review

Structure and binding of unconjugated bilirubin: relevance for physiological and pathophysiological function

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INTRODUCTION

There is still much uncertainty concerning the structure, physical chemistry, transport, and metabolism of unconjugated bilirubin (UCB), in both physiological and pathological states. We believe that this may be due, in large part, to the consideration of UCB-IXα as a single entity, whereas it actually exists in body fluids and tissues as three different species: the uncharged diacid (H_2B), two isomeric monoanions (HB^-), and the dianion (B^{2-}) (1–3). Thus, akin to the "trinity of isomers" (IIIα, IXα, and XIIIα) stressed in the past (4), there exists a more fundamentally important "trinity of species," each of which may exist either unbound (free) or bound to a variety of other molecules or membranes, and may have a different conformation in the bound and free state. Prior reviews have often stressed the multiple conformations and configurations of UCB (5–7), but the importance of the three differently charged species has seldom been discussed (8–10). In this review, we aim to define the proportions of each of the three UCB species, bound and unbound, in plasma, membranes, hepatocytes and bile, and critically summarize what is known about the roles of these species in the transport, metabolism and toxicity of UCB, proposing hypotheses and experiments where information is unavailable.

STRUCTURE

UCB is a nearly symmetrical tetrapyrrole, consisting of two rigid, planar dipyrrole units (dipyrrinones), joined by a methylene ($-CH_2-$) bridge at carbon 10 (**Fig. 1**) (5, 6). The structure thus resembles a two-bladed propellor, in which the blades could theoretically be joined at different angles and each blade could rotate about its bond to the methylene bridge (6, 9, 11), yielding a panoply of potential conformations. In the preferred "ridge-tile" conformation (5, 6, 11, 12), the two diyprrinones are synperiplanar, as in a partially opened book, and the angle (θ) between the two planes is about 95° (5). This minimum energy conformation is further stabilized by two trios of internal hydrogen bonds formed between the carboxymethyl sidechain of each dipyrrinone and the -CO-NH- and >NH groups of the other dipyrrinone (Fig. 1).

This rigid biplanar structure, with its internal hydrogen bonds, was first demonstrated in the crystalline state by X-ray diffraction (13), but is also the preferred conformation in solutions of UCB in water, alcohols, and chloroform (11, 14–22). Due to the different placement of the vinyl sidechains on the two end rings (endo-vinyl on ring A and exo-vinyl on ring D), the structure is slightly asymmetric and thus has optical activity, so that two enantiomers co-exist in solution (5, 6) (Fig. 1). The enantiomers rapidly and reversibly interconvert by transient breakage of all six of the hydrogen bonds, despite their tenacity (12), forming a flexible, unbonded intermediate (6, 21, 23–25) (Fig. 1). By "fully opening the book," this unbonded transient passes through a flat conformation to complete inversion of the angle of jointure between the two dipyrrinones, and then reforms the hydrogen bonds in the mirror-image conformation (6, 11, 23, 24). In the unbound (free) phase of any system the two enantiomers should be equally prevalent and have the same carboxylic ionization constants (pK'a), but interactions with the solvent or other solutes (15, 21, 26, 27), such as albumin (28–37) may favor one enantiomer over the other and/or

Abbreviations: UCB, unconjugated bilirubin; CD, circular dichroism; K_a, association constant; TC, taurocholate; TDHC, taurodehydrocholate; TCDC, taurochenodeoxycholate; CMC, critical micellar concentration; TDC, taurodeoxycholate; BSP, bromosulfonphthalein; TBS, tetrabromosulfonephthalein; BMG, bilirubin monoglucuronide.

