed has been reported to increase fecal bile acids and decrease serum cholesterol (149). This agent lacks the bile salt binding property, its effect being probably due to trapping of bile acids into a large gelatinous fecal mass.

Neomycin precipitates bile acids *in vitro* (291), even from human bile (292). In view of these findings, it seemed logical to infer that earlier findings on neomycin-induced increase in fecal bile acid elimination (293–295) would have been caused by intraluminal precipitation of bile salts, perhaps associated with the antibacterial action of neomycin and its toxic effect on the intestinal mucosa. More recent studies have shown that neomycin (88,296) or its *N*-methylated derivative without antibacterial action (297) may increase fecal neutral sterols to a larger extent than bile acids. Neomycin treatment decreases the bile salt pool, has little effect on cholic acid half-life, and is virtually ineffective on the duodenal micellar bile salt and fatty acid concentrations after a fat meal, suggesting that bile salt loss has not been sufficient to impair the intraluminal phase of fat digestion (298). However, administration of neomycin simultaneously with the fat meal clearly disturbs micelle formation by precipitating fatty acids and somewhat less consistently bile acids and, probably secondarily, cholesterol (299). These findings offer an explanation for the neomycin-induced steatorrhea (292), augmented fecal bile acid and particularly neutral sterol excretion, and fall in the serum cholesterol level (300).

REFERENCES

1. H. Danielsson and K. Einarsson, *in* "The Biological Basis of Medicine" (E. E. Bittar and N. Bittar, eds.), Vol. 5, p. 279, Academic Press, London, New York (1969).
2. I. M. Weiner and L. Lack, *in* "Handbook of Physiology" (C. F. Code and W. Heidel, eds.), Vol. III, Section 6, p. 1439, Williams & Wilkins, Baltimore (1968).
3. S. Bergström, H. Danielsson, and B. Samuelsson, *in* "Lipid Metabolism" (K. Bloch, ed.), p. 291, Wiley, New York (1960).
4. H. Danielsson, *Advan. Lipid Res.* **1,** 335 (1963).
5. G. A. D. Haslewood, *Biol. Rev.* **39,** 537 (1964).
6. A. F. Hofmann, *Gastroenterology* **50,** 56 (1966).
7. A. F. Hofmann, *Gastroenterology* **52,** 752 (1967).
8. E. R. Schiff and J. M. Dietschy, *Am. J. Digest. Dis.* **14,** 432 (1969).

9. B. W. D. Badley, *Can. Med. Ass. J.* **102,** 159 (1970).
10. B. Borgström, A. Dahlqvist, T. Lindberg, and E. Hess Thaysen, *in* "The Biological Basis of Medicine" (E. E. Bittar and N. Bittar, eds.), Vol. 5, p. 385, Academic Press, London, New York (1969).
11. T. A. Miettinen, *Ann. Clin. Res.* **2,** 300 (1970).
12. I. Björkhem, H. Danielsson, K. Einarsson, and G. Johansson, *J. Clin. Invest.* **47,** 1573 (1968).
13. E. Staple and J. L. Rabinowitz, *Biochim. Biophys. Acta* **59,** 735 (1962).
14. J. B. Carey, Jr., and G. A. D. Haslewood, *J. Biol. Chem.* **238,** PC855 (1963).
15. J. B. Carey, Jr., *J. Clin. Invest.* **43,** 1443 (1964).
16. K. Anderson, E. Kok, and N. B. Javitt, *J. Clin. Invest.* **49,** 4a (1970).
17. J. L. Rabinowitz, R. H. Herman, D. Weinstein, and E. Staple, *Arch. Biochem. Biophys.* **114,** 233 (1966).
18. R. F. Hanson and J. B. Carey, Jr., *J. Clin. Invest.* **49,** 39a (1970).
19. T. Scherstén, *Acta Chir. Scand. Suppl.* **373,** 1 (1967).
20. J. Sjövall, *Proc. Soc. Exptl. Biol. Med.* **100,** 676 (1959).
21. R. H. Palmer, *Proc. Natl. Acad. Sci.* **58,** 1047 (1967).
22. B. A. Gordon, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem. Physiol.* **41,** 77 (1963).
23. J. Sjövall, *Clin. Chim. Acta* **5,** 33 (1960).
24. B. W. D. Badley, G. M. Murphy, and I. A. D. Bouchier, *Lancet* **2,** 400 (1969).
25. B. W. D. Badley, G. M. Murphy, I. A. D. Bouchier, and S. Scherlock, *Gastroenterology* **58,** 781 (1970).
26. G. M. McLeod and H. S. Wiggins, *Lancet* **1,** 873 (1968).
27. T. A. Miettinen and M. Siurala, *Z. Klin. Chem. Klin. Biochem.* **9,** 47 (1971).
28. B. W. Van Deest, J. S. Fordtran, S. G. Morawski, and J. D. Wilson, *J. Clin. Invest.* **47,** 1314 (1968).
29. A. F. Hofmann, *Biochem. J.* **89,** 57 (1963).
30. J. M. Dietschy, *J. Lipid Res.* **9,** 297 (1968).
31. B. Borgström, G. Lundh, and A. F. Hofmann, *Gastroenterology* **45,** 229 (1963).
32. W. J. Simmonds, A. F. Hofmann, and E. Theodor, *J. Clin. Invest.* **46,** 874 (1967).
33. I. G. Hislop, A. F. Hofmann, and L. J. Schoenfield, *J. Clin. Invest.* **46,** 1070 (1967).
34. T. Cronholm and J. Sjövall, *Europ. J. Biochem.* **2,** 375 (1967).
35. S. Lindstedt, *Acta Physiol. Scand.* **40,** 1 (1957).
36. S. Lindstedt, *Clin. Chim. Acta* **7,** 1 (1962).
37. S. Lindstedt, *in* "Atherosclerosis," Proceedings of the Second International Symposium (R. J. Jones, ed.), p. 262, Springer-Verlag, Berlin–Heidelberg–New York (1970).
38. H. Danielsson, P. Eneroth, K. Hellström, S. Lindstedt, and J. Sjövall, *J. Biol. Chem.* **238,** 2299 (1963).
39. P. Eneroth, B. Gordon, R. Ryhage, and J. Sjövall, *J. Lipid Res.* **7,** 511 (1966).
40. P. Eneroth, B. Gordon, and J. Sjövall, *J. Lipid Res.* **7,** 524 (1966).
41. A. Norman, *Brit. J. Nutr.* **18,** 173 (1964).
42. S. M. Grundy, E. H. Ahrens, Jr., and G. Salen, *J. Lipid Res.* **9,** 374 (1968).
43. R. S. Rosenfeld and L. Hellman, *Arch. Biochem. Biophys.* **97,** 406 (1962).
44. J. Sjövall, *Acta Chem. Scand.* **13,** 711 (1959).
45. K. Hellström and J. Sjövall, *Acta Physiol. Scand.* **51,** 218 (1961).
46. A. Norman and R. H. Palmer, *J. Lab. Clin. Med.* **63,** 986 (1964).
47. T. Hoshita, H. Yashima, S. Okada, and T. Yamada, *Hiroshima J. Med. Sci.* **17,** 105 (1968).
48. H. S. Mekhjian, S. F. Phillips, and A. F. Hofmann, *Gastroenterology* **54,** 1256 (1968).

49. P. Samuel, G. M. Saypol, E. Meilman, E. H. Mosbach, and M. Chafizadeh, *J. Clin. Invest.* **47,** 2070 (1968).
50. P. H. Ekdahl and J. Sjövall, *Acta Chir. Scand.* **114,** 439 (1957).
51. J. B. Carey, Jr., and G. Williams, *J. Clin. Invest.* **42,** 450 (1963).
52. D. H. Sandberg, J. Sjövall, K. Sjövall, and D. A. Turner, *J. Lipid Res.* **6,** 182 (1965).
53. A. F. Hofmann, L. J. Schoenfield, B. A. Kottke, and J. R. Poley, *in* "Methods in Medical Research" (R. E. Olson, ed.), Vol. 12, p. 149, Year Book Medical Publishers, Inc., Chicago (1970).
54. L. J. Schoenfield, J. Sjövall, and E. Perman, *Nature* **213,** 93 (1967).
55. S. M. Grundy, E. H. Ahrens, Jr., and T. A. Miettinen, *J. Lipid Res.* **6,** 397 (1965).
56. S. S. Ali, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem.* **44,** 957 (1966).
57. P. Eneroth, K. Hellström, and J. Sjövall, *Acta Chem. Scand.* **22,** 1729 (1968).
58. E. Evrard and G. Janssen, *J. Lipid Res.* **9,** 226 (1968).
59. L. Hellman, R. S. Rosenfeld, W. Insull, Jr., and E. H. Ahrens, Jr., *J. Clin. Invest.* **36,** 898 (1957).
60. R. B. Moore, J. T. Anderson, A. Keys, and I. D. Frantz, Jr., *J. Lab. Clin. Med.* **60,** 1000 (1962).
61. S. M. Grundy and E. H. Ahrens, Jr., *J. Clin. Invest.* **45,** 1503 (1966).
62. T. A. Miettinen, R. Pelkonen, E. A. Nikkilä, and O. Heinonen, *Acta Med. Scand.* **182,** 645 (1967).
63. T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **24,** Suppl. 110, 48 (1969).
64. T. A. Miettinen, *Europ. J. Clin. Invest.* **1,** 452 (1971)
65. S. Lindstedt and A. Norman, *Acta Physiol. Scand.* **38,** 121 (1956).
66. M. M. Stanley and B. Nemchausky, *J. Lab. Clin. Med.* **70,** 627 (1967).
67. W. E. Meihoff and F. Kern, Jr., *J. Clin. Invest.* **47,** 261 (1968).
68. L. Lack and I. M. Weiner, *Gastroenterology* **52,** 282 (1967).
69. B. A. Kottke, *Circulation* **40,** 13 (1969).
70. K. Hellström and S. Lindstedt, *J. Lab. Clin. Med.* **63,** 666 (1964).
71. K. Hellström, *Acta Physiol. Scand.* **63,** 21 (1965).
72. S. Lindstedt, J. Avigan, D. S. Goodman, J. Sjövall, and D. Steinberg, *J. Clin. Invest.* **44,** 1754 (1965).
73. K. Hellström and S. Lindstedt, *Am. J. Clin. Nutr.* **18,** 46 (1966).
74. J. Wollenweber, B. A. Kottke, and C. A. Owen, Jr., *J. Lab. Clin. Med.* **69,** 584 (1967).
75. W. G. M. Hardison and I. H. Rosenberg, *New Engl. J. Med.* **277,** 337 (1967).
76. W. I. Austad, L. Lack, and M. P. Tyor, *Gastroenterology* **52,** 638 (1967).
77. K. W. Heaton, W. I. Austad, L. Lack, and M. P. Tyor, *Gastroenterology* **55,** 5 (1968).
78. R. Abaurre, S. G. Gordon, J. G. Mann, and F. Kern, Jr., *Gastroenterology* **57,** 679 (1969).
79. B. Josephson, *Physiol. Rev.* **21,** 463 (1941).
80. E. Theodor, N. Spritz, and M. H. Sleisenger, *Gastroenterology* **55,** 183 (1968).
81. M. Blum and N. Spritz, *J. Clin. Invest.* **45,** 187 (1966).
82. L. A. Turnberg and G. Grahame, *Gut* **11,** 126 (1970).
83. K. W. Heaton and T. S. Low-Beer, *in* "Bile-Salt-Meeting" (P. Back, ed.), p. 69, Freiburg (October 5, 1970).
84. T. A. Miettinen and J. Perheentupa, *Scand. J. Clin. Lab. Invest.* **27,** Suppl. 116, 36 (1971).
85. E. H. Ahrens, Jr., *in* "Fourteenth International Conference on the Biochemistry of Lipids," p. 13, Lund (1970).
86. J. D. Wilson and C. A. Lindsey, Jr., *J. Clin. Invest.* **44,** 1805 (1965).
87. S. M. Grundy, E. H. Ahrens, Jr., and J. Davignon, *J. Lipid Res.* **10,** 304 (1969).
88. T. A. Miettinen, unpublished observations.

89. T. A. Miettinen, *in* "Atherosclerosis," Proceedings of the Second International Symposium (R. J. Jones, ed.), p. 508, Springer-Verlag, Berlin–Heidelberg–New York (1970).
90. T. A. Miettinen, *in* "Atherosclerosis," Proceedings of the Second International Symposium (R. J. Jones, ed.), p. 558, Springer-Verlag, Berlin–Heidelberg–New York (1970).
91. S. Shefer, S. Hauser, I. Bekersky, and E. H. Mosbach, *J. Lipid Res.* **11,** 404 (1970).
92. J. M. Dietschy and J. D. Wilson, *New Engl. J. Med.* **282,** 1128, 1179, 1241 (1970).
93. D. M. Tennent, S. A. Hashim, and T. B. Van Itallie, *Fed. Proc.* **21,** Suppl. 11, 77 (1962).
94. S. M. Grundy, A. F. Hofmann, J Davignon, and E. H. Ahrens. Jr., *J. Clin. Invest.* **45,** 1018 (1966).
95. R. B. Moore, I. D. Frantz, Jr., R. L. Varco, and H. Buchwald, *in* "Atherosclerosis," Proceedings of the Second International Symposium (R. J. Jones, ed.), p. 295, Springer-Verlag, Berlin–Heidelberg–New York (1970).
96. J. L. Thistle and L. J. Schoenfield, *New Engl. J. Med.* **284,** 177 (1971).
97. T. A. Miettinen, *Progr. Biochem. Pharmacol.* **4,** 68 (1968).
98. G. Salen, E. H. Ahrens, Jr., and S. M. Grundy, *J. Clin. Invest.* **49,** 84a (1970).
99. N. B. Myant and H. A. Eder, *J. Lipid Res.* **2,** 363 (1961).
100. J. B. Carey, Jr., and G. Williams, *Gastroenterology* **56,** 1249 (1969).
101. R. Fiasse, R. Eyssen, C. Dive, C. Harvengt, P. J. Kestens, and C. Nagant de Deuxchaisnes, *in* "Advance Abstracts," Fourth World Congress of Gastroenterology (P. Riis, P. Anthonisen, and H. Baden, eds.), p. 354, Copenhagen (1970).
102. A. F. Hofmann, *in* "Bile-Salt-Meeting" (P. Back, ed.), p. 1, Freiburg (October 5, 1970).
103. I. Sperber, *Pharmacol. Rev.* **11,** 109 (1959).
104. R. Preisig, H. Bucher, H. Stirnemann, and J. Tauber, *Rev. Franc. Etud. Clin. Biol.* **14,** 151 (1969).
105. T. Scherstén, S. Nilsson, E. Cahlin, M. Filipson, and G. Brodin-Persson, *Europ. J. Clin. Invest.* **1,** 242 (1971).
106. L. Swell and C. C. Bell, Jr., *Am. J. Digest. Dis.* **13,** 1077 (1968).
107. S. Nilsson and T. Scherstén, *Gastroenterology* **57,** 525 (1969).
108. S. Nilsson and T. Scherstén, *Europ. J. Clin. Invest.* **1,** 109 (1970).
109. J. H. Payne, L. T. DeWind, and R. R. Commons, *Am. J. Surg.* **106,** 273 (1963).
110. J. G. Maxwell, R. C. Richards, and D. Albo, Jr., *Am. J. Surg.* **116,** 648 (1968).
111. I. E. Danhof, *Am. J. Clin. Nutr.* **18,** 343 (1966).
112. A. F. Hofmann and B. Borgström, *J. Clin. Invest.* **43,** 247 (1964).
113. J. M. Johnston, *in* "Handbook of Physiology" (C. F. Code and W. Heidel, eds.), Vol. III, Section 6, p. 1353, Williams & Wilkins, Baltimore (1968).
114. J. R. Senior, *J. Lipid Res.* **5,** 495 (1964).
115. R. G. H. Morgan and B. Borgström, *Quart. J. Exptl. Physiol.* **54,** 228 (1969).
116. B. Borgström, *J. Clin. Invest.* **39,** 809 (1960).
117. C. R. Treadwell and G. V. Vahouny, *in* "Handbook of Physiology" (C. F. Code and W. Heidel, eds.), Vol. III, Section 6, p. 1407, Williams & Wilkins, Baltimore (1968).
118. B. Arnesjö, Å. Nilsson, J. Barrowman, and B. Borgström, *Scand. J. Gastroenterol.* **4,** 653 (1969).
119. T. A. Miettinen, *Lancet* **ii,** 358 (1968).
120. M. Bergmann, *Wien. Klin. Wchschr.* **64,** 704 (1952).
121. W. Forth, W. Rummel, and H. Glasser, *Naunyn-Schmiedbergs Arch. Exptl. Pathol. Pharmakol.* **254,** 364 (1966).
122. S. F. Phillips, H. S. Mekhjian, and A. F. Hofmann, *in* "Advance Abstracts," Fourth World Congress of Gastroenterology (P. Riis, P. Anthonisen, and H. Baden, eds.), p. 563, Copenhagen (1970).
123. G. G. Rowe, *Gastroenterology* **53,** 1006 (1967).

124. A. F. Hofmann and J. R. Poley, *New Engl. J. Med.* **281,** 397 (1969).
125. K. Sickinger, *Deutsch. Med. Wchschr.* **94,** 1151 (1969).
126. G. Bahls, *Z. Gastroenterol.* **8,** 41 (1970).
127. T. A. Miettinen and M. Lempinen, *Scand. J. Clin. Lab. Invest.* **25,** Suppl. 113, 55 (1970).
128. M. A. Eastwood and R. H. Girdwood, *Lancet* **ii,** 1170 (1968).
129. T. A. Miettinen and P. Peltokallio, *Scand. J. Gastroenterol.* **6,** 543 (1971).
130. J. R. Poley, J. C. Dower, C. A. Owen, Jr., and G. B. Stickler, *J. Lab. Clin. Med.* **63,** 838 (1964).
131. A. M. Bongiovanni, *J. Clin. Endocrinol. Metab.* **25,** 678 (1965).
132. J.-C. Encrantz and J. Sjövall, *Acta Chem. Scand.* **11,** 1093 (1957).
133. J.-C. Encrantz and J. Sjövall, *Clin. Chim. Acta* **4,** 793 (1959).
134. D. H. Sandberg, *Pediat. Res.* **4,** 262 (1970).
135. T. A. Miettinen and T. Luukkainen, *Acta Chem. Scand.* **22,** 2603 (1968).
136. T. A. Miettinen, *Circulation.* **44,** 842 (1971).
137. R. B. Moore, J. T. Anderson, H. L. Taylor, A. Keys, and I. D. Frantz, Jr., *J. Clin. Invest.* **47,** 1517 (1968).
138. T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **25,** Suppl. 113, 28 (1970).
139. T. A. Miettinen, *Clin. Chim. Acta* **19,** 341 (1968).
140. M. M. Stanley, *Metabolism* **19,** 865 (1970).
141. T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **21,** Suppl. 101, 20 (1968).
142. J. Avigan and D. Steinberg, *J. Clin. Invest.* **44,** 1845 (1965).
143. S. S. Ali, A. Kuksis, and J. M. R. Beveridge, *Can. J. Biochem.* **44,** 1377 (1966).
144. N. Spritz, E. H. Ahrens, Jr., and S. M. Grundy, *J. Clin. Invest.* **44,** 1482 (1965).
145. S. M. Grundy and E. H. Ahrens, Jr., *J. Clin. Invest.* **49,** 1135 (1970).
146. W. E. Connor, D. T. Witiak, D. B. Stone, and M. L. Armstrong, *J. Clin. Invest.* **48,** 1363 (1969).
147. P. D. S. Wood, R. Shioda, and L. W. Kinsell, *Lancet* **2,** 604 (1966).
148. H. S. Sodhi, P. D. S. Wood, G. Schlierf, and L. W. Kinsell, *Metabolism* **16,** 334 (1967).
149. D. T. Forman, J. E. Garvin, J. E. Forestner, and C. B. Taylor, *Proc. Soc. Exptl. Biol. Med.* **127,** 1060 (1968).
150. D. M. Small, *New Engl. J. Med.* **284,** 214 (1971).
151. H. S. Sodhi, personal communication (1971).
152. S. Lindstedt and E. H. Ahrens, Jr., *Proc. Soc. Exptl. Biol. Med.* **108,** 286 (1961).
153. R. B. Moore, C. A. Crane, and I. D. Frantz, Jr., *J. Clin. Invest.* **47,** 1664 (1968).
154. C. D. Moutafis and N. B. Myant, *Clin. Sci.* **37,** 443 (1969).
155. C. D. Moutafis, N. B. Myant, and S. Tabaqchali, *Clin. Sci.* **35,** 537 (1968).
156. R. B. Moore, I. D. Frantz, Jr., and H. Buchwald, *Surgery* **65,** 98 (1969).
157. R. Blomstrand, *Proc. Soc Exptl. Biol. Med.* **107,** 126 (1961).
158. S. M. Grundy and E. H. Ahrens, Jr., *J. Lipid Res.* **10,** 91 (1969).
159. D. Kritchevsky, *Metabolism* **9,** 984 (1960).
160. N. B. Myant, *in* "The Biological Basis of Medicine" (E. E. Bittar and N. Bittar, eds.), Vol. 2, p. 193, Academic Press, London, New York (1968).
161. K. Hellström and S. Lindstedt, *J. Lab. Clin. Med.* **63,** 666 (1964).
162. T. A. Miettinen, *J. Lab. Clin. Med.* **71,** 537 (1968).
163. R. B. Failey, Jr., E. Brown, and M. E. Hodes, *Am. J. Clin. Nutr.* **11,** 4 (1962).
164. W. R. J. Middleton, *Gut* **12,** 172 (1971).
165. J. R. Poley, cited by A. F. Hofmann, *in* "Bile-Salt-Meeting" (P. Back, ed.), Freiburg (October 5, 1970).
166. E. A. Nikkilä, *in* "Advances in Lipid Research" (R. Paoletti and D. Kritchevsky, eds.), Vol. 7, p. 63, Academic Press, New York, London (1969).

