# Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem

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**Abstract** The structure of the bile acid molecule is described and correlated with physicochemical properties of bile acids such as solubility, ionization, and micelle formation. Recent measurements of the critical micellar concentration (CMC) of a large number of bile acids indicate that the CMC is influenced by both side chain and nuclear structure. Bile acids with hydroxy substituents on both sides of the steroid nucleus are non-amphipathic and do not form micelles, and decreasing the length of the side chain causes an exponential increase in the CMC. Bile acid ionization, measured by titration in alcohol–water mixtures, is shown to be uninfluenced by nuclear substituents; the $pK_a$ of all unconjugated bile acids is about 5. Interactions of bile acid solutions with $Ca^{2+}$ are discussed; recent work indicates that cholyl conjugates bind $Ca^{2+}$ as monomers in solution. Model systems relevant to biological processes are classified, as are some of the physicochemical parameters of these systems. Biological processes involving bile acids are tabulated, and corresponding model systems are assigned to each. Some biological processes such as bile acid transport show marked species differences, suggesting that physicochemical parameters are insufficient to explain biological differences. It is recommended that the physical chemist study a variety of bile acids, that the biologist study a variety of species, and that both collaborate to attempt to factor out the extent to which physicochemical properties of bile acids can explain their biological properties.—**Hofmann, A. F., and A. Roda.** Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem. *J. Lipid Res.* 1984. **25:** 1477–1489.

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## Introduction

The occasion of the 25th anniversary of the *Journal of Lipid Research* is a stimulus to stop and reflect on progress in the field of one's interest, to summarize achievements, and to attempt to organize the remaining challenges. In this brief review, we would like to focus on the relationship between the physicochemical properties and the biological properties of bile acids. That such a relationship does exist has become a working hypothesis—the biological properties of bile acids can be explained by their physiochemical properties. Why has this belief arisen, and why has there been such an emphasis on relating physicochemical properties to biological properties for bile acids in contrast to many other molecules, such as hormonal steroids, prostaglandins, antibiotics, etc.? We suggest that there are at least three reasons. First, bile acids are present in bile and intestinal content in millimolar concentrations in the physical form of mixed micelles; obviously micelle formation is related to physicochemical properties, and there is a large body of fundamental knowledge about the physicochemical properties of detergents and other types of amphipathic molecules. Second, bile acid molecules are "friendly" molecules; they are stable; they possess few reactive groups and derivatives can be prepared easily; and they are available in large quantities at low cost. Third, even among the few bile acids that are readily available, there is a considerable range in physicochemical properties and biological properties; it is obviously challenging to relate the one to the other in order to develop quantitative structure activity relationships that would lead to predictive equations.

## A bit of history

Twenty-five years ago, the field of bile acids ("cholanology") was in its youth, although studies on bile and bile acids had been a continuing area of activity in chemical (1, 2), biochemical (3, 4), physiological (5), pharmacological (6), and clinical (7) laboratories throughout the world during the preceding century (for a review of the work before 1940, see the monograph of Sobotka (8)). The major bile acids had been isolated from bile and their chemical structure was almost cor-

Abbreviations: CMC, critical micellar concentration; CMT, critical micellar temperature; CMpH, critical micellar pH; OWDR, octanol–water distribution ratio; HPLC, high performance liquid chromatography.

rectly elucidated by the laboratories of Windaus and Wieland; assignment of the correct structures was straightforward once the correct structure of the cyclopentane-perhydrophenanthrene ring was clarified by Rosenheim and King. By 1940, cholic acid, which is readily isolated from ox bile, had become a readily available commercial product. With the discovery of the remarkable anti-inflammatory effects of cortisone at the Mayo Clinic, immense synthetic efforts were undertaken to develop a route for conversion of deoxycholic acid to anti-inflammatory steroids; these were a stimulus to bile acid chemistry, but activity subsided when microbiological routes to steroid synthesis were perfected (1). Chenodeoxycholic acid, the major bile acid of human bile, was not available, although its synthesis from cholic acid was not difficult (9). In Japan, Iwasaki (10) established the chemical structure of ursodeoxycholic acid in 1936. This work stimulated the Japanese pharmaceutical industry to develop a synthesis for ursodeoxycholic acid (11), and to explore its physiological and therapeutic properties (reviewed in 12). Ursodeoxycholic acid was known to be present in the bile of the bear, and bear bile had been considered to have curative properties for centuries.

The modern era of bile acid biochemistry began when isotopes became available. In one of the earlier experiments using isotopically labeled precursors, Bloch, Berg, and Rittenberg (13) had shown that bile acids were formed from cholesterol, confirming a speculation made many decades before by Ruzicka (14) and laying to rest a longstanding controversy. The discovery and availability of $^{14}C$ and $^{3}H$, as well as liquid scintillation counters, stimulated Bergstrom and his associates in Lund (Danielsson, Samuelsson, Eriksson, Sjövall, Norman, Lindstedt, Tryding, among others) (15) and Doisy and his associates in St. Louis (4) to undertake a series of studies on the metabolism of bile acids in animals and man.