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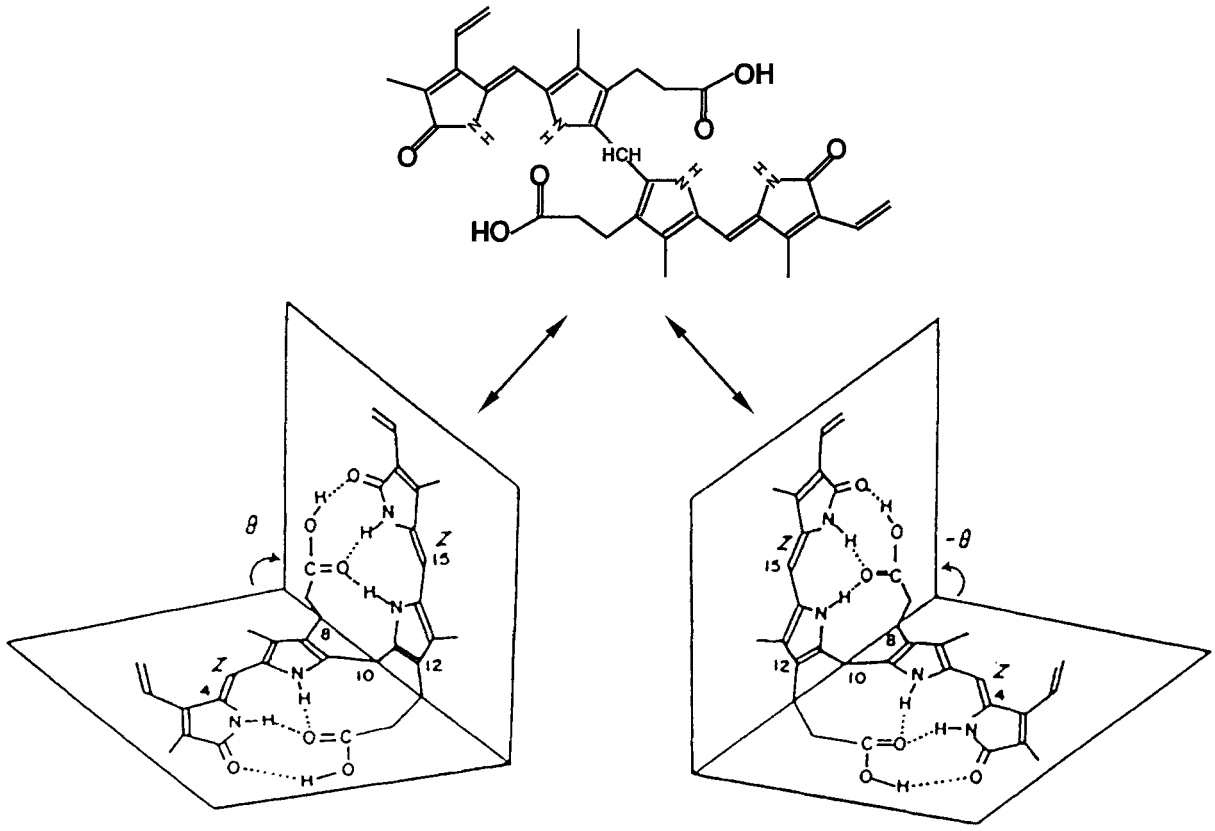

Fig. 1. The structure of unconjugated bilirubin IXα-Z,Z, diacid (H_2B), which consists of two slightly asymmetrical, rigid, planar dipyrrinone chromophores, connected by a central $-CH_2-$ bridge. At the bottom are the two optical enantiomers of H_2B in their rigid, folded, ridge-tile conformations, stabilized by three hydrogen bonds (----) between each sidechain carboxyl group and the >C=O and two >NH groups of the opposite dipyrromethenone. These two enantiomers are in rapid equilibrium via the unfolded, planar intermediate, shown at top, in which all six hydrogen bonds are transiently ruptured, conferring flexibility about the central $-CH_2-$bridge. (Adapted, with permission from Pu, Y. M., and D. A. Lightner. The conformation of bilirubin dianion. *Tetrahedron.* **47:** 6163-6170. Copyright 1991 by Pergamon Press (21)).

deform the ridge-tile. The resulting Cotton effects on circular dichroism (CD) spectra may also be induced by asymmetry in the microenvironment of bilirubin.

BILIRUBIN IONIZATION AND AQUEOUS SOLUBILITY

Ionization of UCB IXα

At physiological pH values in plasma (7.4), tissues (7.0), and bile (6.0 to 8.0) there is significant ionization of the -COOH groups of the natural IXα isomer of UCB (3), so that, in addition to the diacid (H_2B), a proportion of UCB is present as the monoanion (HB^-) and dianion (B^{2-}). As in the diacid, internal hydrogen bonds stabilize the rigid, ridge-tile structure of the unbound UCB anions (11, 21, 28, 38) (**Fig. 2**), and reversible disruption of these bonds, with enantiomeric interconversion via a flexible, fully planar intermediate, still occurs.