167. B. Isaksson, *Acta Soc. Med. Uppsal.* **59,** 296 (1954).
168. W. H. Admirand and D. M. Small, *J. Clin. Invest.* **47,** 1043 (1968).
169. D. M. Small, *New Engl. J. Med.* **279,** 588 (1968).
170. I. A. D. Bouchier and J. W. Freston, *Lancet* **1,** 340 (1968).
171. T. Scherstén, S. Nilsson, and E. Cahlin, *Scand. J. Gastroenterol.* **5,** 473 (1970).
172. Z. R. Vlahcevic, C. C. Bell, Jr., and L. Swell, *Gastroenterology* **59,** 62 (1970).
173. D. M. Small and S. Rapo, *New Engl. J. Med.* **283,** 53 (1970).
174. Z. R. Vlahcevic, C. C. Bell, Jr., I. Buhac, J. T. Farrar, and L. Swell, *Gastroenterology* **59,** 165 (1970).
175. E. Thureborn, *Acta Chir. Scand. Suppl.* **303** (1962).
176. H. Sarles, J. Hauton, N. E. Planche, H. Lafont, and A. Gerolami. *Am. J. Digest. Dis.* **15,** 251 (1970).
177. K. W. Heaton and A. E. Read, *Brit. Med. J.* **3,** 494 (1969).
178. S. Cohen, M. Kaplan, L. Gottlieb, and J. Patterson, *Gastroenterology* **60,** 237 (1971).
179. L. J. Schoenfield, J. Sjövall, and K. Sjövall, *J. Lab. Clin. Med.* **68,** 186 (1966).
180. J. F. Davidson, *Am. J. Med. Sci.* **244,** 703 (1962).
181. T. A. Miettinen and M. Siurala, *Scand. J. Gastroenterol.* **6,** 527 (1971).
182. T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **27,** Suppl. 116, 56 (1971).
183. L. A. Turnberg and G. Grahame, *Gut* **11,** 126 (1970).
184. G. D. Friedman, W. B. Kannel, and T. R. Dawber, *J. Chronic Dis.* **19,** 273 (1966).
185. H. Dam, *World Rev. Nutr. Dietet.* **11,** 199 (1969).
186. Y. Hikasa, S. Matsuda, M. Nagase, M. Yoshinaga, T. Tobe, I. Maruyama, R. Shioda, H. Tanimura, R. Muraoka, H. Muroya, and M. Togo, *Arch. Jap. Chir.* **38**(1), 107 (1969).
187. J. L. Thistle and L. J. Schoenfield, *J. Lab. Clin. Med.* **74,** 1020 (1969).
188. J. B. Carey, Jr., I. D. Wilson, F. G. Zaki, and R. F. Hanson, *Medicine* **45,** 461 (1966).
189. P. Holsti, *Nature* **186,** 250 (1960).
190. P. Holsti, *Acta Pathol. Microbiol. Scand.* **54,** 479 (1962).
191. W. Erb, J. Schreiber, and E. Böhle, *in* "Advance Abstracts," Fourth World Congress of Gastroenterology (P. Riis, P. Anthonisen, and H. Baden, eds.), p. 184, Copenhagen (1970).
192. J. B. Carey, Jr., *J. Lab. Clin. Med.* **56,** 797 (1960).
193. G. Neale, B. Lewis, V. Weaver, and D. Panveliwalla, *Gut* **12,** 145 (1971).
194. P. -H. Ekdahl, *Acta Chir. Scand.* **115,** 208 (1958).
195. J. B. Carey, Jr., *J. Clin. Invest.* **37,** 1494 (1958).
196. D. Rudman and F. E. Kendall, *J. Clin. Invest.* **36,** 530 (1957).
197. J. Roovers, E. Evrard, and H. Vanderhaeghe, *Clin. Chim. Acta* **19,** 449 (1968).
198. I. Makino, S. Nakagawa, and K. Mashimo, *Gastroenterology* **56,** 1033 (1969).
199. J. A. Gregg, *Am. J. Clin. Pathol.* **49,** 404 (1968).
200. J. B. Carey, Jr., R. F. Hanson, and B. K. Monson, *Gastroenterology* **56,** 1142 (1969).
201. G. Paumgartner, *in* "Alkohol und Leber" (W. Gerek, K. Sickinger, and H. H. Henneheuser, eds.), p. 435, F. K. Schattauer Verlag, Stuttgart and New York (1971).
202. J. B. Carey, Jr., I. D. Wilson, G. Onstad, and F. G. Zaki, *J. Clin. Invest.* **46,** 1042 (1967).
203. T. A. Miettinen and M. Siurala, *Scand. J. Clin. Lab. Invest.* **19,** Suppl. 95, 69 (1967).
204. T. A. Miettinen and M. Siurala, *in* "Advance Abstracts," Fourth World Congress of Gastroenterology (P. Riis, P. Anthonisen, and H. Baden, eds.), p. 358, Copenhagen (1970).
205. T. B. Van Itallie and S. A. Hashim, *Med. Clin. N. Am.* **47,** 629 (1963).
206. K. Sjövall and J. Sjövall, *Clin. Chim. Acta* **13,** 207 (1966).
207. M. Rautureau, B. Chevrel, and J. Caroli, *Rev. Med.-Chir. Mal. Foié* **42,** 167 (1967).

208. B. Lewis, D. Panveliwalla, S. Tabaqchali, and I. D. P. Wootton, *Lancet* **1,** 219 (1969).
209. E. H. Ahrens, Jr., M. A. Payne, H. G. Kunkel, W. J. Eisenmenger, and S. H. Blondheim, *Medicine* **29,** 299 (1950).
210. J. B. Carey, Jr., and G. Williams, *Science* **150,** 620 (1965).
211. C. N. Williams, R. Kaye, and J. R. Senior, *Gastroenterology* **60,** 178 (1971).
212. J. B. Carey, Jr., *Gastroenterology* **46,** 490 (1964).
213. M. Modai and E. Theodor, *Gastroenterology* **58,** 379 (1970).
214. B. Frosch and H. Wagener, *Klin. Wchschr.* **46,** 913 (1968).
215. B. Frosch and H. Wagener, *Deutsch. Med. Wchschr.* **93,** 1754 (1968).
216. T. Cronholm, A. Norman, and J. Sjövall, *Scand. J. Gastroenterol.* **5,** 297 (1970).
217. A. Norman, B. Strandvik, and R. Zetterström, *Acta Paediat. Scand.* **58,** 59 (1969).
218. G. Paumgartner, G. Grabner, H. Brunner, and K. Hauth, *Wien. Z. Inn. Med. Grenzg.* **5,** 171 (1969).
219. E. C. Osborn, I. D. P. Wootton, L. C. da Silva, and S. Sherlock, *Lancet* **2,** 1049 (1959).
220. B. Frosch, H. Wagener, and H. Immich, *Clin. Chim. Acta* **22,** 294 (1968).
221. P.-H. Ekdahl, A. Gottfries, and T. Scherstén, *Bull. Soc. Int. Chir.* **25,** 83 (1966).
222. T. A. Miettinen, *Clin. Chim. Acta* **20,** 43 (1968).
223. J. M. Dietschy and W. G. Gamel, *J. Clin. Invest.* **50,** 872 (1971).
224. J. A. Glomset, *J. Lipid Res.* **9,** 155 (1968).
225. K. B. Turner, G. H. McCormack, Jr., and A. Richards, *J. Clin. Invest.* **32,** 801 (1953).
226. E. Gjone and J. P. Blomhoff, *Scand. J. Gastroenterol.* **5,** 305 (1970).
227. R. A. Cooper and J. H. Jandl, *J. Clin. Invest.* **47,** 809 (1968).
228. A. Kappas and R. H. Palmer, *Pharmacol. Rev.* **15,** 123 (1963).
229. C. L. Krone, E. Theodor, M. H. Sleisenger, and G. H. Jeffries, *Medicine (Baltimore)* **47,** 89 (1968).
230. W. L. W. Go, J. R. Poley, A. F. Hofmann, and W. H. J. Summerskill, *Gastroenterology* **58,** 638 (1970).
231. W. Creutzfeldt and H. Schmidt, *Scand. J. Gastroenterol.* **5,** Suppl. 6, 47 (1970).
232. W. M. Capper, *Ann. Roy. Coll. Surg. Engl.* **40,** 21 (1967).
233. J. Rhodes, D. E. Barnardo, S. F. Phillips, R. A. Rovelstad, and A. F. Hofmann, *Gastroenterology* **57,** 241 (1969).
234. B. S. Bedi, H. T. Debas, G. Gillespie, and I. E. Gillespie, *Gastroenterology* **60,** 256 (1971).
235. T. Midtvedt, A. Norman, and K. Nygaard, *Scand. J. Gastroenterol.* **5,** 237 (1970).
236. A. Norman, *Scand. J. Gastroenterol.* **5,** 231 (1970).
237. R. M. Donaldson, Jr., *J. Clin. Invest.* **44,** 1815 (1965).
238. S. Tabaqchali and C. C. Booth, *Lancet* **ii,** 12 (1966).
239. I. H. Rosenberg, W. G. Hardison, and D. M. Bull, *New Engl. J. Med.* **276,** 1391 (1967).
240. J. S. Stewart, D. J. Pollock, A. V. Hoffbrand, D. L. Mollin, and C. C. Booth, *Quart. J. Med.* **36,** 425 (1967).
241. J. S. Fordtran, F. C. Rector, Jr., T. W. Locklear, and M. F. Ewton, *J. Clin. Invest.* **46,** 287 (1967).
242. A. M. Dawson and K. J. Isselbacher, *J. Clin. Invest.* **39,** 730 (1960).
243. J. D. Hamilton, N. H. Dyer, A. M. Dawson, F. W. O'Grady, A. Vince, J. C. B. Fenton, and D. L. Mollin, *Quart. J. Med.* **39,** 265 (1970).
244. S. Tabaqchali, *Scand. J. Gastroenterol.* **5,** Suppl. 6, 139 (1970).
245. A. Norman and M. S. Shorb, *Proc. Soc. Exptl. Biol. Med.* **110,** 552 (1962).
246. A. Norman and R. Grubb, *Acta Pathol. Microbiol. Scand.* **36,** 537 (1955).
247. O. W. Portman, S. Shah, A. Antonis, and B. Jorgensen, *Proc. Soc. Exptl. Biol. Med.* **109,** 959 (1962).

248. B. S. Drasar, M. J. Hill, and M. Shiner, *Lancet* **i,** 1237 (1966).
249. M. J. Hill and B. S. Drasar, *Gut* **9,** 22 (1968).
250. T. Midtvedt and A. Norman, *Acta Pathol. Microbiol. Scand.* **71,** 629 (1967).
251. K. Shimada, K. S. Bricknell, and S. M. Finegold, *J. Infect. Dis.* **119,** 273 (1963).
252. R. H. Dowling and D. M. Small, *Gastroenterology* **54,** 1291 (1968).
253. A. F. Hofmann and D. M. Small, *Ann. Rev. Med.* **18,** 333 (1967).
254. S. Tabaqchali, J. Hatzioannou, and C. C. Booth, *Lancet* **ii,** 12 (1968).
255. S. L. Gorbach and S. Tabaqchali, *Gut* **10,** 963 (1969).
256. G. Egger and J. Kessler, *Schweiz. Med. Wchschr.* **99,** 609 (1969).
257. P. C. Bewes, G. A. D. Haslewood, and R. A. Roxburgh, *Brit. J. Surg.* **53,** 709 (1966).
258. D. Panveliwalla, S. Tabaqchali, I. D. P. Wootton, and B. Lewis, *in* "Nutrition," Proceedings of the Eighth International Congress (J. Masek, K. Osancová, and D. P. Cuthbertson, eds.), No. 213, p. 448, Excerpta Medica, Amsterdam (1970).
259. P. Panveliwalla, B. Lewis, I. D. P. Wootton, and S. Tabaqchali, *J. Clin. Pathol.* **23,** 309 (1970).
260. J. T. Garbutt, K. W. Heaton, L. Lack, and M. P. Tyor, *Gastroenterology* **56,** 711 (1969).
261. E. Hess Thaysen, A. Bruusgaard, and B. Eriksen, *Scand. J. Gastroenterol.* **5,** 39 (1970).
262. A. Bruusgaard and E. Hess Thaysen, *Acta Med. Scand.* **188,** 547 (1970).
263. A. F. Hofmann and S. M. Grundy, *Clin. Res.* **13,** 254 (1965).
264. W. G. M. Hardison and I. H. Rosenberg, *Am. J. Clin. Nutr.* **18,** 312 (1966).
265. W. G. Hardison and I. H. Rosenberg, *Clin. Res.* **13,** 536 (1965).
266. M. M. Stanley and B. Nemchausky, *Clin. Res.* **14,** 306 (1966).
267. R. J. Usenius and T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **21,** Suppl. 101, 78 (1968).
268. A. F. Hofmann, J. R. Poley, H. S. Mekhjian, and S. F. Phillips, *J. Clin. Invest.* **49,** 44a (1970).
269. H. Buchwald and R. L. Varco, *J.A.M.A.* **196,** 627 (1966).
270. H. W. Scott, Jr., S. E. Stephenson, Jr., G. H. Shepard, M. Welborn, Jr., J. E. Wimberly, and R. K. Younger, *J. Cardiovasc. Surg.* **9,** 553 (1968).
271. A. F. Hofmann, P. J. Thomas, L. H. Smith, and J. T. McCall, *Gastroenterology* **58,** 960 (1970).
272. W. H. Admirand, D. Earnest, and H. Williams, *Clin. Res.* **19,** 562 (1971).
273. T. A. Miettinen, *Gut* **12,** 632 (1971).
274. I. W. Percy-Robb, K. N. Jalan, J. P. A. McManus, and W. Sircus, *Brit. J. Surg.* **56,** 694 (1969).
275. T. A. Miettinen and P. Peltokallio, *Scand. J. Clin. Lab. Invest.* **25,** Suppl. 113, 55 (1970).
276. I. W. Percy-Robb, W. A. Telfer Brunton, K. N. Jalan, J. P. A. McManus, J. C. Gould, and W. Sircus, *Gut* **10,** 1049 (1969).
277. S. L. Gorbach, L. Nahas, L. Weinstein, R. Levitan, and J. F. Patterson, *Gastroenterology* **53,** 874 (1967).
278. W. D. Mitchell, A. S. Truswell, and B. Bronte-Stewart, *in* "Progerss in Biochemical Pharmacology" (D. Kritchevsky, R. Paoletti, and D. Steinberg, eds.), Vol. 2, p. 365, Basel and New York (1967).
279. W. D. Mitchell, personal communication (1969).
280. S. M. Grundy, E. H. Ahrens, Jr., G. Salen, and E. Quintao, *J. Clin. Invest.* **48,** 33a (1969).
281. T. A. Miettinen, *in* "Proceedings of the Workshop on Metabolic Effects of Nicotinic Acid and Its Derivatives" (K. F. Gey and L. A. Carlson, eds.), p. 835, Hans Huber Publ., Bern and Stuttgart (1971).
282. T. A. Miettinen, *Scand. J. Clin. Lab. Invest.* **27,** Suppl. 116, 17 (1971).

283. J. B. Carey, Jr., and G. Williams, *J.A.M.A.* **176,** 432 (1961).
284. S. A. Hashim and T. B. Van Itallie, *J.A.M.A.* **192,** 289 (1965).
285. S. A. Hyun, G. V. Vahouny, and C. R. Treadwell, *Proc. Soc. Exptl. Biol. Med.* **112,** 496 (1963).
286. S. D. Splitter, G. D. Michaels, G. Schlierf, P. D. S. Wood, and L. W. Kinsell, *Metabolism* **17,** 1129 (1968).
287. A. H. Juul and W. Van der Linden, *Acta Physiol. Pharmacol. Neerl.* **15,** 469 (1969).
288. H. Sarles, C. Crotte, A. Gerolami, A. Mule, N. Domingo, and J. Hauton, *Scand. J. Gastroenterol.* **5,** 603 (1970).
289. T. M. Parkinson, K. Gundersen, and N. A. Nelson, *J. Atherosclerosis Res.* **11,** 531 (1970).
290. M. A. Eastwood and D. Hamilton, *Biochim. Biophys. Acta* **152,** 165 (1968).
290a. K. W. Heaton, S. T. Heaton, and R. E. Barry, *Scand. J. Gastroenterol.* **6,** 281 (1971).
291. P. De Somer, H. Vanderhaeghe, and H. Eyssen, *Nature* **204,** 1306 (1964).
292. W. W. Faloon, I. C. Paes, D. Woolfolk, H. Nankin, K. Wallace, and E. N. Haro, *Ann. N.Y. Acad. Sci.* **132,** 879 (1966).
293. G. A. Goldsmith, J. G. Hamilton, and O. N. Miller, *Arch. Int. Med.* **105,** 512 (1960).
294. R. C. Powell, W. T. Nunes, R. S. Harding, and J. B. Vacca, *Am. J. Clin. Nutr.* **11,** 156 (1962).
295. J. G. Hamilton, O. N. Miller, and G. A. Goldsmith, *Fed. Proc.* **19,** 187 (1960).
296. W. W. Faloon, A. Rubulis, and M. Rubert, *Clin. Res.* **17,** 158 (1969).
297. J. F. Van den Bosch and P. J. Claes, *in* "Progress in Biochemical Pharmacology" (D. Kritchevsky, R. Paoletti, and D. Steinberg, eds.), Vol. 2, p. 97, S. Karger, Basel and New York (1967).
298. W. G. M. Hardison and I. H. Rosenberg, *J. Lab. Clin. Med.* **74,** 564 (1969).
299. G. R. Thompson, J. Barrowman, L. Gutierrez, and R. H. Dowling, *J. Clin. Invest.* **50,** 319 (1971).
300. P. Samuel, C. M. Holtzman, and J. Goldstein, *Circulation* **35,** 938 (1967).

Chapter 8

EFFECT OF HORMONES ON BILE ACID METABOLISM*

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I. INTRODUCTION

This chapter deals largely with the action of thyroid hormones on bile acid metabolism, because few definitive studies with other hormones (e.g., pituitary, adrenal, gonadal) have been carried out so far.

II. THYROID HORMONES

In most mammalian species, serum cholesterol concentrations vary inversely with thyroid function. However, the rate of cholesterol synthesis is reduced in the hypothyroid state and enhanced in hyperthyroidism. These apparently contradictory observations have been explained in terms of changes in steady-state conditions. For example, increased cholesterol synthesis in hyperthyroid animals would be expected to lead to hypercho-

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lesterolemia. Since this does not occur, it has been assumed that the excretion of cholesterol and bile acids must also be increased in the hyperthyroid state. The end result of these competing processes (increase in synthesis and an even greater increase in removal) is a new steady state in which the serum cholesterol concentration is reduced. Conversely, the increase of serum cholesterol in hypothyroidism is attributed to a decrease in metabolism and excretion in relation to synthesis (1).

The effects of thyroid hormone on cholesterol metabolism have been studied in bile fistula rats: Such preparations exhibit an inverse relationship between serum cholesterol levels and biliary cholesterol concentrations. The level of biliary cholesterol is directly related to thyroid activity, and the biliary excretion of cholesterol increases in the hyperthyroid state and decreases in the hypothyroid state (2,3).

Since, in the rat, cholesterol is eliminated largely in the form of bile acids, it was expected that bile acid secretion in bile would be increased in the hyperthyroid state. Early experiments to test this point indicated that biliary bile acid secretion was actually normal or below normal (2,3). These results can be explained in terms of the inadequate analytical procedures then in use. Only cholate secretion was measured, and the levels of chenodeoxycholate were not taken into account. When both of these bile acids were determined, it was shown that, in the bile fistula rat, the total production of bile acids was about the same in the hyperthyroid as in the euthyroid state, and lower in the hypothyroid state (4). In addition, in the hyperthyroid state, the normal ratio of cholate/chenodeoxycholate was reversed from approximately 3:1 to 1:3—cholic acid synthesis was decreased, and chenodeoxycholic acid synthesis was increased two- to threefold (4). Identical results were obtained in the bile fistula rat treated with noncalorigenic doses of D-triiodothyronine (5,6), suggesting that these effects are not necessarily a function of the basal metabolic rate.

Experiments comparing bile fistula rats with intact rats are difficult to interpret. In the bile fistula rat, the enterohepatic circulation of bile acids is interrupted, feedback inhibition by the circulating pool is abolished, and bile acid synthesis is presumably at a maximum. The effects of thyroid hormone are thus superimposed on a system that is operating already at or near maximal capacity. The stimulation of bile acid output by thyroid hormone can thus be seen more readily in the intact rat, where bile acid synthesis is under feedback regulation by the circulating bile acid pool.

In intact rats (180–250 g), treatment with thyroid hormones to induce the hyperthyroid state resulted in an increase in total bile acid output. Normal rats produced an average of 3.9 mg of cholate and 1 mg of chenodeoxycholate per day, while hyperthyroid animals secreted 5.3 mg of cholate and 2.9 mg of chenodeoxycholate per day (7). The synthesis of chenodeoxy-

cholate was stimulated to a greater extent, resulting in a change in the cholate/chenodeoxycholate ratio similar to that found in bile fistula rats. The average size of the cholate pool was determined by analysis of the intestinal contents. In the thyroid-treated animals, the pool (14.5 mg) was similar to that of the normal controls (12.5 mg). The size of the chenodeoxycholate pool increased from 3.2 to 8.1 mg, resulting in an increase of the total bile acid pool from 15.7 to 22.7 mg. The half-lives of the bile acids in normal and thyroid hormone treated animals did not differ significantly.

Thyroid activity also influences bile acid conjugation. In ten hypothyroid patients, the mean ratio of glycine-conjugated/taurine-conjugated bile acids was 8.8. The mean value after treatment with desiccated thyroid or thyroxine to restore a euthyroid state was 3.4, corresponding quite closely to the mean value of 3.1 for normal subjects (8).