The extensive studies of Haslewood (16), which combined imaginative organic chemistry with a deep knowledge of biological taxonomy, mapped out the remarkable variation in bile acids occurring in different species. These investigations showed that the chemical structure of bile acids provided information on the evolution of species. Haslewood's work also provided strong experimental evidence for a biochemical analogy of Haeckel's law of phylogeny recapitulating ontogeny: the end products of bile acid biosynthesis in lower vertebrates are the precursors of end products of bile acid biosynthesis in higher vertebrates.

Chemical work recommenced. The new and powerful techniques for structure elucidation, such as NMR and mass spectroscopy, have been used to identify uncommon bile acids, and, for the first time, there are efforts to design new bile acids that have desired biological properties (17).

The broad outlines of the enterohepatic circulation of bile acids were filled in by the thorough clinical studies of Josephson (18), but the next major advance in bile acid physiology was made just a quarter century ago. Lack and Weiner (19) showed that the terminal ileum actively transports bile acids, thus providing a physiological explanation for the efficiency of intestinal reabsorption of bile acids. This was not a new idea: Tappeiner, over a century before, had shown that bile acids are absorbed more rapidly from the ileum than the jejunum (20). The significance of his observations were largely overlooked, however, in part because the idea of regional localization of transport systems in the intestine had not yet been advanced.

Discovery of the key role of the ileum in maintaining the enterohepatic circulation of bile acids enabled quantitative descriptions and modeling of the enterohepatic circulation (21). Understanding the enterohepatic circulation of bile acids permitted the determinants of serum bile acid levels to be clarified (22), and this had considerable clinical impact, since it occurred concomitantly with the development of sensitive methods for measuring serum bile acids, such as enzymatic methods or competitive binding assays (for review, see 23). The end result was that serum bile acids could be measured accurately, and that serum bile acid measurements were shown to provide clinically useful information (24, 25).

Discovery of the key role of the ileum in maintaining the enterohepatic circulation of bile acids was important in gastroenterology because ileal disease is common, because bile acid synthesis is regulated by a negative feedback mechanism (26, 27), and because dihydroxy bile acids possess cathartic activity, inducing electrolyte and water secretion by the large intestine (28). Patients with ileal dysfunction were shown to malabsorb bile acids causing diarrhea, and the diarrhea was shown to respond to therapy with bile acid sequestrants (29).

In therapeutics, the administration of two dihydroxy bile acids, chenodeoxycholic (30, 31) and ursodeoxycholic acid (32, 33), has been shown to lower the relative proportion of cholesterol in bile. As a consequence, bile which is commonly supersaturated in patients with cholesterol gallstones becomes unsaturated in cholesterol; cholesterol gallstones, if their surface is accessible to the unsaturated bile, slowly dissolve (34, 35).

The first article on bile acids to appear in the *Journal of Lipid Research* was by Tennent et al (36), and described the development of cholestyramine and its use to lower serum cholesterol in chickens and dogs (36). Twenty-five years later, the results of the Coronary Primary Prevention Trial indicate that cholestyramine administration to hypercholesterolemic patients lowers serum

cholesterol and decreases the incidence of coronary events (37).

Another concept, which has slowly developed during the past 25 years, has been that individual bile acids have specific biological or pathological activities. This idea of course is a very old one, since the early bile acid chemists distinguished bile acids by their taste. The names of the bile acids often reflected the biological source from which they were isolated, for example, urso(deoxy)cholic acid (from the bear) or lithocholic acid (from a gallstone). Neubauer, in 1923, showed that dehydrocholate, the oxidation product of cholate, had remarkable choleretic properties and was non-hemolytic (38). Holsti, in 1962, observed that the feeding of chenodeoxycholic acid to rabbits induced cirrhosis (39), and eventually lithocholate was the bile acid shown to be responsible (reviewed in 40). Swell et al. (41), working in the laboratory of Treadwell, showed that trihydroxy bile acids had a specific effect on cholesterol absorption, an observation recently confirmed and extended by Watt and Simmonds in this journal (42). As noted, studies in the past decade have shown that chenodeoxycholate and ursodeoxycholate decrease biliary cholesterol saturation, but that other common bile acids do not (43, 44). Thus individual bile acids have particular biological effects.

Physicochemical studies on bile acids had been largely restricted to chemistry laboratories, such as those of McBain (45), Ekwall (46, 47), and Hartley (48, 49), although Mellander and Stenhagen (50), working in the Medicinal Chemistry Department of Uppsala University, showed by conductivity measurements that bile acids aggregate to form micelles. Nonetheless, communication between these laboratories and physiological laboratories was scanty, and the physical chemists tended to study one or two bile acids only and did not attempt to relate physical properties to biological properties. Then, in 1959, the year of the founding of the *Journal of Lipid Research*, Sune Bergstrom arranged for Norman, who had already carried out important studies on the chemistry (51) and biology (52) of bile acids, to take a postdoctoral year in the physical chemistry laboratory of Ekwall in Abo (now Turku), Finland. Interdisciplinary communication began (53, 54). This tradition was continued when Ingelfinger, an American gastroenterologist, sent Small to work with Dervichian in a Biophysics Lab at the Institut Pasteur (55).