The pK'a values of the -COOH groups on the two carboxymethyl (propionyl) sidechains determine the proportions of the free UCB species at any given pH. The -COOH group of propionic acid has a pK'a value below 5.0, fostering the prevailing view that this was true also of the -COOH groups of UCB in aqueous solution, so that B^{2-} was presumed to be the predominant species present in body fluids at physiological pH values (1, 39, 40). These conclusions were based on data regarding the effects of pH on the solubility of H_2B crystals in aqueous solutions (1, 39, 41, 42). Those studies were, however, limited by: *a)* poor accuracy of the measurement of low concentrations of UCB; *b)* surface-active impurities in the UCB; *c)* failure to reach equilibrium due to incubation of a relatively small excess of crystals for insufficient time periods (39, 42); and *d)* vastly greater (metastable) solubilities obtained after rapid acidification of the dissolved disodium salt of B^{2-} (Na_2B) (43-46). More recent studies, which yielded similar solubilities from dissolution of H_2B crystals and acidification of dissolved Na_2B, suggested, rather, that the two pK'a values of UCB were both above 6.0 and differed by about 3 pH units (2, 47, 48). This wide disparity between pK'_1 and pK'_2, however, was inconsistent with the wide spatial separation between two -COOH groups in UCB (8), which should yield little effect of the electrical field of one $-COO^-$ group on the ionization of the other -COOH (49).

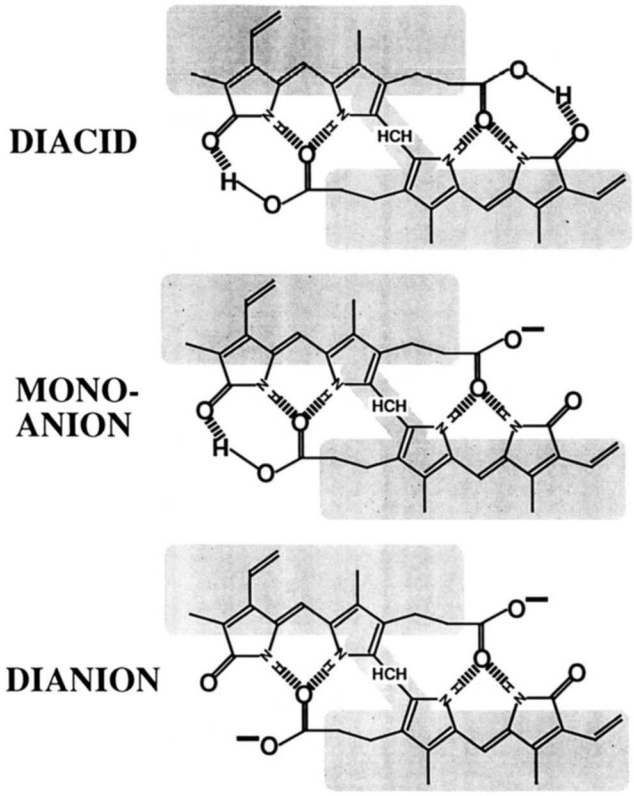

Fig. 2. Two-dimensional representations of the structures of unconjugated bilirubin IXα-Z,Z diacid (H_2B, top), monoanion (HB^-, middle) and dianion (B^{2-}, bottom), showing the hydrogen bonds (bars) and the hydrophobic domains of each dipyrromethenone chromophore and the central methylene bridge (shaded areas). On each dipyrrinone, a string of 10 or 11 nonpolar groups extends from the exo-methyl or exo-vinyl substituent on the end-ring, through the ring carbons and the connecting methene bridge, to the proximal carbons of the carboxymethyl sidechain on the inner ring. One hydrogen bond is lost with each -COOH ionization, but the residual hydrogen bonds maintain the rigid, folded ridge-tile conformation of the molecule. However, enantiomeric interconversion via a fully unbonded planar intermediate (as in Fig. 1) is facilitated by the decrease in hydrogen bonding, so that formation of the planar intermediate is more likely for the more ionized species. (Adapted, with permission, from Carey, M. C., and W. Spivak. Physical chemistry of bile pigments and porphyrins with particular reference to bile. *In* Bile Pigments and Jaundice; Molecular, Metabolic and Medical Aspects. J. D. Ostrow, editor. 81–132. Copyright 1986 by Marcel Dekker, New York (8)).