In patients with hypothyroidism, cholic acid has a longer half-life (3. 8 days) and a slower turnover (0.22 g/day) than in patients with hyperthyroidism (half-life of 2.1 days and turnover of 0.33 g/day) (9). Treatment of the hypothyroid subjects with desiccated thyroid, L-triiodothyronine, or L-thyroxine resulted in an increase in the turnover of cholic acid amounting to 0.15 g/day. The faster bile acid turnover was accompanied by decreases of serum cholesterol. The ratio of glycine conjugates/taurine conjugates was also determined. It was found that the glycine/taurine ratio for euthyroid patients was 3.2, for patients with hyperfunctional thyroid 1.9, and for hypofunctional thyroid 6.3. When the hypothyroid patients were treated with thyroid hormone, the ratio was reduced to 1.9, confirming a regulatory effect of thyroid hormone on the conjugating mechanism.

Beher *et al.* (10) studied sterol and bile acid excretion in normal, thyroidectomized, hypophysectomized, and hypophysectomized thyroid hormone treated rats. They found that hypophysectomized rats eliminated cholesterol at a rate markedly lower than normal. In these animals, after a dose of DL-mevalonic acid-2-^{14}C, the specific activity of the β-sterol pool was high (i.e., sterol synthesis was low); consequently, bile acid synthesis was low. Since a major pathway of cholesterol elimination was thus blocked, the expected accumulation of cholesterol was found in the serum, organs, and carcass. The thyroidectomized animals again exhibited a sterol and bile acid excretion similar to normal. The hypophysectomized thyroid hormone treated animals showed a reduction of serum cholesterol concentration, an increased conversion of sterol to bile acids, and a proportional increase of bile acid excretion, demonstrating that of the pituitary hormones, TSH was probably the most important in maintaining a normal rate of bile acid production and excretion.

Beher *et al.* (11) further studied the rate of conversion of cholesterol to bile acids and the excretion of bile acids in hypophysectomized rats and

in hypophysectomized rats treated with thyroid hormone. Hypophysectomized animals had a reduced rate of fecal bile acid excretion. This was restored to about normal levels when thyroid hormone was administered. They postulated that the major factor producing a reduced turnover of bile acids in hypophysectomized rats is the slow rate of excretion of bile acids from the pool.

Early *in vitro* studies showed that mitochondria from livers of hyperthyroid rats did not oxidize cholesterol-26-^{14}C to $^{14}CO_2$ at a faster rate than similar preparations from normal animals (12). A more recent study (13) led to the conclusion that the effects of thyroid hormones on bile acid metabolism must take place at a biosynthetic step preceding side-chain oxidation, perhaps involving hydroxylation of the steroid nucleus. However, it must be realized that the normal substrate for side-chain oxidation leading to the formation of cholic acid from cholesterol is not cholesterol itself but presumably 3α,7α,12α-trihydroxy-5β-cholestane (14,15), and the substrate for the side-chain oxidation leading to chenodeoxycholate is, presumably, 3α,7α-dihydroxy-5β-cholestane (16). Thus results of *in vitro* experiments in which cholesterol is employed as the substrate must be interpreted with caution, since mitochondria do not have the enzyme system required for formation of the triol and diol from cholesterol.

The effect of thyroid hormone on the biosynthesis of bile acids was investigated further by Berséus (17). He confirmed that in the bile fistula rat the total bile acid output remains the same in both the normal and hyperthyroid states but that the cholate/chenodeoxycholate ratio changes from a normal value of 3:1 to 1:3 in the hyperthyroid state. It seemed likely that thyroid hormone acts specifically on an enzyme, catalyzing one of the steps on the pathway of bile acid biosynthesis in such a way that it favors the production of chenodeoxycholate. To test this hypothesis, 3α,7α,12α-trihydroxy-5β-cholestane was used as substrate for 26-hydroxylation, which initiates the oxidation of the side chain. It was found that liver homogenates from hyperthyroid rats had a greater capacity to oxidize 5β-cholestane-3α,7α,12α-triol than those from normal rats. However, a comparison with 5β-cholestane-3α,7α-diol was not made. Thyroid hormone thus may have a stimulating effect on side-chain oxidation, and it is possible that the oxidation of the diol might proceed more rapidly than that of the triol, accounting for the increased chenodeoxycholate/cholate ratio.

More recently, it has been shown that the 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one (an intermediate in cholic acid synthesis) is increased in homogenates from hypothyroid rats and decreased in homogenates from hyperthyroid rats (18). Thyroid hormone, therefore, may exhibit a specific inhibitory effect on the hydroxylation of 7α-hydroxycholest-4-en-3-one. This could explain, in part, the increased chenodeoxycholate

synthesis and consequent changes in the cholate/chenodeoxycholate ratio found in the hyperthyroid state: Less 5β-cholestanetriol would be available for the formation of cholic acid, and a relatively larger proportion of 5β-cholestanediol would be available for chenodeoxycholate synthesis.

III. PITUITARY HORMONES

In hypophysectomized rats, the synthesis of cholesterol from acetate (19,20)—but not from mevalonate (21)—is inhibited, indicating that pituitary hormones have an effect on a metabolic step between acetate and mevalonate, probably on hydroxymethylglutaryl–coenzyme A reductase. In terms of tissue cholesterol concentrations, the hypophysectomized rat differs little from the normal. Although bile acid synthesis and excretion are reduced, these animals reach a steady state in which normal cholesterol concentrations in plasma and tissue are maintained (21,22). This is true, however, only when the hypophysectomized rat is maintained on a low-cholesterol diet. When cholesterol intake is increased, both serum and tissue cholesterol reach high levels, presumably because of the decreased ability of the hypophysectomized rat to eliminate that sterol by conversion to bile acids (10, 11,23).

The major pathway of cholesterol elimination in the rat is via fecal excretion of bile acids. This pathway may be divided into two related events: (a) the catabolism of cholesterol to bile acids and (b) the excretion of bile acids from the pool. Pituitary hormones may thus affect one or both of these processes.

By injecting normal and hypophysectomized rats with ^{14}C-labeled cholate and/or chenodeoxycholate, the half-life, turnover rates, and bile acid pool sizes were determined (23–25). In hypophysectomized rats, bile acid synthesis (calculated from the $t_{1/2}$ and turnover rates) and excretion are reduced to about half when compared to normals. The decrease in bile acid synthesis is reflected in drastically reduced levels of chenodeoxycholate: Apparently, the hypophysectomized rat loses its ability to synthesize this bile acid (23,24). Typical values for daily bile acid synthesis expressed in mg/day/100 g rat were 0.55 mg cholate, 0.19 mg chenodeoxycholate for normals, and 0.31 mg cholate, no detectable chenodeoxycholate for hypophysectomized rats.

In the normal rat, increased cholesterol intake resulted in increased bile acid synthesis and excretion. The increased synthesis was reflected primarily in an increased production of chenodeoxycholate (from 0.2 mg/day/100 g rat to 0.8 mg/day/100 g rat). It was postulated that this increase in bile acid output was a protective mechanism to eliminate the excess

dietary cholesterol (23,24). On high-cholesterol intakes, hypophysectomized rats were able to increase their bile acid synthesis and excretion above the rates observed in hypophysectomized rats on cholesterol-free diets. When compared to cholesterol-fed intact rats, the hypophysectomized cholesterol-fed animals had lower rates of bile acid excretion, total bile acid synthesis, and chenodeoxycholate synthesis, and slower turnover rates (24).

Beher *et al.* (23,24) concluded that pituitary hormones act on sterol and bile acid elimination rather than on bile acid synthesis. They supported this argument by studies using cholestyramine (23,26), a bile acid sequestering anion exchanger, and psyllium hydrocolloid (27), which provides dietary bulk and lowers tissue cholesterol. Both of these agents decreased the $t_{1/2}$ and increased the synthesis and excretion of the bile acids in hypophysectomized rats but did not affect bile acid pool size. Thus "the faster bile acid synthesis and excretion rates in MK-135 [cholestyramine] treated animals are due to an increased rate of elimination of bile acids from their pools." Since bile acid pool size did not change in the hypophysectomized rats, they concluded that "the defect in sterol metabolism in these animals [hypophysectomized] is concerned not with the conversion of liver sterols to bile acids but with the rate of elimination of bile acids from their pools." (23)

This conclusion must be considered tentative at present. The half-life of a bile acid refers to the interval from secretion by the liver to excretion in the feces. It cannot take into account the number of enterohepatic cycles undergone by a bile acid molecule before it is excreted. Presumably, it is the rate of the enterohepatic circulation of bile acids that controls the rate of bile acid biosynthesis (28), and we do not know at present what factor or factors determine the rate of excretion. In the experiments in which the enterohepatic circulation was interrupted by administering the ion exchanger, it may be postulated that synthesis was stimulated by partial removal of the bile acid pool; the mechanism of this effect is not unequivocal since the circulation rate of the pool was not known.

IV. GONADAL HORMONES

In animals as well as man, castration causes increased serum cholesterol levels. Androgens have variable effects on serum cholesterol, while estrogens induce a decrease (29). It has been postulated that the mechanism of action of estrogens on serum cholesterol levels might involve increased uptake of cholesterol by the Kupffer cells of the liver, with a subsequent increase in catabolism of cholesterol (30).

In cockerels (31), small doses of Premarin (a conjugated equine estrogen) increased the cholesterol content of gallbladder bile while decreasing plasma

cholesterol and bile acid excretion. With large doses, opposite effects were observed. There appeared to be a decrease of cholesterol catabolism to bile acids with relatively small doses of estrogen, an increase in bile acid synthesis with higher levels of estrogen. However, the precise effect of estrogens on bile acid metabolism remains to be investigated.

The side-chain oxidation of cholesterol was greater in the presence of liver mitochondria of female rats than in that of male rats (32,33). In the same studies, it was shown that the presence of estrogens increased and androgens decreased the oxidation of cholesterol.

A more recent study (34) showed that in gonadectomized male and female rats, both serum and liver cholesterol increased. A lowering of cholesterol levels and increased bile acid synthesis were observed in both males and females only with estrogen administration. It was postulated that estrogen acts on the conversion of cholesterol to bile acids by increasing mitochondrial cholesterol oxidation. Tracer studies (cholesterol-^{14}C) showed that a significant increase in cholesterol conversion to bile acids was produced by estrogen. No such effects were observed with androgen.

V. ADRENAL HORMONES

Very little work has been done on the effects of adrenal hormones on bile acid metabolism. In the adrenalectomized bile fistula rat, total bile acid production was only slightly decreased. However, the ratio of glycine-conjugated/taurine-conjugated bile acids was about 1, as compared to a normal ratio of 0.1 (35). The administration of cortisone caused the ratio to revert to normal. It was found that the livers of both intact and adrenalectomized rats contained the same amounts of taurine, indicating that the change in glycine/taurine conjugation was not due to a decrease in the availability of taurine. Unconjugated cholic acid in the bile from adrenalectomized rats amounted to 2–5% (less than 1% in intact rats) of total bile acids. This points to a defective conjugation mechanism at the enzyme level.

It is difficult to study hormonal factors regulating bile acid synthesis in intact animals because effective stimuli are counteracted by homeostatic mechanisms. Recently (36), effects of cortisone on bile acid production and biosynthesis were studied using the isolated, perfused rabbit liver (37), which offers a less complex system in which bile is easily sampled. When the donor rabbits were pretreated with cortisone acetate (3 mg/kg, intramuscularly) for 3 days, secretion of glycocholate during a 2.5-hr perfusion increased two- to threefold. The incorporation of acetate-1-^{14}C into bile acids was likewise increased, suggesting that part of the stimulatory effect was due to an increase in bile acid biosynthesis. Biliary cholesterol concentration (but not *de novo*

synthesis) was increased about five times above the control levels. Similar, though less striking, effects were observed when hydrocortisone was added directly to the perfusion fluid.

VI. CONCLUSION

This survey of information currently available on the effect of hormones on bile acid metabolism makes it clear that much additional work is needed. Of particular interest would be work dealing with the effect of individual hormones on specific enzymatic steps of bile acid biosynthesis. In addition, it would be desirable to have more detailed information on the action of specific hormones on the size and composition of the bile acid pool and on its rate of enterohepatic cycling.

REFERENCES

1. D. Kritchevsky, *Metabolism* **9,** 984 (1960).
2. J. C. Thompson and H. M. Vars, *Proc. Soc. Exptl. Biol. Med.* **83,** 246 (1953).
3. J. C. Thompson and H. M. Vars, *Am. J. Physiol.* **179,** 405 (1954).
4. S. Eriksson, *Proc. Soc. Exptl. Biol. Med.* **94,** 582 (1957).
5. O. Strand, *Proc. Soc. Exptl. Biol. Med.* **109,** 668 (1962).
6. T. H. Lin, R. Rubinstein, and W. L. Holmes, *J. Lipid Res.* **4,** 63 (1963).
7. O. Strand, *J. Lipid Res.* **4,** 305 (1963).
8. K. Hellström and J. Sjövall, *J. Atherosclerosis Res.* **1,** 205 (1961).
9. K. Hellström and S. Lindstedt, *J. Lab. Clin. Med.* **63,** 666 (1964).
10. W. T. Beher, G. D. Baker, M. E. Beher, A. Vulpetti, and G. Semenuk, *Proc. Soc. Exptl. Biol. Med.* **117,** 738 (1964).
11. W. T. Beher, M. E. Beher, and G. Semenuk, *Metabolism* **15,** 181 (1966).
12. D. Kritchevsky, M. C. Cottrell, and S. A. Tepper, *J. Cell. Comp. Physiol.* **60,** 105 (1962).
13. D. Kritchevsky and S. A. Tepper, *J. Cell. Comp. Physiol.* **66,** 91 (1965).
14. S. Bergström, K. Paabo, and J. A. Rumpf, *Acta Chem. Scand.* **8,** 1109 (1954).
15. H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.* **237,** 338 (1962).
16. I. Björkhem, *Acta Chem. Scand.* **21,** 2561 (1967).
17. O. Berséus, *Acta Chem. Scand.* **19,** 2131 (1965).
18. K. A. Mitropoulos, M. Suzuki, N. B. Myant, and H. Danielsson, *FEBS Letters* **1,** 13 (1968).
19. G. M. Tomkins, I. L. Chaikoff, and L. L. Bennett, *J. Biol. Chem.* **199,** 543 (1952).
20. J. W. Bauman, R. Hill, N. S. Nejad, and I. L. Chaikoff, *Endocrinology* **65,** 73 (1959).
21. W. T. Beher, G. D. Baker, and D. G. Penney, *Proc. Soc. Exptl. Biol. Med.* **114,** 195 (1963).
22. W. T. Beher, B. Rao, M. E. Beher, and J. Bertasius, *Proc. Soc. Exptl. Biol. Med.* **124,** 1193 (1967).
23. W. T. Beher, B. Rao, M. E. Beher, G. Semenuk, J. Bertasius, and N. Vulpetti, *Henry Ford Hosp. Med. J.* **15,** 107 (1967).

24. W. T. Beher, K. K. Casazza, A. B. Filus, M. E. Beher, and J. Bertasius, *Atherosclerosis* **12,** 383 (1970).
25. W. T. Beher, M. E. Beher, and B. Rao, *Life Sci.* **6,** 863 (1967).
26. W. T. Beher, M. E. Beher, and B. Rao, *Proc. Soc. Exptl. Biol. Med.* **122,** 881 (1966).
27. W. T. Beher and K. K. Casazza, *Proc. Soc. Exptl. Biol. Med.* **136,** 253 (1971).
28. S. Shefer, S. Hauser, I. Bekersky, and E. H. Mosbach, *J. Lipid Res.* **10,** 646 (1969).
29. D. Kritchevsky, "Cholesterol," p. 203, Wiley, New York (1958).
30. G. S. Boyd, *Fed. Proc.* **21,** 86 (1962).
31. G. B. Clarke, R. Pick, P. Johnson, and L. N. Katz, *Ciro. Res.* **9,** 564 (1966).
32. D. Kritchevsky, E. Staple J. L. Rabinowitz, and M. W. Whitehouse, *J. Physiol.* **200,** 519 (1961).
33. D. Kritchevsky, S. A. Tepper, E. Staple, and M. W. Whitehouse, *J. Lipid Res.* **4,** 188 (1963).
34. S. Mukherjee and S. Gupta, *J. Atherosclerosis Res.* **7,** 435 (1967).
35. K. Hellström and O. Strand, *Acta Endocrinol.* **43,** 305 (1963).
36. E. H. Mosbach, M. A. Rothschild, L. L. Abell, and M. Oratz, *Gastroenterology* **60,** 748 (1971).
37. E. H. Mosbach, M. A. Rothschild, I. Bekersky, M. Oratz, and J. Mongelli, *J. Clin. Invest.* **50,** 1720 (1971).

Chapter 9

ENZYMES IN BILE ACID METABOLISM*

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I. INTRODUCTION

The metabolic relationship of cholesterol to bile acids was first suggested by Lifschutz (1) in 1914 on the basis of their occurrence together in bile and their behavior toward a ferric chloride reagent. Lifschutz's assumption could not be experimentally verified until, in 1943, Bloch, in association with Berg and Rittenberg (2), succeeded in labeling cholesterol isotopically by exchange with deuterium. These workers established the origin of cholic acid from cholesterol by isolating deuterium-labeled cholic acid from the urine of a dog which was given deuterated cholesterol. With the advent of ^{14}C-labeled cholesterol, the quantitative relationship between cholesterol and bile acids was established unequivocally, showing that bile acids form the principal end products of cholesterol metabolism. The mechanisms of bile acid biogenesis from cholesterol, the structural intermediates involved in this pathway, and the enzymes catalyzing these reactions are described by Danielsson in Chapter 1 of this volume (3).

The primary bile acids, which are derivatives of cholanoic acid, are

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generally conjugated with glycine and taurine before being secreted into the bile. These conjugated bile acids undergo further structural alterations under the influence of intestinal microflora as they are propelled through the gut together with intestinal contents. Discussion of the enzymes catalyzing these reactions will form the principal content of this chapter.

II. FORMATION OF THE PEPTIDE BOND OF CONJUGATED BILE ACIDS

The terminal step in the biosynthesis of bile acids from cholesterol is represented by the enzymatic synthesis of water-soluble bile acid conjugates of taurine and/or glycine through a peptide bond, as shown in Fig. 1.

The ability to synthesize peptide bonds is an inherent propensity of hepatic cells and is a biological phenomenon associated with detoxification processes, in which the end products are generally water-soluble conjugates with amino acids. A classical example is the biosynthesis of hippuric acid by the conjugation of benzoic acid with glycine.

The demonstration by Bergström *et al.* (4,5) in 1953 of the conversion of deoxycholic acid to taurocholic acid in the rat *in vivo* and by rat liver slices paved the way for studies on 7α-hydroxylation and conjugation of bile acids. The early work on the synthesis of bile acid conjugates *in vitro* utilized slices or homogenates of rat and human liver, and the enzymatic reaction was followed by the incorporation of radioactivity from carboxyl-^{14}C-labeled bile acids into the corresponding taurine and glycine conjugates (6,7). The

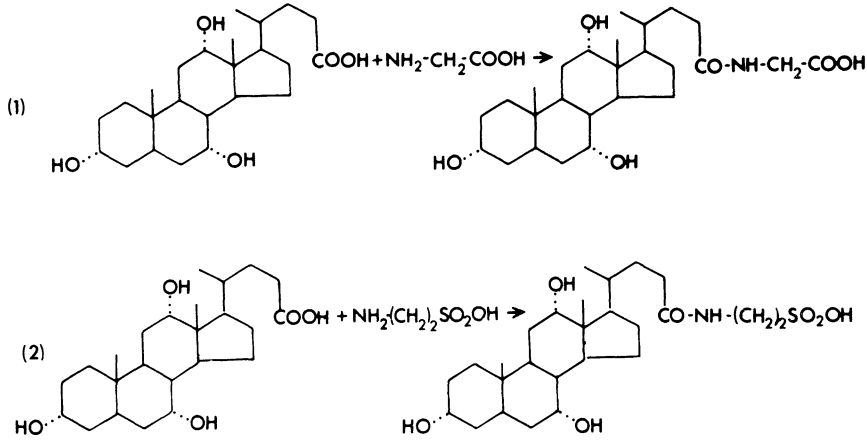

Fig. 1. Biosynthesis of conjugated bile acids. (1) Glycocholic acid. (2) Taurocholic acid.

incubations were carried out in 0.12 M sucrose, buffered with phosphate at *p*H 7.4, and the reaction products were separated for radioactivity measurements by the paper chromatographic procedure of Sjövall (8). The enzymatic reaction was inhibited by free bile acids in the concentration range of 10–30 mg % of the homogenate, although hydroxylation of taurodeoxycholic acid proceeded uninhibited. In human liver homogenates, without the addition of exogenous amino acids, taurocholic acid represented 60% of the total conjugates formed, while the rest of it was made up of glycocholic acid. However, the relative proportions of the two conjugates could be readily altered in favor of taurocholic acid (90%) by the addition to the incubation mixture of 3 moles of taurine per mole of bile acid. In contrast, the system was not significantly influenced by the addition of glycine in the concentration range of 3–30 moles per mole of bile acid.

A. Properties of the Enzyme System

During the period 1955–1957, the nature and characteristics of the enzymatic steps involved in the biosynthesis of bile acid conjugates were independently investigated by Bremer, Gloor, Elliott, and Siperstein. Bremer, using ^{35}S-labeled taurine and a butanol extraction procedure, investigated the characteristics of the enzyme in liver subcellular fractions (9–11). In rat liver, the full conjugating activity of the homogenate is retained in the postmitochondrial supernatant (12,000 *g* supernatant). Although the microsomes and supernatant are inactive by themselves, when they are combined, full activity is restored in the presence of ATP. When fresh microsomes are incubated with the boiled supernatant, the enzyme is partially reactivated by the addition of ATP. As possible cofactors, ATP, DPN, Mg^{2+}, EDTA, and TPN enhance the activity of the enzyme in the postmitochondrial supernatant. In the absence of ATP, cyanide strongly inhibits the conjugation. When ATP is added, cyanide has no effect, and the reaction is almost quantitative at low taurine and cholic acid concentrations.