This review has two purposes. The first is to give an overview of the current concepts of the physicochemical properties of bile acids in dilute aqueous solution in order to highlight certain advances that have occurred during the past 25 years. The second is to attempt to classify the kinds of model systems whose physicochemical aspects are of biological relevance, and to list which properties of these systems appear relevant to certain key biological processes in which bile acids are involved. We also propose that an understanding of the physicochemical properties of bile acids may not always be sufficient to explain their biological properties.

## Overview of the bile acid molecule

The bile acid skeleton has a body (nucleus) and a tail (side chain). Both parts of the molecule have a large number of possible steric arrangements. The nucleus can be altered by expansion or contraction of individual rings, and the side chain can be shortened or lengthened.

In addition, both parts of the bile acid molecule have a large number of possible polar substituents. Ionizing groups may be present on the nucleus or the side chain. Finally, conjugating groups may be present on the nucleus (e.g., sulfate, glucuronate, phosphate) or on the side chain (glycine or taurine or other amino acids, or even sugars). Thus, the number of possible bile acids that can be envisioned is infinite, and the number of bile acids whose physical properties have been studied in detail is miniscule.

This abundance of possible chemical structures contrasts sharply with the scarcity of uncommon bile acids. Only very recently have the common epimers of the natural dihydroxy and trihydroxy bile acids been synthesized (56, 57). Only one or two laboratories have prepared the nor, bis-nor, or homo-derivatives of the natural bile acids. The 5$\alpha$ (allo) bile acids have been prepared occasionally (58), but the synthesis of other ring junction stereoisomers has rarely been attempted. The same situation applies to bile acid conjugates. Pure glycine or taurine conjugates (amidates) of all but the most common bile acids are expensive or unavailable, despite satisfactory methods for chemical synthesis (51, 59, 60). Most sulfates and all glucuronides are unavailable. Thus, if the physical chemist, or the physiologist, wants to obtain uncommon bile acids in high purity and gram amounts, he must arrange his own synthesis. (This situation has precedent: 50 years ago, a biochemist had to synthesize or isolate his own amino acids; 30 years ago, a biochemist had to isolate his own enzymes; 10 years ago, an immunobiologist had to produce his own antibodies.)

## Nomenclature, solubility, and ionization

There are relationships between nomenclature and physical properties. The solubility of long chain fatty acids is so much less than that of soaps that it has been useful to call them by different names despite the identical structure of the organic moiety. Similarly, workers have distinguished bile acids from bile salts (cf. 61). The former term denoted the insoluble protonated acid usually obtained by crystallization from an organic

solvent, whereas the latter term denoted the water-soluble salts of bile acids or bile alcohols occurring in bile. In general, in biology, one uses the term fatty acids to apply to the class of compounds having the chemical structure of fatty acids, and the term does not specify the extent of ionization of the fatty acid. This same convention appears useful for bile acids. Soaps, for example, sodium stearate, may be extremely insoluble, and so are some bile salts. For example, the sodium salt of the simplest bile acid, sodium cholanoate, is quite insoluble in water below 90°C (62). Thus, in our judgment it is most convenient to use the term bile acids for all acidic compounds having the cholane nucleus, irrespective of their physical properties. Nonetheless, as a number of bile acid derivatives that are not acids are being synthesized, for example, sugar derivatives, and there is no single term which can be used for these compounds, in the future, it may be useful to have another name for all compounds resembling bile acids and salts. Perhaps a suitable term would be "cholanoids".

To discuss bile acid solubility, one must distinguish the solubility of the protonated, non-ionized molecule from that of the fully ionized molecule. Aqueous solubility reflects the balance between the affinity of molecules for each other in the crystal and the affinity of molecules for water. The molecular packing in crystals of only a few bile acids has been resolved. Recent studies by Roda and Fini (63) have provided data on the aqueous solubility of the protonated form of a representative group of a substantial number of naturally occurring bile acids. This work has shown that aqueous solubility is influenced by both nuclear and side chain structure. For nuclear substituents, solubility is proportional to the number of hydroxy substituents, but is also influenced by their orientation. For the side chain, solubility increases as the side chain is shortened. What is astonishing is that some bile acids (e.g., "ursocholic acid" 3$\alpha$,7$\beta$,12$\alpha$-trihydroxy-cholanoic acid) are quite soluble in the protonated form. (Thus some "bile acids" may be water-soluble and some "bile salts" may be insoluble!)

For most weak electrolytes that have a major organic component, ionization increases solubility. Since ionization is usually varied by adjusting bulk pH, it is common to plot solubility in relation to pH.