These unsatisfactory pK'a values result from the inherent unreliability of crystal dissolution studies, caused by great variation in solubilities obtained among crystals of different size and orderliness (2, 50, 51). Studies of the relationship of UCB solubility to pH, determined by rapid partition of H_2B from chloroform into aqueous buffers over a pH range from 4.0 to 9.6, avoid these problems (3). As expected, the solubilities at each pH (3) were much lower than those derived with UCB crystals (2), and analysis of the data yielded values for pK'_1 of 8.12 ± 0.23 and pK'_2 of 8.44 ± 0.33 (3). These unexpectedly high pK'a values are probably the result of retarded dissociation of the protons due to the internal hydrogen bonds involving each -COOH group (2, 5, 8, 9). ^{13}C- and ^1H-NMR, Raman resonance, and circular dichroism (CD) spectra of UCB analogues, which contain substituents that would preclude this hydrogen bond, are similar to those of UCB in chloroform and aqueous alkaline solutions, suggesting that the remaining hydrogen bonds, between the ring >NH groups and the $-COO^-$ group, are preserved in the ionized UCB species in simple aqueous solutions (6, 14, 19, 20, 27, 38, 52–54) (Fig. 2). When this internal bonding is broken, e.g., by dissolution of UCB in dimethylformamide (55, 56) or dimethyl sulfoxide (57, 58), titrimetric and ^{13}C-NMR studies yield, as expected, pK'a values for UCB between 4.5 and 5.3.

Using the partition-derived experimental pK'a values of 8.12 and 8.44 (3), the calculated proportions of the individual UCB species in aqueous solution at pH values from 6.0 to 8.0 show that H_2B is the dominant species and HB^- the dominant anion (**Fig. 3**). The fraction of B^{2-} is minimal at pH values below 7.2 and less than 14% at pH values up to 8.0. The dominant features are a decrease in the proportion of H_2B and increase in the proportion of HB^- with increasing pH. There is no significant self-association of any UCB species, although self-association of B^{2-} becomes prominent only above pH 8.3, at which concentrations of B^{2-} are very much higher (3, 16). At pH 7.4 (e.g., plasma), H_2B constitutes 83%, HB^- 16%, and B^{2-} less than 1.5% of total UCB; at pH 7.0 (e.g., cytosol), the proportions are H_2B 93% and HB^- 7%, whereas B^{2-} is undetectable (Fig. 3).

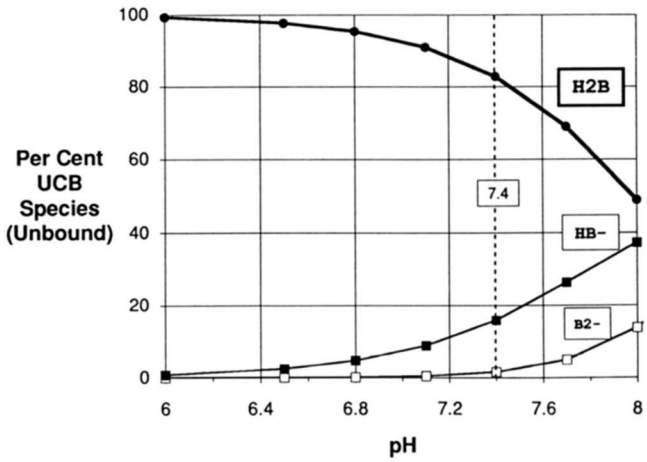

Fig. 3. Proportions of unbound species of unconjugated bilirubin at pH 6.0 to 8.0, derived from partitions of UCB from chloroform into buffered NaCl at ionic strength 0.15, yielding pK'a values of 8.12 and 8.44 by computer-modelling of the data. The fully protonated diacid (H_2B) is the dominant species, and the monoanion (HB^-) the dominant anion over this physiological pH range. The bilirubin dianion (B^{2-}) is a significant fraction only at pH 7.2 or above, and there is no significant self-association of B^{2-} into dimers. (Adapted, with permission, from Hahm, J. S., J. D. Ostrow, P. Mukerjee, and L. Celic. Ionization and self-association of unconjugated bilirubin, determined by rapid solvent partition from chloroform, with further studies on bilirubin solubility. *J. Lipid Res.* **33:** 1123–1137. Copyright 1993 by Lipid Research, Inc. (3)).