In the taurocholic acid synthesizing system of rat liver microsomes, the fact that the soluble cytoplasmic fraction could be substituted for, by CoA and ATP, appeared to indicate the existence of cholyl-CoA as an activated intermediate (12). This was independently observed by a number of investigators (13–17). In Bremer's studies (13), the conjugation of cholic acid with taurine by rat liver microsomes at *p*H 7.3–7.6 required coenzyme A, ATP, Mg^{2+}, Mn^{2+}, and fluoride to inhibit ATPase activity. In some cases, cysteine and glutathione stimulated the activity. In this report, a reaction mechanism involving cholyl-S-CoA as an intermediate and AMP and pyrophosphate as reaction products was proposed, in which the transfer of the cholyl group from CoA to taurine and glycine is irreversible. Since hepatic microsomes

derived from various animal species differ in their relative abilities to transfer the bile acid moiety from CoA to taurine and/or glycine, the question has been raised concerning the existence of two distinct enzymes, acyl transferases corresponding to taurine and glycine. On the basis of these studies, the following reaction sequence was proposed:

$$\text{Cholic acid} + \text{ATP} + \text{CoA-SH} \xrightarrow[\substack{\text{activating enzyme} \\ + Mg^{2+} \text{ or } Mn^{2+} \\ + \text{ATP}}]{\text{bile acid}} \text{Cholyl-S-CoA} + \text{AMP} + \text{Pyrophosphate}$$

$$\text{Cholyl-S-CoA} + \text{taurine} \xrightarrow[\text{acyl transferase}]{\text{cholyl taurine}} \text{taurocholic acid} + \text{CoA-SH}$$

$$\text{Cholyl-S-CoA} + \text{glycine} \xrightarrow[\text{acyl transferase}]{\text{cholyl glycine}} \text{glycocholic acid} + \text{CoA-SH}$$

In a similar investigation, using guinea pig liver microsomes, Elliott (18) demonstrated the formation of taurocholic acid when the microsomes were incubated in phosphate buffer in the presence of Mg^{2+}, CoA, ATP, potassium cholate, taurine, cysteine, and potassium fluoride. The complete incubation system consisted of 0.2 ml of 0.25 M phosphate buffer, *p*H 7.4; 0.1 ml of 0.1 M $MgSO_4$; 100 units of CoA; 0.25 ml of 0.08 M ATP; 0.1 ml of 0.04 M potassium cholate; 0.1 ml of 0.1 M taurine; 0.05 ml of 0.2 M cysteine; 0.1 ml of 0.3 M potassium fluoride; and 0.5 ml of microsomal suspension.

Scherstén *et al.* (19–22) have investigated more extensively the synthesis of taurocholic and glycocholic acids by homogenates of human liver. The yields of taurocholic and glycocholic acids in 1:1 molar proportion were linear, with increasing amounts of cholic acid in the incubation mixture up to a concentration of 22.32 mμmoles of cholic acid per 40 mg of hepatic tissue. With the addition of taurine and glycine in equimolar amounts together with cholic acid, there was a preferential synthesis of taurocholic acid up to 95% of the total conjugates formed in the absence of any exogenous glycine. On the other hand, when only glycine was added without any taurine, glycocholic acid represented 80% of the total conjugated bile acids. Among the subcellular fractions derived from human liver, only a combination of the microsomal fraction and a crude lysosomal (L) fraction exhibited about 70% of the original activity present in the total homogenate (21,22). The L-fraction, which appeared to stimulate microsomal synthesis of bile acid conjugates in the presence of optimum concentrations of cofactors, had no effect on the synthesis of cholylhydroxamic acid, which was exclusively related to the amounts of microsomal protein present in the incubation mixture. Purification of the factor in the L-fraction by solubilization or sucrose gradient centrifugation gave an active light L-fraction associated with high acid phosphatase activity but with no ability to form cholylhydroxamic acid with

hydroxylamine. Since earlier studies had shown that the participation of the bile acid activating enzyme (cholyl-CoA synthetase) is obligatory to the synthesis of cholylhydroxamic acid, the light L-fraction was assumed to possess the other component, the acyl transferases, that mediate the transfer of the activated bile acid moiety to either taurine or glycine.

B. Assay of Bile Acid Conjugating Activity in Human Liver Homogenates

Assay of bile acid conjugating activity is based on the procedure described by Ekdahl *et al.* (23) and Gottfries *et al.* (19). Specimens of human liver obtained by biopsy or during laparatomy are placed in ice-cold 0.1 M phosphate buffer, *p*H 7.4. After removal of the capsule and the connective tissue, the liver is homogenized in 4 vol of 0.1 M phosphate buffer, *p*H 7.4. Aliquots of the homogenate, corresponding to 40 mg of tissue, are transferred to test tubes containing 2.32 mμmoles of cholic acid-24-^{14}C (sp. act. 16.3 μCi/mg), 0.6 μmole of ATP, 4 μmoles of nicotinamide, 1 μmole of $MgCl_2$, and nonradioactive cholic acid in graded amounts from 2 mμmoles in the first tube to about 70 mμmoles in the last one. The total volume of the incubation mixture is 0.5 ml, and the final *p*H is adjusted to 7.4. The tubes are incubated in a water bath at 37° C for 120 min, and the reaction is terminated by the addition of ethanol or by placing the tubes in boiling water for 5 min.

The ethanol-precipitated protein is removed by centrifugation, and the supernatant is evaporated to dryness under a stream of dry nitrogen in a separate test tube. The residue is dissolved in 0.2 ml of methanol containing 40 μg each of cholic, glycocholic, and taurocholic acids and subjected to thin-layer chromatography (24). The zones corresponding to taurocholic and glycocholic acids are scraped, and radioactivity is measured by liquid scintillation counting. The activities are usually expressed as mμmoles synthesized per milligram of protein.

C. Characteristics of the Peptide-Bond Synthesizing Systems

The naturally occurring bile acids in higher animals are considered to be derivatives of cholanoic acid, a C_{24} bile acid. To a large extent, therefore, the bile acid conjugating system of hepatic cells utilizes bile acids with 24 carbon atoms as the substrate for the synthesis of the corresponding conjugates. However, when tritium-labeled 3α,7α,12α-trihydroxycoprostanic acid, a C_{27} bile acid intermediate, was incubated with rat liver homogenates, Bergström *et al.* (25) isolated from the incubation mixture, in addition to labeled taurocholic acid, a small proportion of labeled trihydroxycoprostanic acid in the form of its conjugate with taurine. Since trihydroxycoprostanic acid is a primary bile acid among members of the *Crocodylidae* and *Amphibia,*

the ability to utilize this C_{27} bile acid as a substrate for conjugation by mammalian liver cells possibly reflects the vestigial remains of a functional capacity once fully expressed in the early stages of biochemical evolution.

The conjugation of bile acids with added hydroxylamine by rat liver microsomes in the presence of ATP, CoA, and fluoride to yield bile acid hydroxamates has been used to demonstrate the formation of cholyl-CoA as an intermediate in the enzymatic system catalyzing the synthesis of the natural conjugates (12,13). In pig liver, there appear to be two distinctly different enzyme systems capable of the synthesis of cholylhydroxamic acid (15). A partially solubilized preparation from pig liver catalyzed the rapid formation of cholylhydroxamic acid in the presence of cholic acid and hydroxylamine but in the absence of ATP and CoA. This is in contrast to the synthesis of hydroxamates of bile acids by the microsomes, which required both ATP and CoA. Since fluoride inhibits the system that functions in the absence of ATP and CoA, the reaction appears to be similar to that of a lipase (15)

The activation of bile acids by liver microsomes to form the corresponding CoA esters in the presence of ATP, CoA, and Mg^{2+} has been shown to be the first step in the conjugation of bile acids with glycine or taurine. Recently, Shah and Staple (26) studied the relative specificities of the microsomal enzymes to form CoA esters of several dihydroxycholanoic acids, such as hyodeoxycholic, deoxycholic, and chenodeoxycholic acids. In these studies, the formation of CoA esters was measured by trapping the thioester with hydroxylamine and determining the hydroxamates spectrophotometrically as the ferric ion color complex. The enzyme system in rat liver microsomes was about 50% as active for hyodeoxycholic acid as it was for cholic acid. The corresponding activities with deoxycholate and chenodeoxycholate were in the range of 83–85%.

There are several biological mechanisms for the formation of peptide bonds (27). The bile acid conjugates (glycocholic and taurocholic acids) and hippuric acid are two classes of compounds possessing peptide bonds that are formed in the liver enzymatically. Although the identity and catalytic properties of the various enzyme(s) systems capable of peptide bond synthesis would appear to be similar, the one catalyzing the synthesis of bile acid conjugates is exclusively a microsomal enzyme, compared to the one forming hippuric acid, which has a mitochondrial localization (9).

D. Factors That Determine Bile Acid Conjugation

The intrinsic ability of the liver to conjugate bile acids and the relative proportions of the two principal forms, the glycine and taurine conjugates, are determined and regulated by several factors, hepatic and extrahepatic.

In the newborn infant, the bile acids are almost exclusively conjugated with taurine (28,29), while after the first week there is a steady rise in the amounts of glycine conjugates in bile and duodenal contents, reaching adult values during the course of the first year. The average ratio of glycine conjugates/taurine conjugates in the normal human adult is about 3.2:1, although some variations are observed that are ascribed to dietary and other external influences. For example, normal healthy human subjects when fed taurine of up to 1.5 g/day responded with an increase in taurine conjugates (30), although glycine in amounts of up to 21 g/day did not alter the conjugation pattern. In disease states, such as in myxedema, there is an increase in the G:T ratio to about 7 (31). Replacement therapy with thyroid hormone restores the conjugation pattern to normal. The relationship to thyroid status is further exemplified by the decrease in the glycine/taurine ratio to about 2 in thyrotoxicosis (32).

Administration of nicotinic acid for the reduction of serum cholesterol also reduces the proportion of bile acids conjugated with glycine (33), although the converse is not true. In other words, alteration of the glycine/taurine ratio in favor of taurine conjugates by dietary means, such as the feeding of taurine, does not lower blood cholesterol levels. In vitamin B_6 deficient rats, there is an increase in glycine conjugates (34,35). There are a variety of pathological conditions that would bring about an alteration in the relative amounts of the conjugates in man. Extensive studies conducted by Ekdahl, Sjövall, and others in Sweden are reviewed by Scherstén (20) and by Bergström *et al.* (36).

Recent studies on bile salts in duodenojejunal samples from subjects suffering from tropical sprue have shown a significantly lowered glycine/taurine ratio compared to ratios in control or protein–caloric malnutrition subjects (37).

In contrast, in ileal absorptive disorders, Garbutt *et al.* (38) observed an increase in G:T ratios of both cholate and deoxycholate, which was attributed to a decrease in enterohepatic recirculation of taurocholate and deoxycholate. It is also interesting to note that a selective conjugation of cholic acid with L-ornithine could be induced in the rat and guinea pig liver by the injection of a toxic capsular polysaccharide of *Klebsiella pneumoniae* (39). Partial hepatectomy has also been shown to result in a shutdown in the hepatic synthesis of glycine conjugates of bile acids (40).

III. PEPTIDE BOND HYDROLASES OF BILE ACID CONJUGATES

In Section II, we have discussed the enzymatic synthesis of the peptide bond of bile acid conjugates by mammalian liver. The further metabolism of

conjugated bile salts during their passage through the intestinal tract, as far as we know, is almost exclusively brought about by the action of microflora indigenous to the gut. Among the several structural alterations, the most widely studied appears to be the hydrolytic cleavage of the peptide bond of bile acid conjugates by bacterial enzymes.

Since most of the fecal bile acids are in the unconjugated form, the observation by Norman (41) that rats treated with antibiotics excreted only conjugated bile acids suggested a role for intestinal microorganisms in the deconjugating process. This was further supported by the fact that in germ-free animals all of the fecal bile acids were in the conjugated form (42). Frankel (43), in 1936, reported the first successful isolation of a bacterium from human feces capable of hydrolyzing conjugated bile acids in cultures, although this organism was unable to grow at 37° C. Among the early studies on peptide-bond hydrolysis by microbial cultures, the one by Norman and Grubb (44) is notable. Their approach was to allow microorganisms from rat fecal suspensions to grow in a variety of culture media previously fortified with either labeled or unlabeled bile acid conjugates. After the organisms had grown for 24–52 hr, the existence of free bile acids in the growth medium was demonstrated by chromatographic analysis of the broth. Strains of *Clostridium* and *Enterococcus* were capable of peptide-bond hydrolysis, although they differed in some respects. The clostridia were especially active on the taurine conjugates, and effected a more complete splitting of the conjugates than the enterococci. Incidentally, enterococci are one of the few species of gram-positive organisms that can survive in the presence of bile. In 1964, Ogura and Ozaki (45) reported from Japan the isolation of a strain of *Aerobacter aerogenes* capable of splitting hippuric acid and to a lesser extent the conjugates of bile acids (45).

It should be emphasized that until about this time our knowledge of the existence of bacterial bile salt hydrolases was limited and only indirectly inferred from the fact that certain organisms, when allowed to grow in culture media fortified with bile salts, were capable of hydrolyzing them. Most attempts at isolating or purifying a cell-free enzyme preparation were largely unsuccessful. In 1964, a group of investigators from the author's laboratory initiated a systematic study of known peptidases and proteases in a search for a natural source of an enzyme capable of splitting bile salt conjugates (46). These studies led us to two significant conclusions: First, the C–N bond in bile salt conjugates is unique in its ability to withstand cleavage by all known proteolytic enzymes examined by us and in its requirement of drastic alkaline conditions (15% NaOH in 50% ethanol at 115–200° C for several hours in a Parr bomb) for its chemical hydrolysis. Second, microbial splitting of the C–N bond in these compounds is an attribute of bacterial metabolism during multiplication in media enriched with bile salt conjugates and normal-

ly does not represent the production of a specific enzyme that is discharged into the growth medium. Therefore, in order to obtain a source for a cell-free enzyme, it was necessary to screen several strains of organisms in an attempt to isolate a mutant in which the gene coding for the enzyme(s) was fully derepressed. Abscesses of the gallbladder and bile ducts and the urine from patients with jaundice were obvious sources of our organisms, since these mutants were likely to have developed biochemical mechanisms that would allow them to survive in an environment rich in bile salts. These studies culminated in the isolation of a strain of *Clostridium perfringens* (ATCC-19574, Sinai-43-F-4) in 1965 that yielded cell-free extracts containing a hydrolytic enzyme (47). The crude enzyme obtained from this strain exhibited a *p*H optimum at 5.8, unlike the extracellular enzyme (48) obtained from the clear supernatant fluid of rat intestinal contents, which showed an optimum between *p*H 6.5–8.0.

A. Cultivation of *C. perfringens*

C. perfringens (ATCC-19574, Sinai-43-F-4) (49) was isolated from a human subject with a biliary abscess using trypticase–soy–blood–agar (47). Cells are generally grown aerobically at 37° C in freshly prepared Brewer modified thioglycollate broth without indicator (No. 01-135C, Baltimore Biological Laboratories) for 16 hr and harvested by *centrifugation at room temperature*. Accidental cooling of the broth below room temperature can result in the formation of a white precipitate that interferes with the preparation of the cell-free extract. The cells are washed five times with physiological 0.9% NaCl at a temperature of 4° C.

B. Characteristics of Clostridial Cholylglycine Hydrolase

The partially purified enzyme from *C. perfringens* (ATCC-19574, Sinai-43-F-4) (49,50) has a broad *p*H optimum between 5.6–5.8 and in acetate buffer at *p*H 5.6 gives higher values than those in phosphate buffer at *p*H 5.8. The enzyme is inhibited by sulfhydryl reagents and metal ions such as mercury, copper, and zinc. Cholic acid, the product of the enzymatic reaction, exhibits competitive inhibition of the hydrolytic process. Similarly, glycodehydrocholic acid, the triketo analogue of the standard substrate, glycocholic acid, is not only inactive but is inhibitory to the enzymatic cleavage of the latter. Electroconvection studies showed that the enzyme has an isoelectric point between *p*H 7.3–7.4. The enzyme has a molecular weight in excess of 200,000 and is completely excluded from Sephadex G-200.

C. Assay of Cholylglycine Hydrolase Activity

The reaction mixture containing sodium glycocholate (10 μmoles), acetate buffer (pH 5.6, 10 μmoles), β-mercaptoethanol (20 μmoles), disodium EDTA (20 μmoles), and dilute enzyme in a final volume of 1.0 ml is incubated at 37° C for 5 min in a shaking water bath. The reaction is terminated by the addition of 1.0 ml 20% TCA. The supernatant is assayed for free glycine, along with substrate and enzyme blanks, by the procedure of Troll and Cannan (51).

D. Relationship of Substrate Structure to Enzyme Activity

The glycine and taurine conjugates of the four most common naturally occurring bile acids are good substrates, as would be expected. Conjugates of cholic and deoxycholic acids possessing negatively charged end groups with the exception of the one with δ-aminovaleric acid are active as substrates, in contrast to those with positively charged end groups, which are not attacked by the enzyme. The effect of increasing the length of the side chain beyond the C–N bond is exemplified by using poly-L-lysine conjugates of cholic and deoxycholic acids, both of which are inactive. It appears that a side chain longer than two carbon atoms (beyond the C–N bond) renders the substrate inactive to the altered spatial relationships between the points of attachment of the substrate to the active sites on the enzyme (50). From the limited studies conducted so far, substrate activity is related to (a) the presence of free hydroxyl groups on the bile acid moiety and (b) the presence of a negatively charged end group on a side chain not longer than two carbon atoms, beyond the C–N bond.

E. Bile Salt Hydrolases of Other Bacterial Genera

Aries and Hill (52), of the Wright-Fleming Institute of St. Mary's Hospital Medical School in London, extended the earlier studies from this author's laboratory by examining several organisms for extracellular and intracellular bile salt hydrolases. They described the characteristics of enzymes from two strains of each of the genera *Bacteroides, Bifidobacterium, Clostridium,* and *Enterococcus*. Except for the bifidobacteria, all organisms produced an active intracellular enzyme capable of hydrolyzing glycodeoxycholic and taurodeoxycholic acids. One of the enterococcal strains had an intracellular enzyme specific for glycodeoxycholic acid. Among these organisms, the bifidobacteria were unique in that they produced a hydrolase that was specific for glycine conjugates and was mostly extracellular, a small proportion being bound to the wall and membranous fragments. The eight hydrolases describ-

ed by Aries and Hill had characteristics similar to those of the clostridial cholylglycine hydrolase of Nair *et al.* (49), with the exception of their chromatographic behavior on DEAE-cellulose. Furthermore, the enzymes of the British workers seem to have molecular weights in the range of 50,000–100,000, unlike that of the clostridial enzyme of about 200,000 (50). Until the subunit structure of this constellation of hydrolases is elucidated, one can only speculate on the nature and specificity of the individual units in this cluster.

IV. HYDROXYSTEROID OXIDOREDUCTASES AND RELATED ENZYMES

A. Oxidoreductases Acting on 3-Hydroxy/3-Ketocholanoic Acids

Rat liver 3α-hydroxysteroid dehydrogenase is known to reversibly dehydrogenate 3α-hydroxycholanoic acids as readily as those in C_{19}- or C_{21}-steroids, NAD being required as a cofactor (53). The existence of similar dehydrogenases in other tissues such as the intestines and kidney and in intestinal bacteria has been reported (54–56). Kallner (57) reported his studies on the reduction *in vitro* of 3-keto groups of a series of substituted cholanoic acids by a 100,000 *g* supernatant of rat liver homogenate. Incubation of the monohydroxymonoketo bile acids with the mammalian soluble enzyme led to the formation of small amounts of 3β-hydroxy acids in addition to the usual 3α-hydroxy epimer. While the microsomal fraction catalyzed the reduction of Δ^4-3-keto bile acids to the corresponding 3-keto-5α bile acids, the enzymes in the supernatant specifically catalyzed the reduction of Δ^4-3-keto bile acids to the corresponding 3-keto-5β bile acids. Aries and Hill (58) studied cell-free preparations of strains of *Clostridium welchii* and *Bifidobacterium* exhibiting both 3α- and 12α-hydroxycholanyl dehydrogenase activities. These enzymes are NADP dependent, and the 3α-hydroxycholanyl dehydrogenase of *C. welchii* did not oxidize 3β-hydroxy-5β-cholanic acid, 3α-hydroxy-5α-androstan-17-one, 5β-cholestan-3β-ol, or cholesterol, thus showing a high degree of specificity for the substrate.

B. Miscellaneous Oxidoreductases and Dehydroxylases

Aries and Hill (58), during the course of their investigations of degradation of steroids by intestinal bacteria, prepared partially purified extracts of several microorganisms, such as *Clostridium, Bacteroides, Bifidobacterium,* and *Enterobacterium,* possessing enzymatic activity catalyzing the oxidoreduction of the 7α- and 12α-hydroxy groups in bile acids, as well as the 7-

dehydroxylase. The enzymes from *C. welchii* were NADP dependent, while those from *Escherichia coli* and *Bacteroides* utilized NAD. The presence of 7-dehydroxylase activity could be demonstrated only in cells grown in the presence of bile acid substrate in a growth medium with a final *p*H above 6.5. Enzymatic activity was enhanced by the addition of a reducing agent (0.05% cysteine HCl) or blood (1%) to the growth medium.

V. CONCLUSION

In this chapter, the author has chosen to describe only those reactions on bile acids that have been demonstrated by the use of cell-free preparations. Because of this, a large number of other metabolic reactions carried out by, for example, microorganisms during their growth and multiplication in media enriched with bile salts are not included here, although admittedly many of them are enzyme-mediated reactions. The constraints placed by the definition in the first sentence of this paragraph has made this chapter rather selective but also serves to alert the reader that this area of bile salt metabolism is still in its infancy and is open to further investigation.