Three situations should be distinguished for bile acids. The first is when there is no self-association, i.e., when micelle formation does not occur because a bile acid is non-amphipathic. In this situation, the solubility (actually the logarithm of the solubility) increases linearly with pH. The second situation is when, under conditions of partial ionization, some kind of large aggregate forms which actually has a lower solubility than the protonated acid. This is likely to be the case with deoxycholate which forms large, viscous aggregates, probably helical in arrangement under conditions of incomplete ionization (64). (Fatty acids of course form acid soaps, whose solubility is less than that of the fatty acid (65).) Thus, in this situation, solubility decreases as pH is increased from acidic to neutral, and then increases at alkaline pH values. The third situation is when self-association to form micelles occurs. As solution pH is raised, solubility increases until the critical micellar concentration (CMC) of the bile acid is reached. Micelles form, and these micelles solubilize the protonated species. The protonated species can then be titrated as a micellar solubilizate. Thus solubility rises markedly over an extremely small pH range. (We have suggested it may be useful to coin a term "critical micellar pH" (CMpH) which is the pH at which the solubility increases markedly (66).)

Temperature also influences solubility. The effect will be small, unless temperature induces cooperativity effects. If a change in temperature increases the solubility of the monomer to the CMC, then molecules pass from a crystalline phase to a micellar phase. A phase change (from crystalline to micellar) occurs over a narrow range of temperature, at least in concentrated systems. Some years ago, the term "critical micellar temperature" (CMT) was suggested, and this term appears to have been generally accepted (62). The older term "Krafft point" has been redefined (67) and is now used less frequently.

These cooperativity effects explain the uncommon solubility behavior of the common bile acids. The three common bile acids in man (cholate, deoxycholate, chenodeoxycholate) all have CMT values below O°C; thus their solubility is independent of temperature. On the other hand, lithocholate, a monohydroxy bile acid, has a CMT of about 65°C (62, 64). Its solubility increases markedly above this temperature. (The CMC of sodium lithocholate is about 1 mM. When the solubility of lithocholate reaches 1 mM, micelles form, and there is marked increase in solubility; this occurs at about 65°C.)

The effect of pH on solubility has been studied for the common dihydroxy and trihydroxy bile acids, all of which form micelles at a relatively low concentration and all of which have a relatively well defined CMpH, above which solubility increases markedly. This value is about 7.0 for chenodeoxycholate (68), 7.8 for deoxycholate, and 8.0 for ursodeoxycholate (69). This is the value at which the monomeric solubility of the ion reaches the CMC. The solubility of the monomeric ion is determined by crystal lattice structure and interaction with water; the CMC value is determined by the hydrophobicity of the molecule. For trihydroxy bile acids such as cholate, the CMpH is quite low, about 6, since the monomeric solubility of this molecule is high and its

CMC is quite low. On the other hand, for a molecule such as dehydrocholate whose CMC is above 250 mM, its high aqueous solubility reflects high monomer solubility and will not show any abrupt change over a narrow pH range.

Thus, to rationalize the behavior of any bile acid in water, one would like to know the aqueous solubility of the protonated form, its $pK_a$ value, its CMC, its CMT, and its CMpH.

### Ionization properties of bile acids

Schmidt-Nielsen reviewed early work on the ionization properties of long chain fatty acids and came to the conclusion that the intrinsic $pK_a$ value of all long chain fatty acids was about 5 (70). He noted that their extremely low aqueous solubility precluded any accurate measurement of this value, but that a reasonably accurate estimate of $pK_a$ could be made by alcohol–water titrations. Recently, this approach has been taken by Fini, Roda, and DeMaria (71) who have determined the $pK_a$ values of a number of bile acids in ethanol–water mixtures and then estimated the $pK_a$ value in water by extrapolating the compounds with known $pK_a$ values. In brief, they concluded that the $pK_a$ values for all common bile acids is about 5.0. Thus the ionization properties of the side chain of bile acids is that of isopentanoic acid; the nuclear substituents do not influence ionization.

Other work using the heterogeneous titration method of Back and Steenberg (72) had concluded that the $pK_a$ values of bile acids varied considerably for bile acids with differing nuclear substituents. Unfortunately, this method does not seem to give valid results, since micellar solutions must be titrated, and the $pK_a$ values of bile acids, as any micellar solubilizate, are increased above that in monomeric solution (73, 74).

Amidation of bile acids with glycine or taurine lowers the $pK_a$ value by about 2.4 and 5 units, respectively, because of the inductive effect of the carbonyl group of the amide bond (analogous compounds would be propionic acid, $pK_a = 5$; N-acetyl glycine, $pK_a = 2.6$). Taurine-conjugated bile acids have a $pK_a$ of about 0, because of the extremely acidic nature of the sulfonic acid group (75). The end result of amidation is to form compounds that have superior biological and physicochemical properties. Conjugation results in compounds that are strongly ionized at physiological pH and thus undergo little back-diffusion during their passage down the biliary tree; in the small intestine, conjugated bile acids are more resistant to precipitation by low pH or divalent cations such as $Ca^{2+}$ during digestion. It is beyond the scope of this review to discuss this point further, but the biological aspects of bile acid conjugation have recently been reviewed (76).

*CMC.* Bile acids (salts) self-associate to form micelles over a narrow range of concentration, which is usually termed a CMC. As compared to that of typical ionic detergents, bile salt self-association occurs over a broader concentration range, and the pattern of association is more step-wise (77). Early measurements of the CMC of bile acids used conductivity, dye solubilization, or equilibrium surface tension techniques (reviewed in 78). The first method is imprecise, and the last method may be biased by small amounts of surface active impurities that concentrate on the surface (79). The spectral shift of cationic dyes has also been used, despite warnings in the literature that pre-micellar association of a large cation and a large anion may give erroneous results (80). Recently, Roda, Hofmann, and Mysels (81) used a sensitive dynamic method for measuring surface tension (a maximum bubble pressure device) developed by Karol Mysels to measure the CMC of a large number of bile acids. This work showed that the structure of both the nucleus and the side chain influences the CMC. For the nucleus, the key factor influencing self-association appeared to be the contiguous hydrophobic area. Any decrease in this hydrophobic area raised the CMC, such that bile acids in which both sides of the molecule were hydrophilic did not form micelles even at high concentrations. Decreasing the side chain increased the CMC exponentially. Amidation of the bile acid with glycine or taurine had little effect on the CMC value: the polarity of the amide bond compensated for the increased length of the side chain caused by glycine or taurine. Thus amidation altered the change on the side chain, but not the amphipathicity.

The significance of this work was to show that the natural bile acids possess relatively low CMC values (<10 mM) because of the striking assymetry of their nuclear structure and because of their $C_5$ side chain. The work also showed that a variety of bile acids with essentially no amphipathic properties was available to the physiologist, provided he could arrange their chemical synthesis. In **Table 1,** we have tabulated the water solubility (of the protonated form) and the CMC of some of the common bile acids.

Measurement of the properties of bile acid micelles (for example, size, molecular arrangement, hydration, charge, etc.) continues to be an area of great activity (reviewed in 78; see also 82). The limited information available suggests that bile acid structure influences the size of the micelle. Techniques are extremely difficult (83).

*CMT.* The CMT of bile acids has not received a great deal of attention inasmuch as the CMT of most bile acids is below room temperature. A striking exception to this is the unnatural bile acid, 7$\alpha$,12$\alpha$-dihydroxy cholanate, which has a CMT of about 40°C (62). The

TABLE 1. Physicochemical properties of some bile acids: solubility (in protonated form) and CMC of sodium salt[a]

| Trivial Name of Bile Acid | Position and Orientation of Hydroxyls | Water Solubility | CMC | |
|---|---|---|---|---|
| | | | Water | 0.15 M Na[b] |
| | | *mM* | | *mM* |
| I. Effect of different nuclear substituents | | | | |
| Lithocholic | 3α | 0.00005 | 0.9[c] | 0.5[c] |
| Iso-lithocholic | 3β | 0.00079 | | |
| Iso-lithocholic | 7α | 0.00020 | | |
| Iso-lithocholic | 7β | | 2.8 | 0.6 |
| Chenodeoxycholic | 3α,7α | 0.027 | 9 | 4 |
| Urosdeoxycholic | 3α,7β | 0.0009 | 19 | 7 |
| Deoxycholic | 3α,12α | 0.028 | 10 | 3 |
| Hyodeoxycholic | 3α,6α | 0.015 | 14 | 6 |
| Hyocholic | 3α,6α,7α | 0.045 | 17 | 8 |
| Cholic | 3α,7α,12α | 0.273 | 13 | 11 |
| Ursocholic | 3α,7β,12α | 1.67 | 60 | 39 |
| II. Effect of side chain length | | | | |
| nor-Chenodeoxycholic | 3α,7α | 0.380 | 40 | 15 |
| nor-Cholic | 3α,7α,12α | 1.390 | 30 | 21 |

[a] The $pK_a'$ values are not tabulated, as these are about 5.0 for all unconjugated bile acids.
[b] Values measured at 25°C.
[c] CMC measured at 75°C using dye solubilization technique (ref. 81). Other CMC values were determined at 25°C, but the effect of temperature on CMC was small.

monohydroxy bile acids tend to have CMT values above 100°C (62).

*Hydrophobicity.* Bile acids adsorb strongly to hydrophobic surfaces, and if a bile acid solution is passed over a $C_{18}$ octadecyl reversed phase column, all of the common bile acids, whether unconjugated or conjugated with glycine or taurine, adsorb strongly. Addition of a hydrogen bond-breaking solvent such as ethanol to the water permits elution from the column, and each bile acid has a characteristic retention time (84–87). The logarithm of the retention time has been shown to correlate highly with the logarithm of the octanol–water distribution ratio (OWDR), at least for some compounds (88, 89); and there has been a widespread tendency in the last few years to equate the chromatographic behavior of bile acids during reversed phase HPLC to their "hydrophobicity" (87).