REFERENCES

1. I. Lifschutz, *Hoppe-Seylers Z. Physiol. Chem.* **91,** 309 (1914).
2. K. Bloch, B. N. Berg, and D. Rittenberg, *J. Biol. Chem.* **149,** 511 (1943).
3. H. Danielsson, this volume, p.1.
4. S. Bergström, M. Rottenberg, and J. Voltz, *Hoppe-Seylers Z. Physiol. Chem.* **295,** 278 (1953).
5. S. Bergström, A. Dahlquist, and U. Ljungqvist, *Kgl. Fysiograf Sällskap. Lund* **23,** No. 12 (1953).
6. S. Bergström and U. Gloor, *Acta Chem. Scand.* **8,** 1109 (1954).
7. S. Bergström and U. Gloor, *Acta Chem. Scand.* **8,** 1373 (1954).
8. J. Sjövall, *Acta Chem. Scand.* **8,** 329 (1954).
9. J. Bremer, *Acta Chem. Scand.* **9,** 268 (1955).
10. J. Bremer, *Acta Chem. Scand.* **9,** 683 (1955).
11. J. Bremer and U. Gloor, *Acta Chem. Scand.* **9,** 689 (1955).
12. J. Bremer, *Acta Chem. Scand.* **9,** 1036 (1955).
13. J. Bremer, *Acta Chem. Scand.* **10,** 56 (1956).
14. W. H. Elliott, *Biochim. Biophys. Acta* **17,** 440 (1955).
15. W. H. Elliott, *Biochem. J.* **62,** 427 (1956).
16. M. D. Siperstein, *Fed. Proc.* **14,** 282 (1955).
17. M. D. Siperstein and A. E. Murray, *Science* **123,** 377 (1956).
18. W. H. Elliott, *Biochem. J.* **62,** 433 (1956).
19. A. Gottfries, T. Scherstén, and P. H. Ekdahl, *Scand. J. Clin. Lab. Invest.* **18,** 643 (1966).
20. T. Scherstén, *Acta Scand. Suppl.* **373,** (1967).

21. T. Scherstén, *Biochim. Biophys. Acta* **141,** 144 (1967).
22. T. Scherstén, P. Björntorp, P. H. Ekdahl, and S. Björkerud, *Biochim. Biophys. Acta* **141,** 155 (1967).
23. P. H. Ekdahl, A. Gottfries, and T. Scherstén, *Scand. J. Clin. Lab. Invest.* **17,** 104 (1965).
24. H. Gännshirt, F. W. Koss, and K. Morianz, *Arzneimittel-Forschung* **10,** 943 (1960).
25. S. Bergström, R. J. Bridgwater, and U. Gloor, *Acta Chem. Scand.* **11,** 836 (1957).
26. P. P. Shah and E. Staple, *Arch. Biochem. Biophys.* **132,** 349 (1969).
27. H. Borsook, *Advan. Protein Chem.* **8,** 127 (1953).
28. J. Sjövall, *Acta Physiol. Scand.* **46,** 332 (1959).
29. J. Sjövall, *Clin. Chim. Acta* **5,** 33 (1960).
30. J. Sjövall, *Proc. Soc. Exptl. Biol. Med.* **100,** 676 (1959).
31. K. Hellström and K. Sjövall, *J. Atherosclerosis Res.* **1,** 205 (1961).
32. K. Hellström and S. Lindstedt, *J. Lab. Clin. Med.* **63,** 666 (1964).
33. R. B. Failey, Jr., E. Brown, and M. E. Hodes, *Circulation* **20,** 984 (1959).
34. E. A. Doisy, Jr., M. Daniels, and S. M. A. Zimmerman, *Fed. Proc.* **15,** 243 (1956).
35. B. Bergeret and F. Chatagner, *Biochim. Biophys. Acta* **22,** 273 (1956).
36. S. Bergström, H. Danielsson, and B. Samuelsson, *in* "Lipid Metabolism" (K. Bloch, ed.), p. 291, Wiley, New York (1960).
37. P. P. Nair, J. G. Banwell, S. L. Gorbach, C. Lilis, and A. Alcaraz, *Am. J. Clin. Nutr.* **23,** 1659 (1970).
38. J. T. Garbutt, K. W. Heaton, L. Lack, and M. P. Tyor, *Gastroenterology* **56,** 711 (1969).
39. L. Peric-Golia and R. S. Jones, *Proc. Soc. Exptl. Biol. Med.* **110,** 327 (1962).
40. S. P. Bengmark, P. H. Ekdahl, and R. Olsson, *Gastroenterologia* **99,** 327 (1963).
41. A. Norman, *Acta Physiol. Scand.* **33,** 99 (1955).
42. B. S. Gustafsson, S. Bergström, S. Lindstedt, and A. Norman, *Proc. Soc. Exptl. Biol. Med.* **94,** 467 (1957).
43. M. Frankel, *Biochem. J.* **30,** 2111 (1936).
44. A. Norman and R. Grubb, *Acta Pathol. Microbiol. Scand.* **36,** 537 (1955).
45. M. Ogura and K. Ozaki, *Yonago Acta Med.* **8,** 41 (1964).
46. J. Markowitz, M. Gordon, and P. P. Nair, unpublished studies.
47. P. P. Nair, M. Gordon, S. Gordon, J. Reback, and A. I. Mendeloff, *Life Sci.* **4,** 1887 (1965).
48. A. Norman and O. A. Widström, *Proc. Soc. Exptl. Biol. Med.* **117,** 442 (1964).
49. P. P. Nair, M. Gordon, and J. Reback, *J. Biol. Chem.* **242,** 7 (1957).
50. P. P. Nair, *in* "Bile Salt Metabolism" (L. Schiff, J. B. Carey, and J. M. Dietschy, eds.), p. 172, C. C Thomas, Springfield, Ill. (1969).
51. W. Troll and R. K. Cannan, *J. Biol. Chem.* **200,** 803 (1953).
52. V. Aries and M. J. Hill, *Biochim. Biophys. Acta* **202,** 526 (1970).
53. M. Ogura, *J. Biochem. (Tokyo)* **46,** 1077 (1959).
54. K. Hayase, *J. Yonago Med. Ass.* **11,** 55 (1960).
55. T. Usui, H. Ohtagaki, and T. Fujioka, *J. Yonago Med. Ass.* **11,** 154 (1960).
56. W. M. Hohen, L. H. Schmidt, and H. B. Highes, *J. Biol. Chem.* **152,** 59 (1954).
57. A. Kallner, *Ark. Kemi* **26,** 553 (1967).
58. V. Aries and M. J. Hill, *Biochim. Biophys. Acta* **202,** 535 (1970).

Chapter 10

ACTION OF HYPOLIPIDEMIC DRUGS ON BILE ACID METABOLISM*

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I. INTRODUCTION

The effects of pharmacological agents on bile acid metabolism may be assessed in several ways. The total excretion of bile acids in the bile or feces can be measured, the spectrum of bile acids or their conjugates can be determined, or the synthesis of bile acids can be assessed. To date, most experimental approaches have involved the first of these possibilities.

Although it has been known for some time that cholesterol is a precursor of bile acids, only recently have studies of bile acid metabolism been carried out in parallel with studies of cholesterol metabolism. The drugs whose effects on bile acids have been studied have generally been hypocholesterolemic agents.

The effects of hormones on bile acid metabolism are reviewed by Bekersky and Mosbach (1) in Chapter 8 of this volume, and these compounds will not be considered in this discussion.

II. NICOTINIC ACID

The hypocholesterolemic effect of nicotinic acid was first reported by

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Altschul *et al.* (2) in 1955 and has since been confirmed by a number of investigators. The mechanism of action of nicotinic acid has been studied in a number of laboratories from various points of departure. Two recent reviews of the possible mechanisms of action of this compound are available (3,4). One suggestion has been that nicotinic acid accelerates bile acid excretion, affects bile acid conjugation, or does both.

Kottke *et al.* (5,6) have studied the effects of nicotinic acid on bile acid conjugation in dogs made hypercholesterolemic by diet or chemical thyroidectomy. Dietarily induced cholesterolemia, effected by the addition of lard or corn oil to a meat diet, caused a moderate average rise in cholesterol levels (420 mg/dl), and administration of 1 g/day of nicotinic acid reduced this to an average level of 218 mg/dl. In two dogs whose bile ducts were cannulated, the starting cholesterol levels were lower than average. Although there was only a slight change in bile volume, the total bile acid excretion (mg/24 hr) doubled. The increase might have been due to a generally increased endogenous synthesis caused by the bile drainage. In contrast, bile acid excretion in one hypothyroid dog was increased tenfold, but this brought total excretion up to the level displayed by the euthyroid dogs. Nicotinic acid did not increase bile acid pool size, half-life, or turnover in either euthyroid or hypothyroid dogs.

Miller *et al.* (7) reported that nicotinic acid did not increase bile acid excretion in man. Miettinen (8), in a sterol balance study of five hypercho-

TABLE I. Influence of Nicotinic Acid on Lipids and Bile Acid Metabolism in Six Hypercholesterolemic Patients[a]

	Nicotinic acid	
	−	+
Plasma lipids (mg/dl)		
Cholesterol	389	267
Triglycerides	258	123
Phospholipids	452	341
Bile acid ratios		
Glycocholic/taurocholic	4.6	3.9
Cholic/chenodeoxycholic	1.0	1.3
Cholic acid		
Half-life (days)	2.1	3.3
Pool size (g)	0.51	0.63
Turnover (g/day)	0.269	0.230
Chenodeoxycholic acid		
Half-life (days)	2.1	2.1
Pool size (g)	0.38	0.34
Turnover (g/day)	0.159	0.156

[a] After Wollenweber *et al.* (9).

lesterolemic patients, showed average serum cholesterol and triglyceride reductions of 30 and 55%, respectively. Their total fecal steroid output (mg/day) rose from 831 to 1368. Their total neutral steroid rose from 680 to 1129 mg/day, but acidic steroid excretion was relatively unchanged, 151 versus 239 mg/day. He concluded that nicotinic acid exerted its hypocholesterolemic action by increased mobilization of tissue cholesterol and possibly by reduced cholesterol synthesis.

In another study, Wollenweber *et al.* (9) determined half-life, pool size, and turnover of cholic and chenodeoxycholic acids in six hypercholesterolemic patients. Their findings are summarized in Table I. It is apparent that turnover of neither cholic nor chenodeoxycholic acid was affected significantly by administration of nicotinic acid. Failey *et al.* (10) found that the ratio of glycocholamic/taurocholanic acid in seven patients treated with nicotinic acid (2 g/day) fell from 4.0 to 1.7. These authors also reported that subjects given 8 g/day of either benzoic or *p*-aminobenzoic acid showed changes in these ratios of 1.9 to 3.0 and 2.4 to 0.9, respectively. The available data show that nicotinic acid does not affect bile acid metabolism.

III. NEOMYCIN

The antibiotic neomycin is hypocholesterolemic in man (11,12) and in chicks (13). Since the compound is effectively hypocholesterolemic only when administered by the oral route, its presence in the gut is obviously what determines its mode of action. Goldsmith (14) and Powell *et al.* (15) observed increased fecal excretion of bile acids in patients fed this compound. Neomycin even when administered orally retains some antibacterial activity, since Rubulis *et al.* (16) found a marked decrease in fecal coprostanol excretion in patients fed 6 g/day of neomycin; they suggested that cholesterol excretion was increased in these patients. In general, neutral steroid excretion increased slightly. Bile acid excretion increased by about 100% in these patients, and stool weight increased by 150%. There was a change in the spectrum of fecal bile acids. There was a drop in fecal lithocholic acid and a sharp rise in cholic acid; deoxycholic acid excretion was virtually unchanged. The most striking difference was the appearance of appreciable quantities of chenodeoxycholic acid in the feces. Hamilton (17) had previously noted the increase in fecal cholic acid. Cayen (18) has shown that *in vitro* neomycin precipitates only dihydroxy bile acids.

Van den Bosch and Claes (19) have reported that *N*-methyl neomycin, a compound with no antibiotic properties, will lower cholesterol levels in man and in chicks. In man, *N*-methyl neomycin produces a sharp rise in both the fecal bile acids and neutral steroids. The amount of excreted lithocholic and

deoxycholic acids and of coprostanol and cholesterol is increased. These findings show that the bile acid binding activity is a function of the basicity of the compounds and not of their antibiotic properties. The ineffectiveness of neomycin in the rat (20) has not been explained.

IV. ETHYL *p*-CHLOROPHENOXYISOBUTYRATE (CLOFIBRATE, ATROMID-S)

Clofibrate is a widely used hypolipidemic agent whose mode of action, like that of nicotinic acid, has not been unequivocally established (21). In the earliest studies of the effect of this compound on fecal steroid excretion (22), it was concluded that excretion of sterols was increased but that of bile acids was not. The ratio of glycocholanic/taurocholanic acid was unaffected (23). Grundy *et al.* (24) reported that this drug reduced fecal bile acid excretion in their patients.

Horlick *et al.* (25) have studied the effect of clofibrate on fecal sterols in type II (hyperbetalipoproteinemia) and type IV (endogenous hypertriglyceridemia) patients, the typing being made on the basis of the Fredrickson–Levy–Less (26) classification. In three type II patients, there were significant increases in total fecal steroid excretion. Neutral steroid excretion was increased by 90% and acid steroid excretion by 72%. One normal patient showed significantly increased neutral steroid excretion, but there was no change in his acidic fecal steroids. Four type IV patients showed practically no changes in fecal steroids during clofibrate therapy.

Clofibrate appears to have little general effect on bile acid metabolism, but, as Horlick *et al.* (25) have shown, it may affect bile acid excretion in certain types of hyperlipidemias.

V. CHOLESTYRAMINE

Cholestyramine is a high molecular weight anionic exchange resin which, as the chloride salt, is used to absorb bile acids. In the dog (26), this material, when fed at the level of 25 g/day, was shown to increase fecal sterols by 85% and fecal bile acids by 160% and to reduce cholesterol levels by 22%. Rate fed 2% of cholestyramine in the diet showed a three- to fourfold increase in bile acid excretion, and the increase was all in the dihydroxycholanic acid fraction (27). In mice (28), this resin decreased the time for bile acid turnover from 5 to 1.25 days. Fecal neutral sterols were increased by 37%. Pigs fed 2–4% cholestyramine showed a marked increase in bile acid excretion (29).

Bergen *et al.* (30) first demonstrated the hypocholesterolemic effect of

cholestyramine in man. Hashim and Van Itallie (31) found that this compound had variable effects on neutral sterol excretion in man but that bile acid excretion was increased from 224 to 1349 mg/day (three patients). Moore *et al.* (32) and Miettinen (33) also found increases in the level of fecal bile acids but almost no change in neutral sterol excretion. Carey and Williams (34) found the increased bile acid excretion to be attributable to a sharp rise in deoxycholic acid content of the feces.

Mosbach *et al.* (35) have used cholestyramine to confirm that the 7α-hydroxylation of cholesterol is the rate-limiting step in bile acid synthesis. Because of the loss of cholesterol from the enterohepatic circulation, there is a marked increase in cholesterol synthesis during administration of cholestyramine to rats (27) or man (36). Mosbach *et al.*, using perfused rabbit liver, showed that the biliary content of glycocholic acid rose from 0.34 to 3.3 mg, while the content of glycodeoxycholic acid fell from 7.4 to 3.7 mg. The conversion of radioactive acetate, mevalonate, or cholesterol to bile acids was increased from five- to twentyfold, but the conversion rate of 7α-hydroxycholesterol to cholic acid was unchanged. The formation of 7α-hydroxycholesterol from cholesterol is enhanced by treatment with cholestyramine (37,38).

Other resins (39) have been shown to bind bile acids and to lower cholesterol levels by inhibiting its reabsorption; however, it is apparent that these compounds exert a specific effect on bile acid metabolism.

VI. OTHER COMPOUNDS

Heparin (39) and β-sitosterol (40) have been reported to promote bile acid excretion in man. Vitamin B_{12} has been shown to increase bile acid excretion (41), and one possible mechanism of action might involve its effect on increased bacterial growth (42).

Jones *et al.* (43) have observed that a hypocholesterolemic brain extract preparation (44) enhances bile acid excretion in man. A hydrophilic colloid derived from psyllium seed has been shown to effect a 500% increase in bile acid excretion in man (45,46). In the experiments with the colloid bile acid, excretion rose with the first week of feeding.

VII. MITOCHONDRIAL OXIDATION OF CHOLESTEROL

The nuclear changes involved in the conversion of cholesterol to cholic acid (reduction of the 5,6 double bond, epimerization of the hydroxyl group at carbon-3, and insertion of hydroxyl groups at positions 7 and 12) appear to be carried out by microsomal enzyme systems. Side-chain oxidation is effected by mitochondrial enzymes.

It has been shown (47–49) that suitably fortified preparations of mouse or rat liver mitochondria can oxidize the terminal methyl group of cholesterol to CO_2. The acidic products obtained are not identical with common bile acids (50,51). When the substrate is 3α,7α,12α-trihydroxycoprostane, however, the product of mitochondrial oxidation is cholic acid (52–54). Danielsson (55,56) has reviewed much of this work.

The mitochondrial enzyme system does not exhibit a great deal of specificity, being able to oxidize cholesterol, coprostanol, cholestenone, cholestanone, coprostanone (57), and even ergosterol (58) and desmosterol (59).

With use of the system defined by Whitehouse *et al.* (49,60), investigations have been carried out on the effects of cholesteropenic drugs on the oxidation of cholesterol-26-^{14}C to $^{14}CO_2$. While the substrate is not the natural one for mitochondrial oxidation, on the presumption that the natural enzyme system(s) is operating, it was thought that some useful clues to mechanism of action might be obtained.

The first experiments were carried out with nicotinic acid (61), and it was found that mitochondrial oxidation of cholesterol was enhanced in rats fed this compound. It was later found (62) that addition of nicotinic acid (8×10^{-5} moles, 10 mg) to the incubation mixture enhanced oxidation of cholesterol. Of a large series of nicotinic acid and nicotinamide homologues tested, only 3-pyridylacetic acid also stimulated oxidation of cholesterol (62,63). This compound has also been shown to be hypocholesterolemic in man (64,65). Pyridinol carbamate, 2,6-bis(hydroxymethyl)pyridine-di-*N*-methylcarbamate, fed to rats as 1% of their diet also enhanced *in vitro* oxidation of cholesterol by rat liver mitochondria (66).

Experiments with clofibrate, ethyl *p*-chlorophenoxyisobutyrate, showed that there was no difference in cholesterol oxidation when the complete system was used, but, in the absence of cytosol, liver mitochondrial preparations from drug-treated rats exhibited a significantly higher oxidative capacity (67). Two other compounds of related structure have also been tested in this system. With SU-13,437, [2-methyl-2-(*p*-1,2,3,4-tetrahydro-1-naphthylphenoxy)] propionic acid, results similar to those obtained with clofibrate were observed (68). There was no difference when the complete system was used, but the liver mitochondria from drug-treated rats oxidized more cholesterol than did those from control rats when cytosol was omitted. Similar, but less marked, results were obtained in experiments with Halofenate, 2-acetoamidoethyl(*p*-chlorophenyl)(*m*-trifluoromethylphenoxy)acetate (69). All three of these compounds caused hepatomegaly, but this alone cannot be considered the cytosol effect, since two other cholesterol-lowering compounds were also hepatomegalic but did not enhance cholesterol oxidation in the absence of cytosol. These two compounds were W-1372, *N*-α-phenylpropyl-*N*-ben-

zyloxy:acetamide (70), and Biphenabid, 4,4′-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol) (71).

Toki *et al.* (72,73) have shown that certain amides of linoleic acid are effective in reducing the severity of cholesterol-induced atherosclerosis in rabbits. Their mechanism of action appears to involve inhibition of cholesterol absorption (74–76). Liver mitochondrial preparations from rats fed either linolexamide, *N*-cyclohexyl linoleamide (77) or AC-223, *N*-(α-methylbenzyl)linoleamide (76) do not oxidize more cholesterol-26-^{14}C to $^{14}CO_2$ than do similar preparations from livers of control rats. Table II summarizes the effects of these drugs on mitochondrial oxidation of cholesterol. It should be emphasized again that this system is of interest primarily because the proper enzyme system (for cholesterol side-chain cleavage) is involved. For a completely accurate assessment of this system, 3α,7α,12α-trihydroxycoprostane would be the proper substrate.

VIII. CONCLUSION

It is becoming apparent to all investigators interested in control of hypocholesterolemia by drugs or diet that it is important to know the disposition of the sterol which is lost from the plasma. Since the bile acids are the principal degradation products of cholesterol, it becomes important to learn if cholesterol lowering is accompanied by excretion of the sterol or one of its degradation products. As can be seen from the foregoing discussion, the advent of new analytical methods has permitted an assessment of the

TABLE II. Effect of Cholesterol-Lowering Agents on Oxidation of Cholesterol-26-^{14}C to $^{14}CO_2$ by Rat Liver Mitochondria

	Percentage of control				
Compound[a]	Liver weight (g/100 g)	Serum cholesterol	Serum triglyceride	Cholesterol oxidation	References
Nicotinic acid	90	96	—	229	(61)
Pyridinol carbamate	105	110	79	136	(66)
Clofibrate	125	100	68	94[b]; 304[c]	(67)
SU-13, 437	188	132	65	90[b]; 150[c]	(68)
Halofenate	119	55	91	132[b]; 221[c]	(69)
W-1372	163	107	68	95	(70)
Biphenabid	126	46	55	67	(71,80)
Linolexamide	103	107	—	147	(77)
AC-223	127	108	—	99	(76)

[a] See text for chemical names.
[b] With cytosol.
[c] Without cytosol.

amount of bile acid excreted during several different types of drug treatment. Fewer analyses of bile acid spectrum have been carried out, but this information may be necessary for understanding modes of drug action. Portman (78) has shown that 12-hydroxylated bile acids are the most cholesterolemic for rats. It has also been shown (79) that cholic acid causes cholesterolemia in hyperthyroid rats, whereas chenodeoxycholic acid reduces serum cholesterol levels of hypothyroid rats. Thus the mechanism of drug action may depend not only on changes in bile acid content but on bile acid composition as well. We can expect future studies to concern themselves with bile acid content, spectrum, and conjugation.