Prior to the development of HPLC, hydrophobicity was estimated by determining OWDR, for which an immense amount of information is now available (90, 91). It is not simple to determine OWDR for compounds which vary so widely in "hydrophobicity" as bile acids. The distribution ratios are most easily measured using radioactive bile acids, but many bile acids are not available in radioactive form, and even if this approach can be used, it is hazardous since small amounts of radioimpurities may give rise to large errors. The measurement of retention time using HPLC on the other hand is simple, rapid, and is uninfluenced by bile acid purity.

However, we believe that it is premature to equate bile acid "hydrophobicity" to mobility during HPLC (which can be termed "retention factor") if hydrophobicity is a term that should permit prediction of biological properties of bile acids such as binding to serum albumin or passive membrane permeation.

The reasons for our belief may be summarized as follows. First, a number of publications have shown that, for ionizing organic molecules, there is a poor correlation between [the logarithm of the] (OWDR) and the logarithm of retention time (92, 93). Second, for certain pairs of bile acids, the retention factor varies according to the composition of the mobile phase, meaning that the retention factor is not determined solely by bile acid structure but is influenced by the conditions of the chromatographic procedure (86). Third, binding to $C_{18}$ reversed phase silicic acid may involve adsorption of the charged species and the uncharged species, in similar proportions, whereas OWDR probably involves predominantly the protonated form (89). The pH of the mobile phase strongly affects the retention factor for the differing bile acid classes. The effect is marked for unconjugated bile acids, moderate for glycine-conjugated bile acids, and least for taurine-conjugated bile acids. When published data are compared, the retention factor of an unconjugated bile acid and its respective taurine conjugate is strikingly different if the chromatography is carried out at pH 5, whereas at pH 7, they are quite similar.

In a pioneering study, Shaw and Elliott (86) reported a systematic study of the retention factor of a number of bile acids and calculated the relative effect of individual substituents such as hydroxy, or oxo, groups; they also examined the effect of glycine or taurine on the side

chain. They concluded that there was no simple relationship between RRT and substituents. Recently, Armstrong and Carey (87) have carried out similar studies with somewhat different chromatographic conditions and equated retention factor to hydrophobicity and this in turn to cholesterol-solubilizing capacity.

Thus, in our judgment it seems that the factors determining the retention factor or the OWDR of bile acids have not been clarified and studies are needed to define the mechanisms and to obtain accurate estimates of "hydrophobicity". These values can then be correlated with biological properties such as binding to albumin or passive membrane permeation. We think it likely that OWDR and retention factor will provide complementary but not identical information.

### Interactions with additives: the simplest case and its difficulties

The previous discussion has dealt with dilute aqueous solutions containing only a salt of a bile acid and water. Other substances are present in bile and intestinal content, and their effects on dilute aqueous bile salt solutions should be considered briefly. The simplest class of additives involves oppositely charged ions such as sodium or calcium.

Added sodium ion lowers the CMC for bile acids whose CMT is low. For a bile acid such as lithocholate whose CMT is high, the addition of sodium ions decreases the monomeric solubility to a greater extent than the CMC is lowered. The end result is that the CMT is increased (94).

The addition of $Ca^{2+}$ ions to a solution of glycine-conjugated bile acid will cause precipitation of the insoluble calcium salts, when the solubility product is exceeded. However, the effect of calcium ion is strongly dependent on bile acid structure, and bile acid physical state, i.e., whether the bile acid is exclusively in monomeric state or in micellar and monomeric state. In addition, the concentration of sodium ion is important as it influences the activity of the monomer and the CMC of the system. Indeed, in our experience, an adequate description of the interactions between a solution of a sodium salt of a glycine conjugated bile acid and added $Ca^{2+}$ can only be obtained by constructing a formal phase diagram (95). (Taurine-conjugated bile acids are not precipitated by added $Ca^{2+}$, and probably their calcium salts are fully water-soluble.) It should be noted that recent work of Moore et al. (96, 97) indicates that cholyltaurine in monomeric form strongly binds $Ca^{2+}$, so that $Ca^{2+}$ activity in bile acid solutions is influenced by both monomeric and micellar binding.

Generally, the purpose of studying a model system is to shed light on a biological or pathological process. Precipitation of insoluble calcium salts of bile acids occurs rarely in man, but in animals, increased concentrations of "calcium-sensitive" bile acids leads to formation of gallstones containing calcium salts of glycine-conjugated bile acids (reviewed in 98).

### A classification of model systems

It is useful to distinguish different levels of complexity for model systems that appear relevant to biological problems. Two large classes of model systems can be distinguished. The first class involves interaction of bile acid monomers with additions such as $Ca^{2+}$, macromolecules, or supramolecular aggregates. The second class of model systems involves micellar systems, and in such systems, one may have simple micelles composed of bile acids alone or mixed micelles composed of bile acids and other amphipathic lipids. In **Table 2,** we have listed most of the model systems that are relevant to biological processes involving bile acids. Each model system has been assigned to one of the two classes, depending on whether bile acid monomers or micelles are involved. Each of these "systems" has multiple physicochemical parameters of interest to biologists; and in **Table 3,** we have attempted to summarize some of these physicochemical parameters that can be measured in the model systems presented in Table 2.

The designation of "relevance" stems from biological or pathological processes involving bile acids. Many of these are well known, such as transport of cholesterol in bile or lipolytic products in intestinal content. Others are not physiological effects, but are pharmacological effects; that is, they occur only during pharmacological alteration of the enterohepatic circulation by bile acids. Some effects that are assigned to bile acids may not stand careful scrutiny. In **Table 4,** we have listed biological processes involving bile acids and indicated which model system (from Table 2) is relevant. We have also indicated which physicochemical parameter of the model system (as shown in Table 2) appears particularly related to the respective biological process.

### A criterion for distinguishing physicochemical effects from biological effects

Establishing cause and effect in biological systems is difficult. This is especially true for physiological processes such as bile formation, which can only be studied using the intact organ and not with isolated cells. A common approach taken to identify causality is a high correlation between the magnitude of one variable and that of another. For example, with bile acids, it is common to attempt to correlate a physicochemical property, for example, the CMC or an estimate of hydrophobicity, such as relative retention time, against a biological effect such as hepatic uptake, biliary secretion, induction of

TABLE 2. Aqueous model systems with bile acids relevant to biological processes

| | Item |
|---|---|
| Systems involving bile acid monomers | |
| Bile acid alone (bile acid-water) | 1 |
| Bile acid plus additional well-defined simple additives | |
| Bile acid-cations ($Na^+$, $Ca^{2+}$, tetramethyl ammonium) | 2 |
| Macromolecular additives or adsorbents | |
| Proteins | |
| Albumin | 3 |
| Cytosolic organic acid binding proteins or membrane carrier proteins | 4 |
| Enzymes | 5 |
| Apoproteins | 6 |
| Water soluble polymers | 7 |
| Insoluble polymers | |
| Ionic (e.g., cholestyramine, colestipol) | 8 |
| Uncharged (e.g., charcoal, $C_{18}$ silicic acid, XAD) | 9 |
| Bile acids and multiple polymers (fiber) | 10 |
| Bile acids and immiscible lipids (liquid/liquid partition) | 11 |
| Bile acids and organized lipid aggregates | |
| Liposomes | 12 |
| Micelles of other surfactants | 13 |
| Bile acids and organized lipid protein aggregates | |
| Lipoproteins | 14 |
| Membrane receptors | 15 |
| Bile acids and membranes | 16 |
| Systems involving bile acids in simple and mixed micelles | |
| Related to bile | |
| Bile acids alone (bile acid-water) | 17 |
| Bile acid-phospholipid | 18 |
| Bile acid-cholesterol | 19 |
| Bile acid-bilirubin | 20 |
| Bile acid-phospholipid-cholesterol | 21 |
| Bile acid-cholesterol-bilirubin | 22 |
| Bile acid-phospholipid-bilirubin | 23 |
| Bile acid-phospholipid-cholesterol-bilirubin | 24 |
| Additional additives: $Ca^{2+}$ | 25 |
| Related to fat digestion | |
| Bile acid-fatty acid | 26 |
| Bile acid-2 monoglyceride | 27 |
| Bile acid-soap | 28 |
| Bile acid-fatty acid-2 monoglyceride | 29 |
| Bile acid-fatty acid-soap | 30 |
| Bile acid-2 monoglyceride-soap | 31 |
| Bile acid-fatty acid-2 monoglyceride-soap | 32 |
| Additional additives: $Ca^{2+}$ | 33 |
| colipase and/or lipase | 34 |
| phospholipid and/or phospholipase | 35 |

bile flow, induction of biliary lipid secretion, etc. (61, 99).

What has become clear in the past 5 years is that there are marked interspecies differences in the biological effects induced by different bile acids. For example, chenodeoxycholic acid increases cholesterol secretion in the hamster and dog (99, 100), but decreases it in man (101). Deoxycholyltaurine is rapidly extracted by the rabbit liver, but poorly by the guinea pig liver (102). Analogously, hyocholyltaurine is extracted seven times as rapidly by the rat liver as the rabbit liver. These differences, and they are all in relation to a common bile acid such as cholyltaurine, indicate that physicochemical properties alone cannot be the explanation for the different biological effects of individual bile acids. *Indeed, in our opinion, demonstration of a marked species effect in response to a series of bile acids is a priori evidence for an important biological effect.*

It also seems important to note that the solubilization and transport processes occurring in bile or intestinal content occur at extremely high concentrations of bile acids (millimolar) where micelle formation readily occurs, whereas intracellular and plasma transport occur at bile acid concentrations that are about three orders of magnitude lower.