REFERENCES

1. I. Bekersky and E. H. Mosbach, this volume, p. 249.
2. R. Altschul, A. Hoffer, and J. D. Stephen, *Arch. Biochem. Biophys.* **54,** 558 (1955).
3. D. Kritchevsky, *in* "Metabolic Effects of Nicotinic Acid and Its Derivatives" (K. F. Gey and L. A. Carlson, eds.), p. 149, Huber, Berne (1971).
4. W. B. Parsons, Jr., *in* "Treatment of the Hyperlipidemic States (H. R. Casdorph, ed.), p. 335, C. C Thomas, Springfield, Ill. (1971).
5. B. A. Kottke, J. L. Bollman, and J. L. Juergens, *Circ. Res.* **11,** 108 (1962).
6. J. Wollenweber, B. A. Kottke, and C. A. Owen, Jr., *Proc. Soc. Exptl. Biol. Med.* **122,** 1070 (1966).
7. O. N. Miller, J. G. Hamilton, and G. A. Goldsmith, *Am. J. Clin. Nutr.* **8,** 480 (1960).
8. T. A. Miettinen, *Clin. Chim. Acta* **20,** 43 (1968).
9. J. Wollenweber, B. A. Kottke, and C. A. Owen, Jr., *J. Lab. Clin. Med.* **69,** 584 (1967).
10. R. B. Failey, Jr., R. H. Childress, and T. P. McConahay, *Am. J. Clin. Nutr.* **10,** 158 (1962).
11. P. Samuel and A. Steiner, *Proc. Soc. Exptl. Biol. Med.* **100,** 193 (1959).
12. P. Samuel, *Proc. Soc. Exptl. Biol. Med.* **102,** 194 (1959).
13. P. DeSomer, H. Vanderhaeghe, and H. Eyssen, Nature **204,** 1306 (1964).
14. G. A. Goldsmith, *Fed. Proc.* **21,** 81 (1962).
15. R. C. Powell, W. T. Nunes, R. S. Harding, and J. B. Vacca, *Am. J. Clin. Nutr.* **11,** 156 (1962).
16. A. Rubulis, M. Rubert, and W. W. Faloon, *Am. J. Clin. Nutr.* **23,** 1215 (1970).
17. J. G. Hamilton, *Arch. Biochem. Biophys.* **161,** 7 (1963).
18. M. N. Cayen, *Am. J. Clin. Nutr.* **23,** 1234 (1970).
19. J. F. Van den Bosch and P. J. Claes, *in* "Progress in Biochemical Pharmacology" (D. Kritchevsky, R. Paoletti, and D. Steinberg, eds.), Vol. 2, p. 97, Karger, Basel (1967).
20. H. Eyssen, E. Sacquet, E. Evrard, and J. Van den Bosch, *Life Sci.* **7,** 1155 (1968).
21. D. Steinberg, *in* "Atherosclerosis" (R. J. Jones, ed.), p. 500, Springer-Verlag, Heidelberg (1970).
22. W. D. Mitchell, A. S. Truswell, and B. Bronte-Stewart, *in* "Progress in Biochemical Pharmacology" (D. Kritchevsky, R. Paoletti, and D. Steinberg, eds.), Vol. 2, p. 365, Karger, Basel (1967).
23. A. S. Truswell, W. D. Mitchell, and S. McVeigh, *J. Atherosclerosis Res.* **6,** 591 (1966).
24. S. M. Grundy, E. H. Ahrens, Jr., G. Salen, and E. Quintao, *J. Clin. Invest.* **48,** 33a (1969).

25. L. Horlick, B. J. Kudchodkar, and H. S. Sodhi, *Circulation* **43,** 299 (1971).
26. D. M. Tennent, H. Siegel, M. E. Zanetti, G. W. Kuron, W. H. Ott, and F. J. Wolf, *J. Lipid Res.* **1,** 469 (1959).
27. J. W. Huff, J. L. Gilfillan, and V. M. Hunt, *Proc. Soc. Exptl. Biol. Med.* **114,** 352 (1963).
28. W. T. Beher, M. E. Beher, and B. Rao, *Proc. Soc. Exptl. Biol. Med.* **122,** 881 (1966).
29. D. L. Schneider, D. G. Gallo, and H. P. Sarett, *Proc. Soc. Exptl. Biol. Med.* **121,** 1244 (1966).
30. S. S. Bergen, T. B. Van Itallie, D. M. Tennent, and W. H. Sebrell, *Proc. Soc. Exptl. Biol. Med.* **102,** 676 (1959).
31. S. A. Hashim and T. B. Van Itallie, *J.A.M.A.* **192,** 89 (1965).
32. R. B. Moore, C. A. Crane, and I. B. Frantz, Jr., *J. Clin. Invest.* **47,** 1664 (1968).
33. T. A. Miettinen, *Clin. Chim. Acta* **19,** 341 (1968).
34. J. B. Carey, Jr., and G. Williams, *J.A.M.A.* **176,** 432 (1961).
35. E. H. Mosbach, M. A. Rothschild, I. Bekersky, M. Oratz, and J. Mongelli, *J. Clin. Invest.* **50,** 1720 (1971).
36. D. S. Goodman and R. P. Noble, *J. Clin. Invest.* **43,** 2026 (1964).
37. E. H. Mosbach, in "Advances in Experimental Medicine and Biology" (W. L. Holmes, L. A. Carlson, and R. Paoletti, eds.), Vol. 4, p. 421, Plenum Press, New York (1969).
38. G. S. Boyd, N. A. Scholan, and J. R. Mitton, *in* "Advances in Experimental Medicine and Biology" (W. L. Holmes, L. A. Carlson, and R. Paoletti, eds.), Vol. 4, p. 443, Plenum Press, New York (1969).
39. H. Engelberg, *Proc. Soc. Exptl. Biol. Med.* **102,** 365 (1959).
40. K. A. Meschcherskaya and G. P. Borodina, *Farmakol. Toksikol.* **25,** 44 (1962).
41. M. C. Nath and D. M. Brahmankar, *Proc. Soc. Exptl. Biol. Med.* **108,** 337 (1961).
42. H. K. Alimchandini and A. Sreenivasan, *Biochim. Biophys. Acta* **18,** 567 (1955).
43. R. J. Jones, O. K. Reiss, and M. F. Golden, *in* "Drugs Affecting Lipid Metabolism" (S. Garattini and R. Paoletti, eds.), p. 212, Elsevier, Amsterdam (1961).
44. R. J. Jones, *J. Lab. Clin. Med.* **47,** 261 (1956).
45. J. E. Garvin, D. T. Forman, W. R. Eiseman, and C. R. Phillips, *Proc. Soc. Exptl. Biol. Med.* **120,** 744 (1965).
46. D. T. Forman, J. E. Garvin, J. E. Forestner, and C. B. Taylor, *Proc. Soc. Exptl. Biol. Med.* **127,** 1060 (1968).
47. C. B. Anfinsen and M. G. Horning, *J. Am. Chem. Soc.* **75,** 1511 (1953).
48. D. S. Fredrickson, M. G. Horning, and C. B. Anfinsen, *Fed. Proc.* **13,** 212 (1954).
49. M. W. Whitehouse, E. Staple, and S. Gurin, *J. Biol. Chem.* **234,** 276 (1959).
50. W. S Lynn, E. Staple, and S. Gurin, *Fed. Proc.* **14,** 783 (1955).
51. D. S. Fredrickson, *J. Biol. Chem.* **222,** 109 (1956).
52. H. Danielsson, *Acta Chem. Scand.* **14,** 348 (1960).
53. T. Briggs, M. W. Whitehouse, and E. Staple, *J. Biol. Chem.* **236,** 688 (1961).
54. H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.* **237,** 338 (1962).
55. H. Danielsson and T. T. Chen, *in* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. 2, p. 118, Academic Press, New York (1968).
56. H. Danielsson, this volume, p. 1.
57. E. Stevenson and E. Staple, *Arch. Biochem. Biophys.* **97,** 485 (1962).
58. D. Kritchevsky, E. Staple, and M. W. Whitehouse, *Proc. Soc. Exptl. Biol. Med.* **106,** 704 (1961).
59. D. Kritchevsky and E. Staple, *Naturwissenschaften* **49,** 109 (1962).
60. M. W. Whitehouse, E. Staple, and S. Gurin, *J. Biol. Chem.* **236,** 68 (1961).
61. D. Kritchevsky, M. W. Whitehouse, and E. Staple, *J. Lipid Res.* **1,** 154 (1960).
62. D. Kritchevsky and S. A. Tepper, *Arch. Int. Pharmacodyn.* **138,** 349 (1962).

63. D. Kritchevsky and S. A. Tepper, *Arch. Int. Pharmacodyn.* **147,** 564 (1964).
64. G. Ratti and E. deFina, Lancet **ii,** 917 (1959).
65. V. Ferrari and F. Campagnari, *Minerva Med.* **51,** 1147 (1960).
66. D. Kritchevsky and S. A. Tepper, *Arzneimittel-Forschung* **21,** 146 (1971).
67. D. Kritchevsky, S. A. Tepper, P. Sallata, J. R. Kabakjian, and V. J. Cristofalo, *Proc. Soc. Exptl. Biol. Med.* **132,** 76 (1969).
68. D. Kritchevsky and S. A. Tepper, *Experientia* **25,** 699 (1969).
69. D. Kritchevsky and S. A. Tepper, *Proc. Soc. Exptl. Biol. Med.,* **139,** 1284 (1972).
70. D. Kritchevsky and S. A. Tepper, *Arzneimittel-Forschung* **20,** 584 (1970).
71. D. Kritchevsky, H. K. Kim, and S. A. Tepper, *Proc. Soc. Exptl. Biol. Med.* **136,** 1216 (1971).
72. K. Toki and H. Nakatani, *in* "Progress in Biochemical Pharmacology" (D. Kritchevsky, R. Paoletti, and D. Steinberg, eds.), Vol. 2, p. 203, Karger, Basel (1967).
73. K. Toki, T. Fukumaru, H. Nakatani, and H. Fukushima, *J. Atherosclerosis Res.* **7,** 708 (1967).
74. H. Nakatani, H. Fukushima, A. Wakimura, and M. Endo, *Science* **153,** 1267 (1966).
75. H. Fukushima, Y. Nakamura, M. Endo, and T. Ima, *J. Atherosclerosis Res.* **10,** 403 (1969).
76. D. Kritchevsky and S. A. Tepper, *Arzneimittel-Forschung* **21,** 1024 (1971).
77. D. Kritchevsky, P. Sallata, and S. A. Tepper, *Proc. Soc. Exptl. Biol. Med.* **127,** 132 (1968).
78. O. W. Portman, *Fed. Proc.* **21,** 896 (1962).
79. D. Kritchevsky and S. A. Tepper, *Med. Pharmacol. Exp.* **14,** 1 (1966).
80. D. Kritchevsky, *Fed. Proc.* **30,** 835 (1971).

Chapter 11

BILE ACID METABOLISM IN GNOTOBIOTIC ANIMALS*

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I. INTRODUCTION

The bile acid metabolism of conventional animals is an intimately related blend of effects caused by the enzymes of the host and the enzymes of the indigenous microbial flora. In the absence of pathogenic conditions, significant populations of enteric microorganisms will be found in the lower small intestine, and in the colon (and cecum if present) they will reach up to 10^{10} organisms per gram of intestinal contents. This microflora exerts a profound effect on the biology of the host and particularly on those systems which come into intimate contact with it.

During the course of their normal enterohepatic circulation, bile acids are secreted into the lumen of the intestine, where they aid in the emulsification and digestion of fats. In the lower small intestine, they are largely actively reabsorbed and carried back to the liver, where they are again secreted to the upper gut. During this intestinal phase of the circulation, the bile acids are exposed to the action of microbial enzymes which may modify their chemical structure and thus their biological activity in many ways. There is always a small percentage of bile acids not absorbed by the active transport system, and these bile acids will be exposed to the mass of bacterial activity present in the cecum and large intestine. Some passive absorption of the bacterially

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modified bile acid occurs in the lower gut (1) and cecum (2) in most species, and the bile acid which is not absorbed is excreted in the feces. This chapter will discuss comparative bile acid metabolism of germfree and conventional subjects and the activities of pure and mixed cultures of bacteria on bile acids in gnotobiotic species and *in vitro*.

A. Definitions

In order to clarify terminology, the following definitions have been officially adopted:

Germfree animal: a gnotobiote that is free from all demonstrable associated forms of life, including bacteria, viruses, fungi, protozoa, and other saprophytic or parasitic forms.

Gnotobiote: an animal, stock, or strain, derived by aseptic caesarean section or sterile hatching of eggs, which is reared and continuously maintained with germfree techniques under isolator conditions in which the composition of any associated fauna and flora, if present, is fully defined by accepted current methodology.

Defined flora animal; a gnotobiote maintained under isolator conditions in intentional association with one or more known types of microorganisms. Such terms as "monoinfected," "monocontaminated," "monoassociated," and "polycontaminated" have been employed to describe this type of gnotobiote. Of these the terms, "monoassociated" and "polyassociated" are preferable for describing intentional, rather than accidental, association with microbes.

B. Antibiotic Experiments with Conventional Animals

Norman (3) demonstrated that the types of bile acids found in normal rat bile were not the same as those which were excreted in the feces. However, when the rats were fed high levels of antibiotics, the fecal bile acids were excreted essentially unchanged from the biliary bile acids (4). The intestinal bacteria were responsible for the hydrolysis of the biliary taurine-conjugated bile acids to the free bile acids found in the feces. Norman also showed that the dehydroxylation of cholic acid to deoxycholic acid could be prevented by inhibiting the intestinal bacteria. The total amount of fecal bile acid excreted by conventional chicks has been found to be significantly lowered (5) by incorporation of an antibiotic into the diet.

C. Cholesterol Feeding and Liver and Serum Cholesterol Levels of Germfree and Conventional Animals

The intestinal microflora exerts a protective effect in several species

against high tissue accumulations of cholesterol when the subject is fed a cholesterol-containing diet. Eyssen *et al.* (6) reported that both germfree and conventional chicks fed cholesterol-free diets had essentially similar serum and liver cholesterol pools. When the chicks were fed diets containing 0.25% cholesterol, the pool was twice as large for the germfree chicks as for the conventional group. They further showed that the fecal excretion of bile acids was greater in the conventional than in germfree groups on both cholesterol-free and cholesterol-containing diets. This increased catabolism of cholesterol to bile acids would aid in keeping tissue levels of cholesterol low.

Similar data have been reported for liver cholesterol levels in rats (7). Germfree and conventional rats fed a cholesterol-free diet had similar liver cholesterol levels; however, as the amount of cholesterol in the diet was increased, the liver cholesterol levels of the germfree rats rose three times higher than did those of the comparable conventional rats. In these rats, the serum cholesterol levels did not change with microbiological status or dietary cholesterol.

Large changes in serum cholesterol levels have been shown to occur when gnotobiotic swine were fed a cholesterol-containing diet. Gnotobiotic swine fed a cholesterol-containing diet had serum cholesterol levels of about 1000 mg %, but when they were removed from isolators and raised in a nonsterile environment, the serum cholesterol levels fell to 100 mg % within 3 weeks (8). The fall in serum cholesterol could be correlated with the development of an intestinal microflora which metabolized the primary bile acids to secondary bile acids. Similar swine fed an identical but cholesterol-free diet had serum cholesterol levels of about 100 mg % during both periods.

The above experiments indicate that the intestinal microflora is an important variable in qualitative and quantitative bile acid metabolism. In the following sections, some specific aspects of this interaction will be examined in detail.

II. STUDIES OF CHOLESTEROL CATABOLISM TO BILE ACIDS IN GERMFREE ANIMALS

A. Fate of ^{14}C-26 of Cholesterol Molecule

When cholesterol labeled in the 26-position was injected into an intact rat, most of the ^{14}C released by the animal was found as expired $^{14}CO_2$ (9). However, when the identical experiment was performed using ^{14}C-4 labeled cholesterol, none of the isotope was recovered as $^{14}CO_2$. Subsequent experiments (10) established the liver as the site of the side-chain oxidation. *In vitro* experiments with subcellular fractions (11,12) revealed that during the

conversion of cholesterol to bile acids, the three terminal carbons of the cholesterol side chain were oxidatively removed as propionate (12), which was subsequently oxidized via the Krebs cycle to CO_2.

B. Release of Radioactive $^{14}CO_2$ in Germfree and Conventional Animals

Wostmann *et al.* (13) utilized the above findings to monitor the relative rates of cholesterol oxidation to bile acids in germfree and conventional rats. They established that there was a uniform rate of uptake of injected cholesterol-26-^{14}C into blood and tissue in both groups and then studied the distribution of the isotope and the specific activity of cholesterol in various tissues, expired air, urine, and feces. The $^{14}CO_2$ expired by the conventional rats in a 72-hr period was 29.7% (see Table I) of the injected label, while the comparable germfree group expired only 19% of the injected isotope; 54% of the isotope was recovered as unchanged cholesterol-26-^{14}C in the conventional rats versus 67% in the germfree group. There was no significant difference in the total recovery of isotope from the two groups or in the amount of isotope found in the liver or carcass as noncholesterol ^{14}C. The total blood plus liver cholesterol pool was not significantly different in the two groups, indicating that dilution of the isotope was not responsible for the differences observed. Both the liver and carcass cholesterol-^{14}C levels were higher in the germfree group, again supporting the conclusion that the catabolism of cholesterol to bile acid was slower in germfree than in conventional rats.

The ^{14}C recovered in the feces was nearly twice as great in the conventional group as in the germfree, thus indicating that conventional rats ex-

TABLE I. Percentage Distribution of ^{14}C after Intravenous Administration of Cholesterol-26-^{14}C to Germfree and Conventional Male Rats[a]

	Germfree	Conventional	*P*
Expired air	19.0 ± 1.2	29.7 ± 2.0	±0.01
Carcass			
3β-OH sterol	48.9 ± 1.7	42.5 ± 1.7	0.02
Other	3.7 ± 1.1	4.1 ± 1.2	N.S.
Liver			
3β-OH sterol	10.0 ± 0.6	7.5 ± 0.3	0.01
Other	1.7 ± 0.4	2.2 ± 0.5	N.S.
Fecal extract	2.6 ± 0.4	4.6 ± 0.4	0.01
Residual (urine, cecal contents, blood, etc.)	3.2 ± 0.1	2.3 ± 0.1	0.01
Total recovery of original dose	89.1 ± 1.8	92.9 ± 2.0	N.S.

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[a]Mean values ± S.E.M.

creted more fecal neutral sterol than their germfree counterparts. This has subsequently been verified by direct chromatographic experiments by others (14).

III. BILE ACID POOL SIZES, TURNOVER RATES, AND PHYSIOLOGICAL OBSERVATIONS IN GERMFREE VERSUS CONVENTIONAL ANIMALS

The early studies of the relative rate of bile acid excretion, pool size, etc., in germfree and conventional rats suffered from an incomplete knowledge of the qualitative composition of the bile acids which could be biosynthesized by the rat. Studies by workers at St. Louis University (15–20) have shown that intact rats excrete, in addition to chenodeoxycholic and cholic acids, α- and β-murichoilc acids, which can be biosynthesized from chenodeoxycholic acid but not from cholic acid. All of the isotopic studies reported in this section were based on the assumption that administered isotopic cholic acid would uniformally label the bile acid pool. These studies are valid in the context of investigations on the metabolism of cholic acid and its metabolites; however, it should be borne in mind that at least 60% of the germfree bile acid excretion of rats consists of unlabeled muricholic acids (14), and, depending on the techniques of the individual experiments may have been missed.

The first investigation of bile acid metabolism in germfree animals was reported by Gustafsson *et al.* (21) in 1957. They administered an oral dose of cholic acid-^{14}C to germfree, conventional, and gnotobiotic rats 3–6 months old, fed a semisynthetic diet. Feces were collected daily and extracted for bile acids by boiling in 80% ethanol. The ethanol extracts were counted for total radioactivity, and the bile acids were separated by partition chromatography using radioactivity measurements to follow bile acid elution.

As stated above, when conventional rat feces were chromatographed, the ^{14}C from cholic acid was found in a variety of compounds, while in rats treated with chemotherapeutics only taurocholic acid-^{14}C was found—the same compound as was found in the bile when cholic acid was injected. The feces of the germfree rats contained only radioactive taurocholic acid, the same results as when conventional rats were fed antibiotics. This definitely established that intestinal enzymes do not hydrolyze the conjugated bile acids.