This suggestion that physicochemical parameters may sometimes be insufficient to explain certain biological processes involving bile acids does not diminish the scientific value of careful measurement of the physicochemical properties of bile acids. Indeed, a major purpose

TABLE 3. Properties of interest in aqueous model systems with bile acids relevant to biological processes

| | Item |
|---|---|
| Monomeric systems | |
| Monomeric properties: bulk and surface | |
| Solubility of protonated form | A |
| $pK_a$ | B |
| Effect of ionization on solubility (CMpH) | C |
| Solubility of ionized form (CMC, CMT) | D |
| Adsorption to surfaces (oil/water; air/water) | |
| Surface activity | E |
| Arrangement of molecules at interface; surface charge, etc. | F |
| Interaction with cationic additives | |
| Binding by bile acid monomers | G |
| Precipitation of insoluble salt | H |
| Binding to macromolecules | |
| Affinity constants (isotherm), site, mechanisms, specificity, effect on activity, allosteric effects, etc. | I |
| Liquid/liquid distribution ratio (hydrophilicity-hydrophobicity) | J |
| Interaction with lipid aggregates, membranes, etc. | |
| Binding, influence on permeability, enzyme activity, molecular organization | K |
| Micellar Systems | |
| Characteristics of simple and mixed micelles | |
| CMC of system | L |
| Molecular organization, size, hydrophobicity of interior | M |
| Solubilization properties | N |
| Counterion binding | O |
| Osmotic properties | P |
| Phase equilibria | |
| Formation of mesophases at high concentration | Q |
| Molecular organization of mesophases | R |

TABLE 4. Biological processes (P) involving bile acids, relevant model systems, and properties of interest in model systems

| | Process | Relevant Model System and Some Properties of Interest |
|---|---|---|
| | Membrane transport of bile acids | |
| | Hepatocyte | |
| $P_1$ | Active transport by sinusoidal membrane | 1ABEF; 9I; 11J; 15IJK; 16DJK |
| $P_2$ | Passive transport by sinusoidal membrane | 1ABEF; 9I; 11J; 16K |
| $P_3$ | Active transport by canalicular membrane | See $P_1$ |
| $P_4$ | Transcellular transport | 1ABEF; 4I; 11J |
| | Enterocyte (jejunum and ileum) | |
| $P_5$ | Passive transport by brush border membrane | See $P_2$ |
| $P_6$ | Passive transport by basolateral membrane | See $P_2$ |
| $P_7$ | Transcellular transport | See $P_4$ |
| | Enterocyte (terminal ileum) | |
| $P_8$ | Active transport by brush border membrane | See $P_1$ |
| $P_9$ | Active transport by basolateral membrane | See $P_1$ |
| $P_{10}$ | Transcellular transport | See $P_4$ |
| $P_{11}$ | Plasma transport of bile acids | 1ABEF; 3I; 6I; 9I; 14I |
| $P_{12}$ | Modulation of membrane transport by bile acids e.g., receptor mediated endocytosis | 4I; 9I; 15IJK; 16IJK |
| $P_{13}$ | Modulation of cellular metabolism by bile acids, e.g., triglyceride synthesis | 5I |
| $P_{14}$ | Modulation of bile acid and cholesterol synthesis by bile acids | 5I |
| $P_{15}$ | Induction of bile flow by bile acids | 1AB; 2G; 11J; 17–25LMOP |
| $P_{16}$ | Induction of biliary lipid secretion by bile acids | 1AB; 2G; |
| | Transport of lipids in mixed micelles | |
| $P_{17}$ | Cholesterol in bile | 6I; 16–24 L–R |
| $P_{18}$ | Lipolytic products, fat soluble vitamins in intestinal content | 1A–F; 2GH; 16–24L–P |
| $P_{19}$ | Buffering of calcium activity in bile | 1A–F; 2GH; 16–24L–P |
| $P_{20}$ | Modulation of lipase and phospholipase activity | 1EF; 5I; 9I; 11J |
| $P_{21}$ | Modulation of intestinal mucosal permeability | 9I; 11J; 15K; 16K |
| $P_{22}$ | Modulation of intestinal electrolyte secretion | 9I; 11J; 15K; 16K |
| $P_{23}$ | Alteration of circulating bile acids by bile acid administration (for gallstone dissolution) | See $P_1$–$P_{11}$; $P_{16}$; $P_{17}$; $P_{23}$, $P_{24}$ |
| $P_{24}$ | Interruption of enterohepatic circulation of bile acids to induce LDL receptors | 8I; 11J |

of physicochemical studies is to factor out those aspects of biological processes that can be explained solely by physicochemical properties and to distinguish these from those which cannot, and for which an additional biological explanation is required. This view, if correct, means that the physical chemist and the biologist must collaborate to define the point where physical chemistry stops and biology begins. In our judgment, it is wise for the physical chemist to study a variety of bile acids and wise for the biologist to study more than one mammal and even to carry out studies in a variety of vertebrates.

The continuing elucidation of the physicochemical properties of bile acids has been a powerful intellectual stimulus to biomedical research that has constantly sought rational explanations for the many remarkable effects of bile acids. The last 25 years have seen a harmonious duet between the physical chemist and the physiologist. In the next 25 years, we envision a quartet, as the duo is joined by an organic chemist and a cell biologist. If the next 25 years are as exciting as the last 25 years, then the bile acid research community and the *Journal of Lipid Research,* which often publishes its findings, will both have ample justification for another celebration.

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