One rat was monoassociated with *Aspergillus niger* and later diassociated with *A. niger* and *Clostridium perfringens* type E. Monoassociation with *A. niger* did not change the qualitative fecal bile acid excretion; however, addition of *C. perfringens* caused free cholic acid and other compounds to appear. This same rat was then removed from the isolator and placed in the open

TABLE II. Effect of Intestinal Flora on the Distribution, Biological Half-Life, and Daily Excretion of Labeled Bile Acids after Administration of Cholic Acid-24-C^{14}

	Controls				Germfree			Monoinfected with *E. coli*		
Rat	143 X 8	143 X 9	125 X 14	125 X 15	143 X 1	143 X 2	144 X 1	144 X 2	125 X 3	125 X 6
Weight (g)	222	278	297	282	239	226	211	196	307	243
Isotope in the rat after 10 days										
Percent of given amount	7	8			36	44	33	30	32	26
Percentage distribution:										
Liver	3	2	2	3	1	1	1	1	3	1
Small intestine	84	90	91	89	80	86	77	76	78	80
Cecum	10	4	3	5	18	11	19	22	18	19
Colon	3	4	4	3	1	2	3	1	1	1
Labeled bile acids in small intestine (mg)	14.6	14.9[b]	12.7	12.1	17.0	15.0	13.0	17.7	19.0	22.5
Cholic acid (quantitatively determined)	11.8		11.5	11.2	17.0	15.0	11.3	14.3	16.3	20.7
Other labeled acids (calculated from isotope distribution)	2.8		0.9	0.9	—	—	1.7	3.4	2.7	1.8
Labeled bile acids in cecum and colon (mg)	1.1	1.3	0.8[b]	1.0[b]	2.8	2.0	4.3	3.4	2.0	1.4
Cholic acid (quantitatively determined)	—[a]	—[a]			2.8	2.0	3.4	2.7	1.8	1.2
Deoxycholic acid (quantitatively determined)	0.4	0.8			—	—	—	—	—	—

Other labeled acids (calculated from isotope distribution)	0.7	0.5			—	—	0.7	0.7	0.2	0.2
Labeled bile acids in feces, mean daily excretion (mg)	4.8	5.4			2.0	1.8	1.7	2.5	2.0	2.6
Cholic acid (quantitatively determined)	—[a]	—[a]			2.0	1.8	1.1	1.6	1.6	1.8
Deoxycholic acid (quantitatively determined)	1.2	1.6			—	—	—	—	—	—
Other labeled acids (calculated from isotope distribution)	3.6	3.2					0.6	0.9	0.4	0.8
Sampling period (days after administration)	3–10	3–10			1–10	1–10	3–10	2–10	2–9	1–10
Half-life of labeled acids	2.0	2.1			5.8	7.5	5.6	5.0	6.2	4.9
Turnover time ($\bar{T} = t_{1/2}/\ln 2$)	2.9	3.0			8.3	10.7	8.0	7.2	8.9	7.0
Labeled acids in the rat (M, mg)	15.7	16.2	13.5	13.1	19.8	17.0	17.3	21.1	21.0	23.9
Daily excretion of labeled acids calculated ($M/\bar{T}$) (mg)	5.4	5.4			2.4	1.6	2.2	2.9	2.4	3.4
Daily excretion of labeled acids, determined (mg)	4.8	5.4			2.0	1.8	1.7	2.5	2.0	2.6

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[a] Not determined. Included in "other labeled acids."

[b] Not determined. Calculated from isotope distribution in the rat.

laboratory for 10 days to obtain a "normal" intestinal flora. When its feces were examined, the bile acids were present as a complex mixture of many radioactive compounds.

The time required for the conventional rats to excrete one-half of the administered isotope was calculated to be 2 days. The germfree rats required 11.4 days to excrete one-half of their isotope, a similar time as conventional rats fed antibiotics. The rat infected with *A. niger* with or without *C. perfringens* had an excretion half-life similar to its germfree rate; however, after being exposed to the laboratory environment for 10 days, it required only 1.8 days (versus 2 days for conventionally reared) to excrete one-half of its isotope. Thus it rapidly became "normal" in its bile acid excretion. Excretion of the germfree group was estimated to be 0.9 mg/100 g body weight, and the bile acid pool size was 15.4 mg/100 g body weight.

Gustafsson *et al.* (22) reinvestigated this area with improved techniques in a study of the effect of monoassociation with *Escherichia coli* on cholic acid metabolism of rats. In this study, they determined the amount of bile acids excreted by quantitative paper chromatography. These techniques would probably include the muricholic acids. The quantitative results of their study are given in Table II. They, in agreement with earlier work (2), found that in the rat, which does not have a gallbladder, approximately 97% of the bile acids were present in the intestines, with the small intestine having the greatest quantity. In conventional rats, the cecum, a site of maximal bacterial activity on fecal steroids (Kellogg, unpublished data, 23), contains 5–6% (Table II) of the total. The germfree and *E. coli* monoinfected rats (Table II) had nearly three times as much (18%) bile acid in the cecum as did the conventional rats. An enlarged cecum is a normal condition for germfree rats. On the other hand, the bile acid in the *colon* was *less* in the germfree rats, implying that there was greater absorption of bile acid in the germfree and monoinfected rats in the area of the cecum. It has been shown that 50% of a dose of cholic acid-^{14}C injected into the cecum of conventional rats could be recovered in the bile, much of it as secondary bile acids (2).

The estimated pool size of the germfree and monoinfected groups was somewhat larger than that of the conventional group. The half-life of the isotope in the conventional groups was the same as in the previous study, 2.0 days; however, that in the germfree group was shorter, 6.6 days versus 11.4 days in the previous study.

Portman and Murphy (24) reported that the type of diet had an influence on turnover of cholic acid in conventional rats. Gustafsson and Norman (25) have investigated whether this effect is seen in germfree rats. In their study, they concluded that no significant difference existed in the percent of bile acid in the cecum of their rats among different groups of animals kept on different diets or between germfree and conventional ani-

mals. They did find, however, that in germfree rats fed a commercial rat pellet diet, the biological half-life of cholic acid was one-third that in germfree rats fed a semipurified diet. The differences were much less among the conventional rats fed the different diets. Among comparable diet groups, the elimination rates of the germfree rats were always slower than in conventional rats. A possible explanation of the more rapid elimination of bile acid by the germfree group receiving pellets may lie in the observation of a more rapid transit time in this group than in the germfree rats fed the semisynthetic diet. Addition of cellulose to the diet of the latter did not bring it up to the pellet-fed group.

IV. STATE OF BILE ACIDS IN THE INTESTINAL LUMEN

It has been proposed that the differences in excretion rates of bile acid between germfree and conventional rats may lie in the adsorption of the secondary bile acids to the nonfluid sediment in the gastrointestinal tract so that they are less available for absorption. Gustafsson and Norman (23) administered cholic acid-^{14}C to germfree and conventional rats fed a semisynthetic diet *ad libitum*. Forty-eight hours after dosing, the rats were killed and the intestinal tracts removed. The contents of the small intestine, cecum, and colon were removed separately and after homogenization were centrifuged at 25,000g for 60 min. The supernatant and sediment were analyzed for radioactivity, and the bile acids were chromatographed. In the germfree rats, all of the radioactivity was recovered in the supernatant fraction from all sections of the gastrointestinal tract. In the conventional rats, 95% or more of the label was found in the supernatant in the small intestine. This is the site of active bile salt transport and usually has little microbiological activity. In the cecum and large intestine, an average of 35% of the radioactivity was recovered in the sediment. There was proportionally more bile acid without substituents in the C-7 position in the sediment than in the supernatant. Norman (26) conducted a similar study on the distribution of isotope in feces after oral administration of labeled bile acids to human subjects. His conclusions are in agreement with the above report, that "the results show that chemical transformation of cholic acid by intestinal microorganisms also changes 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."

An investigation of the distribution of the dehydroxylation products of cholic and chenodeoxycholic acids by mixed human fecal microorganisms *in vitro* (27) has shown that lithocholic acid (formed by the bacterial dehydroxylation of chenodeoxycholic acid) was recovered almost entirely in the

culture sediment after centrifugation (25,000g, 60 min); however, in this *in vitro* system, deoxycholate was recovered in the supernatant. The authors speculate that lithocholic acid formed *in vivo* by enteromicroorganisms was less available for absorption than deoxycholic acid, and this explains why lithocholic acid is only found in traces in human bile.

It has been shown that the adsorption of bile salts to the "sediment" fraction was greater when the diet was of a natural type than when it was semisynthetic (28). Eastwood and Hamilton (29) have shown that lignin, present in grains, will bind bile acids, with the nonconjugated and 7-dehydroxy forms bound more readily than the more polar bile acids. Since these "less polar" bile acids are formed by bacterial action, their observations may explain both the greater concentration of bile acid in the "sediment" fraction in rats fed crude versus synthetic diets (more lignin in the former) and the increased effect in conventional animals (proportionally more bacterially formed deconjugated and dehydroxylated bile acid, which is bound more strongly).

As will be discussed below, the fecal bile acid excretion of germfree subjects is only one-half that of comparable conventional subjects. Wostmann (unpublished data) has observed that the biliary bile acid flow in the germfree rat was three times greater than in the conventional rat. Since the pool sizes were essentially similar (22), this implies that the absorptive capacity and the number of cycles through the enterohepatic system were markedly greater in germfree rats than in conventional rats.

V. QUANTITATIVE FECAL EXCRETION OF BILE ACIDS BY GERMFREE AND CONVENTIONAL ANIMALS

The quantitative analysis of the fecal bile acids of rats presents a special problem which does not need to be considered in comparable studies with most other species. The presence of appreciable quantities of muricholic acids, over 50% of the total with germfree feces, requires methods which will not cause destruction of these 3,6,7-trihydroxy bile acids if chromatographic techniques are used.

It has been shown that the trifluoroacetates of 3,6,7-trihydroxy bile acids are subject to thermal decomposition in gas chromatographs (30). Oxidation of the bile acids to their keto derivatives and subsequent gas chromatography should also be avoided (31). In our laboratory, we have been unable to gas chromatograph any oxidized 3,6,7 bile acid methyl esters; they are either destroyed or will not elute in a reasonable amount of time.

If isotopic techniques are used, care must be taken to ensure that the entire bile acid pool is uniformally labeled. Use of radioactive cholic acid

will not label the muricholic or chenodeoxycholic acid pools. Radioactive chenodeoxycholic acid would be expected to label its own and the muricholic acid pools, since the liver cannot add a 12-hydroxy group after the side chain has been oxidized. Possibly, a labeled intermediate between cholesterol and an early step in bile acid biosynthesis may prove to be satisfactory, although there is increasing evidence for a branch point at cholesterol and an alternate path of bile acid biosynthesis in some species (32–34).

A. Rats

Gustafsson *et al.* (22) studied the fecal excretion of cholic acid-^{14}C metabolites in conventional and germfree rats. The mean daily excretion of labeled cholic acid and its metabolites was 18.9 mg/kg body weight for the conventional group and 8.2 mg/kg body weight for the germfree group. As mentioned above, their techniques would not uniformly label the bile acid pool; however, their other techniques may have caused inclusion of the muricholic acids in their assay.

Kellogg and Wostmann (14) studied the fecal bile acid excretion of germfree and conventional rats by direct chromatographic procedures. The germfree rats average 11.3 $\pm$ 2.4 mg and the conventional rats 21.4 $\pm$ 9.9 mg of bile acid/kg body wt/day ($p < 0.005$) (Table III and Fig. 1). The coefficients of variation were 21 and 46%, respectively, suggesting that at least half of the quantitative variation seen in fecal bile acid excretion in the conventional animal was derived from effects of the intestinal microflora.

All of the bile acids excreted had TLC mobilities similar to those for

TABLE III. Fecal Bile Acids Excreted by Germfree and Conventional Male Rats

	Germfree (14)	Conventional (11)
Fecal bile acid excretion (mg/kg body wt/day $\pm$ S.D.)	11.3 $\pm$ 2.4	21.4 $\pm$ 9.9[a]
Approximate percent of bacterial modification	None	57.7
Types of bile acids found	Taurocholic	Cholic
	Tauro-β-muricholic	β-Muricholic
	Tauro-α-muricholic	α-Muricholic
	Unidentified compound	Chenodeoxycholic
		Deoxycholic
		Lithocholic
		Many others

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[a]Difference between conventional and germfree means is significant ($p < 0.005$).

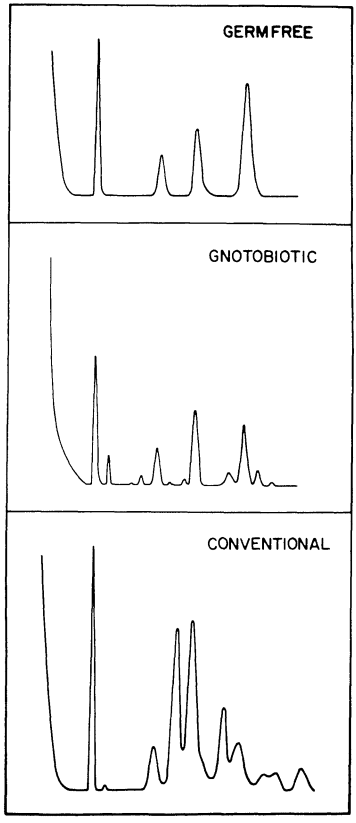

Fig. 1. GLC of fecal bile acids from germfree, gnotobiotic, and conventional rats. The first peak in each chromatogram is the internal standard 5α-cholestane. Instrument, Hewlett-Packard 402 gas chromatograph. Column, 2-m glass Utube packed with 1% SE-30 on 80-120 mesh Gas Chrom Q. Isothermal 220°C. Nitrogen carrier gas 75 ml/min. Flame ionization detector. Bile acids were analyzed as the trimethylsilyl ethers of the bile acid methyl esters. From Kellogg *et al.* (44). Reprinted with permission of the *Journal of Lipid Research.*

taurocholic acids, whereas all of the conventional bile acids were excreted as the free bile acids. Approximately 61% of the total germfree fecal bile acid consisted of β-muricholic acid and some 30% of cholic acid, with possible traces of α-muricholic acid. A small amount of a material which behaved like a monohydroxy bile acid was also observed. Kellogg and Wostmann estimated that 60% of the conventional bile acids had been modified in some form in addition to deconjugation by the intestinal microflora.

The large amount of muricholic acid found in these studies can be partially explained by the results of Shefer *et al.* (35). They found that when cholic acid was infused into intact rats, the *de novo* bile acid synthesis dropped markedly. However, the decrease in bile acid biosynthesis was much sharper for the cholic acid than for the β-muricholic acid, and, after a long-term bile acid infusion, β-muricholic acid became the predominant bile acid biosynthesized in the conventional rat. Data cited above indicate that in the

germfree rat there was a greater reabsorption of bile acid from the gastrointestinal tract and a greater recirculation than in the conventional rat. This would lead to a tighter feedback inhibition control and less new bile acid biosynthesized in the germfree rat. Since Shefer *et al.* (35) have shown that under these conditions in the conventional intact rat β-muricholic acid becomes one of the major newly biosynthesized bile acids; this could explain the predominance of this material in the germfree rat.

B. Chickens

Eyssen (5) showed that germfree chicks excreted much less fecal chenodeoxycholic acid than their conventional counterparts and that the addition of the antibiotic virginiamycin to the diets of conventional chicks significantly reduced fecal bile acid excretion, although the values were never as low as in the germfree birds.

The biliary bile acids of germfree chicks have been shown by Haslewood (34) to be composed of approximately 80% taurochenodeoxycholic acid, 17% taurocholic acid, and 5% tauroallocholic acid.

C. Swine

The bile of germfree swine from two different sources contained the taurine and glycine conjugates of chenodeoxycholic acid, hyodeoxycholic acid, hyocholic acid, and (probably) cholic acid. The hyodeoxycholic acid was present at 4% by weight and could be detected by TLC as its conjugate. It had previously been felt that hyodeoxycholate in swine was solely a microbial dehydroxylation product of hyocholic acid; however, this study shows that it can be biosynthesized by the germfree swine.

D. Rabbits

The bile of germfree rabbits has been shown to be composed of 94% glycocholic acid, 5% glycochenodeoxycholic acid, and 1% glycoallocholic acid (36). Since deoxycholic acid is the predominant bile acid in conventional rabbit bile, this study points up the profound influence of the intestinal microflora in this species.

VI. QUALITATIVE CHANGES IN BILE ACIDS AS A RESULT OF MICROBIOLOGICAL INTERACTION

A. Types of Studies

The marked differences cited above in qualitative and quantitative bile acid metabolism of germfree and conventional species have led many in-

vestigators to study pure and mixed cultures of bacteria in an attempt to determine which microbial species are responsible for the differences observed. These studies are carried out in two fundamental manners. The procedure which yields the most information is to associate otherwise germfree animals with one or more known pure cultures of bacteria and study the effects brought about by the association of these bacteria in the gnotobiotic animal. The intent of these experiments is to procure an animal system in which some, but not all, of the characteristics of the conventional animal are seen. A second approach requiring considerably less investment in time and equipment, however yielding only a partial answer, is to study the effects of microbiological species on bile acids in *in vitro* fermentations. These studies may give us a clearer picture of the microbial activity on the steriod but, of course, do not give us any insight into the interaction of these changes with a host species.

It should be borne in mind with respect to both of these kinds of studies that microorganisms do not always exhibit the same biochemical characteristics under all conditions. The biochemical control mechanisms of repression, induction, and feedback inhibition may cause a given species to exhibit one characteristic *in vivo* and quite another *in vitro*. Further, there are many strains of microorganisms inside an individual species, and one strain isolated from a given source may not possess identical biochemical characteristics with a strain from another source. Only a very small portion of the biochemical and morphological characteristics of a given microorganism is utilized for its taxonomic characterization. It is quite possible that, aside from a given uniform set of taxonomic characteristics, there may be considerable variation in the genetic capabilities to express other characteristics or, indeed, in the conditions under which these characteristics may be allowed to be expressed by the biochemical control mechanisms. It is with these qualifications in mind that in the following discussion an attempt will be made to list and identify some of the activities of the species which have been studied.

B. Observations in Gnotobiotic Animals

The first experiment on bile acid metabolism in germfree animals included one subject, which was associated with known species of bacteria. Gustafsson *et al.* (21) monoassociated a rat with *Aspergillus niger* and, subsequently, also with *Clostridium perfringens* type E. The *A. niger* association did not appear to alter the quantitative or qualitative bile acid metabolism compared to the same animal's germfree state. With the addition of *C. perfringens,* however, the rat feces contained free bile acid and other unidentified metabolites. It has previously been shown that *C. perfringens* could deconjugate bile acids *in vitro* (37). Association with these species did not

cause any marked change in quantitative cholic acid-^{14}C excretion from the rat compared to that in its germfree state. When the rat was removed from the isolator and allowed to acquire a "normal" flora, the rate of isotope excretion increased fourfold.

In a later experiment, the same group (22) studied the effect of monoassociation of rats with *E. coli*. They found that there was no increase in bile acid excretion compared to that of germfree rats and identified 3α,12α-dihydroxy-7-ketotaurocholanic acid and 3α,7β,12α-trihydroxytaurocholanic acid as new bile acids in the feces of the *E. coli* associated rats. Only the 3α,12α-dihydroxy-7-keto derivative was formed *in vitro* by *E. coli*, and this compound was metabolized by the rat to 3α,7β,12α-trihydroxy- and 3α,7α,12α-trihydroxytaurocholanic acids.

In a series of studies, Midtvedt and associates (38-43) isolated species of bacteria capable of deconjugating, 7-dehydroxylating, and oxidizing bile acid. Gustafsson *et al.* (43) investigated the effect of several of these species on bile acid metabolism in gnotobiotic rats. A bacterium capable of 7-dehydroxylating bile acids (called by them strain II) had been shown *in vitro* (see below) to be specific for deconjugated bile acid. These results were confirmed *in vivo*. Strain II, as a monoassociate, could neither deconjugate nor dehydroxylate bile acids, but when it was polyassociated with species which could deconjugate (but not dehydroxylate), both deconjugated and dehydroxylated bile acids (i.e., deoxycholic acid) were recovered.

Kellogg *et al.* (44) utilized gnotobiotic mice to screen for a microorganism which accelerated cholesterol-^{14}C catabolism as compared to a germfree control group. Mice monoassociated with *C. perfringens* type A retained

TABLE IV. Fecal Steroid Excretion of Germfree, *C. perfringens* Associated (Gnotobiotic), and Conventional Rats

Parameter examined	Animal status		
	Germfree	Gnotobiotic	Conventional
Number of rats	12	12	14
Endogenous fecal neutral sterol excretion (mg/kg body wt/day)	12.8 ± 3.0[a]	12.7 ± 4.2[e]	19.5 ± 5.2
Fecal bile acid excretion (mg/kg body wt/day)	11.3 ± 2.4[b]	7.14 ± 3.53[b]	21.4 ± 9.9[b]
Percent fecal bile acids conjugated with taurine	100	None	None
Percent fecal bile acids bacterially modified	None	34.3 ± 14.1[c]	57.7 ± 9.3[c]
Percent fecal bile acids present as cholate and α-muricholate	29.5 ± 9.6	27.3 ± 10.1	—
Percent fecal bile acids as β-muricholate	60.5 ± 9.1[d]	23.6 ± 8.6[d]	

From Kellogg *et al.* (44). Reprinted with permission of the authors and the *Journal of Lipid Research*.

[a,b,c,d,e]Any values with the same superscript are significantly different at $p < 0.50$.

TABLE V. Metabolism of Free and Conjugated Bile Acids by Microorganisms Belonging Within Genera Often Found in the Intestinal Contents of Man and Rat

Name of microorganism[a]	Source[b]	Aerobic (ae) or anaerobic (an) incubations	Splitting of		Transformation of		
			Glyco-cholic acid	Tauro-cholic acid	Cholic acid	Chenodeoxy-cholic acid	Litho-cholic acid
Lactobacillaceae							
Streptococcus faecalis ATCC 8043	ATCC	an	+	+	−	−	−
Streptococcus faecalis M 19	SBL	an	+	+	−	−	−
Streptococcus group D Heazman Williams	SBL	an	+	+	−	−	−
Streptococcus group D F 87	SBL	an	+	+	−	−	−
Streptococcus Manhattan Albany	SBL	an	+	+	−	−	−
Lactobacillus acidophilus 101	RLD	an	−	−	−	−	−
Lactobacillus delbrueckii ATCC 9595	ATCC	an	−	−	−	−	−
Lactobacillus casei ATCC 7469	ATCC	an	−	−	−	−	−
Lactobacillus arabinosus ATCC 8014	ATCC	an	+	+	−	−	−
Lactobacillus brevis CCM 1817	CCM	an	+	−	−	−	−
Eubacterium noiosii Cr 4	IPP	an	+	+	−	−	−
Eubacterium quintum 1961 E	IPP	an	+	+	+	+	−
Eubacterium cadaveris 1098 G	IPP	an	+	+	+	+	+
Eubacterium cadaveris 1609	IPP	an	−	−	−	−	−
Eubacterium tortuosum 1734 E	IPP	an	−	−	−	−	−
Eubacterium aerofaciens 2663	IPP	an	−	−	−	−	−
Eubacterium ventriosum 2405	IPP	an	+	+	+	+	−
Eubacterium minutum 2760 B	IPP	an	+	+	+	+	−
Eubacterium minutum 2895 C	IPP	an	+	+	+	+	−
Eubacterium parvum 171 III	IPP	an	−	−	+	+	+
Eubacterium lentum 1899 B	IPP	an	−	−	+	+	+
Catenabacterium helminthoides 423 A	IPP	an	+	+	−	−	−
Catenabacterium catenaformis 1788G	IPP	an	−	−	−	−	−

Ramibacterium ramosum 2514	IPP	an	+	+	−	−	−
Ramibacterium pleuriticum	IPP	an	−	−	−	−	−
Ramibacterium alactolyticum 204	IPP	an	+	+	−	−	−
Ramibacterium dentium 1855 A	IPP	an	+	+	−	−	−
Lactobacillus bifidus 2811 B[c]	IPP	an	+	+	−	−	−
Lactobacillus appendicitis 1535 B[d]	IPP	an	+	+	−	−	−
Propionibacteriaceae							
Butyribacterium rettgeri 3331[e]	IPP	an	+	+	−	−	−
Corynebacteriaceae							
Corynebacterium diphtheroides 2764	IPP	an	−	−	−	−	−
Bacillaceae							
Bacillus cereus 14/11 63	SBL	ae	−	−	+	+	+
Bacillus cereus 14/11 63	SBL	an	−	−	+	+	+
Bacillus subtilis ATCC 6633	ATCC	ae	−	−	−	−	−
Bacillus subtilis ATCC 6633	ATCC	an	−	−	−	−	−
Clostridium butyricum 9–3–60	SBL	an	+	+	−	−	−
Clostridium difficile ATCC 9689	ATCC	an	−	+	+	+	−
Clostridium perfringens CN 1491	SBL	an	+	+	+	+	+
Clostridium perfringens type B	RVC	an	+	+	+	+	+
Clostridium tetani W. W.	WW	an	−	−	−	−	−
Clostridium histolyticum W. W.	WW	an	−	−	−	−	−
Clostridium histolyticum CN 919	SBL	an	−	−	−	−	−
Clostridium G 62[f]	DGR	an	+	+	−	−	−
Inflabilis rectus PC	IPP	an	−	−	−	−	−
Micrococcaceae							
Staphylococcus aureus Wood	SBL	ae	−	−	−	−	−
Staphylococcus aureus Wood	SBL	an	−	−	−	−	−
Staphylococcus aureus S 209	BLKS	ae	−	−	−	−	−
Staphylococcus aureus S 209	BLKS	an	−	−	−	−	−
Achromobacteriaceae							
Alkaligenes faecalis NCTC 415	NCTC	ae	+	+	−	−	−

Table V (Continued)

Name of microorganism[a]	Source[b]	Aerobic (ae) or anaerobic (an) incubations	Splitting of		Transformation of		
			Glyco-cholic acid	Tauro-cholic acid	Cholic acid	Chenodeoxy-cholic acid	Litho-cholic acid
Enterobacteriaceae							
Escherichia coli S 618	BLKS	ae	−	−	+	+	−
Escherichia coli S 618	BLKS	an	−	−	+	+	−
Escherichia coli 3201	WW	ae	−	−	+	+	−
Escherichia coli 3201	WW	an	−	−	+	+	−
Klebsiella pneumoniae type A 3409/60	WW	ae	−	−	−	−	−
Klebsiella pneumoniae type A 3409/60	WW	an	−	−	−	−	−
Proteus mirabilis 4198	WW	ae	−	−	−	−	−
Proteus mirabilis 4198	WW	an	+	−	−	−	−
Bacteroidaceae							
Bacteroides fragilis 648	ODH	an	+	+	+	+	−
Bacteroides fragilis NCTC 9343	NCTC	an	+	+	+	+	−
Bacteroides necrophorus NCTC 7155	NCTC	an	+	+	+	+	−
Pseudomonadaceae							
Pseudomonas aeruginosa NCTC A 7244	NCTC	ae	−	−	+	+	−
Actinomycetaceae							
Actinomyces israelii B 65/3408	CSC	an	−	−	−	−	−
Cryptococcaceae							
Candida albicans	SBL	ae	−	−	−	−	−

From Midtvedt and Norman (38). Reprinted with the permission of the authors and the *Acta Pathologica et Microbiologica Scandinavica.*

[a] The names of the strains are those given by the reference laboratories.

[b] Abbreviations: ATCC, American Type Culture Collection, Washington, D.C., United States; BLKS, Bacteriological Laboratory, Karolinska Sjukhuset, Stockholm, Sweden; CCM, Czechoslovakian Collection of Microorganisms, Brno, Czechoslovakia; CSC, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; DGR, Department of Germfree Research, Stockholm, Sweden; IPP, Institut Pasteur, Paris, France; NCTC, National Collection of Type Cultures, London, England; ODH, Ontario Department of Health, Toronto, Canada; RLD, Research Laboratory for the Dairies, Prague, Czechoslovakia; RVC, Royal Veterinary College, Stockholm, Sweden; SBL, State Bacteriological Laboratory, Solna, Sweden; WW, Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Oslo, Norway.

[c] Received as *Bifidobacterium bifidum.*

[d] Received as *Bifidobacterium appendicitis.*

[e] Received as *Eubacterium rettgeri.*

[f] An anaerobic, gram-positive, spore-forming rod, with capacity to convert bilirubin to urobilins. Tentatively classified as *Clostridium.*

significantly less isotope 5 days after injection with cholesterol-26-^{14}C than did germfree or six other monoassociated groups. This organism was subsequently studied in monoassociated rats by steroid balance techniques. The fecal bile acids of the *C. perfringens* monoassociated rats were all deconjugated. Figure 1 shows bile acids of germfree, *C. perfringens* gnotobiotic, and conventional rats fed identical diets. Table IV indicates that the quantitative fecal steroid excretion of the gnotobiotic group was not increased by the total deconjugation of the bile acids. Conventional rats also excreted deconjugated bile acids and had a total bile acid excretion twice as large as the germfree and gnotobiotic groups did. It thus appears that the deconjugation seen in conventional rats was not the factor responsible for their increased bile acid excretion.

TABLE VI. Microorganisms Which Modify Bile Acids *in Vitro*

Species	Deconjugation of		Dehydroxylation of	References
	Taurocholate	Glycocholate	cholic acid	
Pseudomonas sp.[a]	−	n.r.[b]	−	(49)
Escherichia coli	−	n.r.	−	(49)
Proteus mirabilis	−	n.r.	−	(49)
Staphylococcus aureus	+	n.r.	−	(49)
Streptococcus pyogenes	−	n.r.	−	(49)
Streptococcus faecalis	+	n.t.	+	(49)
Streptococcus salivarius	−	n.r.	−	(49)
Streptococcus viridans	−	n.r.	−	(49)
Bacteroides sp.	+	n.r.	+	(49)
Clostridium sp.	+	n.r.	+	(49)
Bifidobacterium sp.	+	n.r.	−	(49)
Veilonella sp.	+	n.r.	+	(49)
Lactobacillus sp.	−	n.r.	−	(49)
Candida albicans	−	n.r.	−	(49)
Candida sp.	−	n.r.	−	(49)
Saccharomyces sp.	−	n.r.	−	(49)
Torulopsis sp.	−	n.r.	−	(49)
Streptococcus faecalis E1	+	+	n.r.	(45)
Streptococcus faecalis E12	−	+	n.r.	(45)
Bifidobacterium BBC 18	−	+	n.r.	(45)
Bifidobacterium BBC 29	−	+	n.r.	(45)
Clostridium welchii CC 20	+	+	n.r.	(45)
Clostridium welchii CC 63	+	+	n.r.	(45)
Bacteroides BV 10	+	+	n.r.	(45)
Bacteroides fragilis NCTC 9343	+	+	n.r.	(45)

[a] For number of strains tested, see original paper.
[b] Not reported.

C. Changes Caused by Microbiological Species in *in Vitro* Experiments

Several studies (38–42,45–50) have investigated the occurrence of microorganisms which modify bile acid structure *in vitro*. Table V, reprinted from Midtvedt and Norman (38), lists common intestinal bacteria which they investigated and their activity on some bile acids. Table VI summarizes similar data from several other investigators. Among the less well-known activities observed have been oxidation of hydroxyl groups to ketones, dehydroxylation of 12α-hydroxy groups leading to the formation of secondary chenodeoxycholic acid (48), and aromatization of the A-ring of bile acids (50). A detailed discussion of the effects of 12α-dehydroxylation on various bile acid pools in the rat has been presented by Kellogg *et al.* (14,44).

The data cited in this section and its tables do not represent all the studies done in this area but are selected from recent studies as representative examples of the range of species and activities which have been observed. A detailed review of the entire field of bile acid metabolism by microorganisms *in vitro* has been prepared by Lewis and Gorbach (51).

VII. CONCLUSIONS

Bile acid metabolism in conventional animals is the activity of a balanced ecological system composed of the host, the associated intestinal microflora, and the diet. The host contributes the bile acids themselves and serves to maintain the homeostasis of the gastrointestinal tract. The intestinal microflora alters the molecular structure of the bile acids which it comes into contact with and also profoundly alters the physiological and, to a degree, the anatomical features of the host. The diet contributes the nutrition for both the host and the intestinal microflora and can cause marked changes in the flora's activity toward the bile acids *in vivo* (52). In addition, the amount of dietary sterols may cause the host to change its absorption and/or catabolism of cholesterol to bile acid and thus the rate of bile acid excretion (53).

Conventional animals excrete more bile acids, both qualitatively and quantitatively, than do germfree animals. It appears that the qualitative modification of bile acids *in vitro* is the result of many different microorganisms of widely varying taxonomic types. The studies reported here have characterized the differences in the bile acid metabolism between the two types of animal but have not explained the factor(s) responsible for these differences.

The conventional animal may be thought of as a middle state in a continuum bounded at one end by the germfree animal and at the other by the animal in a state of severe steatorrhea. Thus one could conclude that the conventional animal is in a state of mild "malabsorption," which, since it is the common condition, we define as "normal."

Among the main features of the modification of bile acids by the intestinal microorganism are deconjugation and dehydroxylation. Evidence has been presented that suggests that the deconjugation of bile acids has no effect on the total bile acid excretion from rats. It has been demonstrated *in vivo* and *in vitro* that none of the organisms isolated thus far is capable of dehydroxylating conjugated bile acids unless the same microorganism can also deconjugate. It appears, thus far at least, that the enzyme(s) for dehydroxylation is specific for deconjugated bile acids.

Several experiments have demonstrated the advantage of using germfree animals in studies on the systemic metabolism of the host. Eliminating the "background noise" of the microflora transformations has shown that the host sometimes carries on metabolic activities formerly thought to be exclusively the function of the microflora. It also has been shown that there is considerably less variation in the fecal bile acid excretion in germfree than in conventional rats, thus allowing more statistically sensitive experiments when germfree subjects are used.

REFERENCES

1. P. Samuel, G. M. Saypol, E. Meilman, E. H. Mosbach, and M. Chafizadeh, *J. Clin. Invest.* **47,** 2070 (1968).
2. A. Norman and J. Sjövall, *J. Biol. Chem.* **233,** 872 (1958).
3. A. Norman, *Acta Physiol. Scand.* **33,** 99 (1955).
4. S. Lindstedt and A. Norman, *Acta Physiol. Scand.* **38,** 129 (1956).
5. H. Eyssen, *in* "Ninth International Congress for Microbiology," p. 329, Ivanovski Institute of Virology, Moscow (1966).
6. H. Eyssen, G. Van Messom, and J. Van den Bosch, *in* "Germ-Free Biology" (E. Mirand, ed.), p. 97, Plenum Press, New York (1969).
7. T. F. Kellogg and B. S. Wostmann, *in* "Germ-Free Biology" (E. Mirand, ed.), p. 293, Plenum Press, New York (1969).
8. R. Reiser and G. E. Mott, *Fed. Proc.* **30,** 248 (1971).
9. M. D. Siperstein and I. L. Chaikoff, *J. Biol. Chem.* **194,** 93 (1952).
10. F. M. Harold, J. M. Felts, and I. L. Chaikoff, *Am. J. Physiol.* **183,** 459 (1955).
11. M. W. Whitehouse, E. Staple, and S. Gurin, *J. Biol. Chem.* **236,** 68 (1961).
12. H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.* **237,** 338 (1962).
13. B. S. Wostmann, N. L. Weich, and E. Kung, *J. Lipid Res.* **7,** 77 (1966).
14. T. F. Kellogg and B. S. Wostmann, *J. Lipid Res.* **10,** 495 (1969).
15. F.M. Harold, J. M. Felts, and I. L. Chaikoff, *Am. J. Physiol.* **183,** 459 (1955).
16. J. T. Matschiner, T. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. L. Hsia, and E. A. Doisy, *J. Biol. Chem.* **225,** 771 (1957).
17. T. A. Mahowald, J. T. Matschiner, S. L. Hsia, R. Richter, E. A. Doisy, Jr., W. H. Elliott, and E. A. Doisy, *J. Biol. Chem.* **225,** 781 (1957).
18. T. A. Mahowald, J. T. Matschiner, S. L. Hsia, E. A. Doisy, Jr., W. H. Elliott, and E. A Doisy, *J. Biol. Chem.* **225,** 795 (1957).
19. S. Bergström and J. Sjövall, *Acta Chem. Scand.* **8,** 611 (1954).

20. G. D. Cherayil, S. L. Hsia, J. T. Matschiner, E. A. Doisy, Jr., W. H. Elliott, S. A. Thayer, and E. A. Doisy, *J. Biol. Chem.* **238,** 1973 (1963).
21. B. E. Gustafsson, S. Bergström, S. Lindstedt, and A. Norman, *Proc. Soc. Exptl. Biol. Med.* **94,** 467 (1957).
22. B. E. Gustafsson, A. Norman, and J. Sjövall, *Arch. Biochem. Biophys.* **91,** 93 (1960).
23. B. E. Gustafsson and A. Norman, *Proc. Soc. Exptl. Biol. Med.* **110,** 387 (1962).
24. O. W. Portman and P. Murphy, *Arch. Biochem. Biophys.* **76,** 367 (1958).
25. B. E. Gustafsson and A. Norman, *Brit. J. Nutr.* **23,** 429 (1969).
26. A. Norman, *Brit. J. Nutr.* **18,** 173 (1964).
27. A. Norman and M. S. Shorb, *Proc. Soc. Exptl. Biol. Med.* **110,** 552 (1962).
28. B. E. Gustafsson and A. Norman, *Scand. J. Gastroenterol.* **3,** 625 (1968).
29. M. A. Eastwood and D. Hamilton, *Biochim. Biophys. Acta* **152,** 165 (1968).
30. P. Eneroth and J. Sjövall, *in* "Methods in Enzymology" (R. B. Clayton, ed.), Vol. 15, p. 260, Academic Press, New York (1969).
31. E. Evrard and G. Janssen, *J. Lipid Res.* **9,** 226 (1968).
32. K. A. Mitropoulos and N. B. Myant, *Biochem. J.* **103,** 472 (1967).
33. N. Wachtel, S. Emerman, and N. B. Javitt, *J. Biol. Chem.* **243,** 5207 (1968).
34. G. A. D. Haslewood, *Biochem. J.* **123,** 15 (1971).
35. S. Shefer, S. Hauser, I. Bekersky, and E. Mosbach, *J. Lipid Res.* **11,** 404 (1970).
36. A. F. Hofmann, E. H. Mosbach, and C. C. Sweeley, *Biochim. Biophys. Acta* **176,** 204 (1969).
37. A. Norman and R. Grubb, *Acta Pathol. Microbiol. Scand.* **36,** 537 (1955).
38. T. Midtvedt and A. Norman, *Acta Pathol. Microbiol. Scand.* **71,** 629 (1967).
39. T. Midtvedt and A. Norman, *Acta Pathol. Microbiol. Scand.* **72,** 337 (1968).
40. B. E. Gustafsson, T. Midtvedt, and A. Norman, *J. Exptl. Med.* **123,** 413 (1966).
41. T. Midtvedt, *Acta Pathol. Microbiol. Scand.* **71,** 147 (1967).
42. T. Midtvedt, *Acta Pathol. Microbiol. Scand.* **71,** 313 (1968).
43. B. E. Gustafsson, T. Midtvedt, and A. Norman, *Acta Pathol. Microbiol. Scand.* **72,** 433 (1968).
44. T. F. Kellogg, P. L. Knight, and B. S. Wostmann, *J. Lipid Res.* **11,** 362 (1970).
45. V. Aries and M. J. Hill, *Biochim. Biophys. Acta* **202,** 526 (1970).
46. V. Aries and M. J. Hill, *Biochim. Biophys. Acta* **202,** 535 (1970).
47. B. S. Draser, M. J. Hill, and M. Shiner, *Lancet* **1,** 1237 (1966).
48. M. J. Hill and B. S. Draser, *Biochem. J.* **105,** 55P (1967).
49. M. J. Hill and B. S. Draser, *Gut* **9,** 22 (1968).
50. V. C. Aries and M. J. Hill, *Biochem. J.* **119,** 37P (1970).
51. R. Lewis and S. Gorbach, *Arch. Int. Med.*, in press (1972).
52. T. F. Kellogg, *Fed. Proc.* **30,** 1808 (1971).
53. E. Quintao, S. M. Grundy, and E. H. Ahrens, Jr., *J. Lipid Res.* **12,** 233 (1971).

Note Added in Proof (to Chapter 1)

Since completion of this manuscript in 1970 several reports pertinent to the subject have been published. Some of these will be briefly summarized in the following.

Section II A 1: The properties of cholesterol 7α-hydroxylase have been further studied by Mitton *et al.* (183). Gielen *et al.* (184, 185) have found that the activity of cholesterol 7α-hydroxylase is subject to diurnal variations controlled by the pituitary-adrenocortical system. The differing findings concerning the effect of phenobarbital on rat liver cholesterol 7α-hydroxylase can be ascribed to strain differences (186). Further evidence that free and not esterified cholesterol is the precursor of bile acids has been provided by Ogura *et al.* (187). The differing properties of the hydroxylase systems involved in the formation of bile acids have been further studied by Björkhem (188, 189), who has found that breaking of the C–H bond is rate limiting in some hydroxylations but not in others.

Section II A 6: Anderson *et al.* (190) have reported results indicating that a pathway to cholic acid involving 5-cholestene-3β,26-diol as an intermediate may be of importance in man.

Section II B 2: Okuda and Takigawa (191) have provided further evidence that 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase is identical with liver ethanol dehydrogenase.

Section III: In agreement with the suggested major pathway for formation of chenodeoxycholic acid, Hanson (192) has found that in man cholesterol is converted into 3α,7α-dihydroxy-5β-cholestanoic acid, which in turn is metabolized predominantly into chenodeoxycholic acid. Yamasaki and collaborators (193–196) have provided evidence for the presence in rat of a pathway to chenodeoxycholic acid involving the conversion of 5-cholestene-3β,7α-diol into 3β,7α-dihydroxy-5-cholenoic acid.

Section IV: Mui and Elliott (197) have shown that allochenodeoxycholic acid is converted to a small extent into allocholic acid in the rat.

Section VI: The patterns of diurnal variations in cholesterol synthesis and cholesterol 7α-hydroxylase activity have been found to be practically the

same, indicating a close relationship between cholesterol and bile acid biosynthesis (198). The question whether bile acids exert their effect on liver cholesterol synthesis in the liver or in the intestine has been further studied by Hamprecht *et al.* (199, 200), who have concluded that bile acids influence the activity of hydroxymethylglutaryl-coenzyme A reductase in the liver.

References

183. J.R. Mitton, N.A. Scholan, and G.S. Boyd, *Europ. J. Biochem.* **20,** 569 (1971).
184. J. Gielen, J. van Cantfort, B. Robaye, and J. Renson, *C.R. Acad. Sci. Paris* **269,** 731 (1969).
185. J. Gielen, B. Robaye, J. van Cantfort, and J. Renson, *Arch. Int. Pharmacodyn.* **183,** 403 (1970).
186. S. Shefer, S. Hauser, and E.H. Mosbach, *J. Lipid Res.* **13,** 69 (1972).
187. M. Ogura, J. Shiga, and K. Yamasaki, *J. Biochem.* **70,** 967 (1971).
188. I. Björkhem, *Europ. J. Biochem.* **18,** 299 (1971).
189. I. Björkhem, *Europ. J. Biochem.* **27,** 354 (1972).
190. K.E. Anderson, E. Kok, and N.B. Javitt, *J. Clin. Invest.* **51,** 112 (1972).
191. K. Okuda and N. Takigawa, *Biochim. Biophys. Acta* **220,** 141 (1970).
192. R.F. Hanson, *J. Clin. Invest.* **50,** 2051 (1971).
193. Y. Ayaki and K. Yamasaki, *J. Biochem.* **68,** 341 (1970).
194. S. Ikawa and K. Yamasaki, *Yonago Acta Med.* **15,** 21 (1971).
195. K. Yamasaki, Y. Ayaki, and H. Yamasaki, *J. Biochem.* **70,** 715 (1971).
196. Y. Ayaki and K. Yamasaki, *J. Biochem.* **71,** 85 (1972).
197. M.M. Mui and W. H. Elliott, *J. Biol. Chem.* **246,** 302 (1971).
198. H. Danielsson, *Steroids* **20,** 63 (1972).
199. B. Hamprecht, C. Nüssler, G. Waltinger, and F. Lynen, *Europ. J. Biochem.* **18,** 10 (1971).
200. B. Hamprecht, R. Roscher, G. Waltinger, and C. Nüssler, *Europ. J. Biochem.* **18,** 15 (1971).

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