Solubility of UCB

The low aqueous solubility of UCB diacid is probably due both to its many hydrophobic groups and the internal hydrogen bonding of all its polar groups (Fig. 2, top), precluding their interaction with water (5–8, 10, 12, 17, 19, 20, 38, 54, 59–62). Experimental solubilities near neutral pH, however, derived from studies of crystal dissolution (1, 2, 39, 41, 42, 47, 48), or acidification of Na_2B (2, 43–46), have varied extensively (10 nM to 15 mM). Much more reliable values, determined by chloroform-to-water partition (3), indicate that the maximum aqueous solubility of H_2B, at 25°C and ionic strength 0.15, is about 70 nM (**Fig. 4**). This is much higher than the usually quoted value of 10 nM, which was derived from extrapolation of data obtained at pH 7.5–9.0 by dissolution of UCB crystals for only 4 h (39, 42). This incubation time was much too short to achieve equilibrium, and later data showed that the log-linear extrapolation was invalid. The partition data (3) show that total UCB solubility increases with increasing pH to only 80 nM at pH 6.8, and to 170 nM at pH 8.0. If UCB had a pK'a of 5.0, its solubility would increase much more rapidly in the physiological pH range. Above pH 8.0, as the two -COOH groups ionize, the solubility rises steeply and is close to 1 mM at pH 9.0 and 60 mM above pH 9.5 (Fig. 4). At concentrations of unbound UCB that vastly exceed these solubility limits, the expected supersaturation may lead to formation of microaggregates and even precipitation of the pigment (3, 16, 47, 63, 64). This limits interpretation of many published studies of UCB interactions with proteins, membranes, cells, and bile salts, which were conducted under greatly supersaturated conditions, though minor degrees of metastable supersaturation may be acceptable.

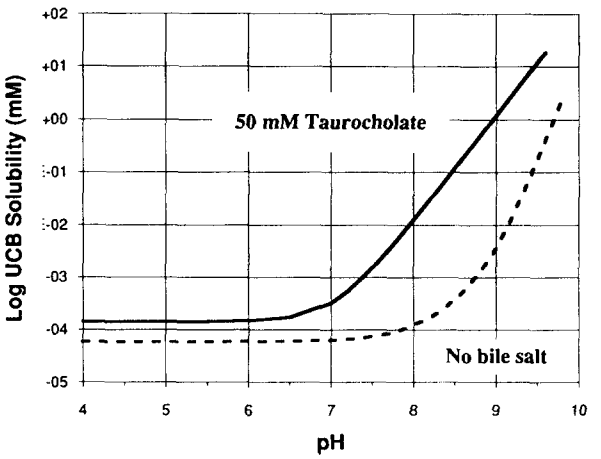

Fig. 4. Solubilities of unconjugated bilirubin in buffered solutions of 50 mM sodium taurocholate (—) and NaCl (----) from pH 4.0 to 10.0, at 25°C, ionic strength = 0.15, determined by solvent partition from chloroform. The maximum solubility of bilirubin in chloroform was 1.14 mM. (Data from Hahm, J. S. et al. 1990 (150)).

Since the solubility of H_2B is the same at all pH values, the increase in overall UCB solubility with increasing pH is due to successive ionization of H_2B to HB^- and B^{2-}. Ionization removes the proton that, in H_2B, hydrogen bonds the -COOH group to the lactam >C=O of the opposite dipyrrole and renders these groups available for interaction with water, and also generates a negatively charged $-COO^-$ group (Fig. 2).

Isomers of UCB IXα with altered conformations

The importance of the internal hydrogen bonding in determining the ionization and consequent solubility of UCB is well illustrated by the properties of several conformational isomers. In UCB-IXα, the natural isomer formed by oxidation of the α-methene bridge of heme, complete internal hydrogen-bonding is possible only if the -COOH groups are protonated and the C5 and C15 methene bridges, that connect the two pyrrole rings in each dipyrrinone half, are each in the Z-conformation (5–7, 28). In geometric photoisomers, formed by the action of light energy on UCB, the C5 and/or C15 Z-bridge(s) is flipped to the E-conformation (**Fig. 5A**) (65). In side-chain UCB isomers, which result from cleavage of heme at its β, γ, or δ bridge, the carboxymethyl sidechains are attached at different sites on the dipyrrole rings (Fig. 5B) (66). In each of these classes of isomers, the altered spatial relationships between the dipyrrole (A or D ring) and the opposite carboxymethyl sidechain render the formation of internal hydrogen bonds sterically impossible (7). The partial loss of hydrogen-bonding in both these classes of isomers lowers the pK'a values and exposes some of the polar groups of UCB to the aqueous medium, resulting in much higher water-solubility (65–67).

BINDING

General considerations

The above concepts describe the behavior of UCB and its species in simple aqueous systems which contain no other compounds that bind UCB. In body fluids and tissues, the situation is complicated by the presence of additional compounds, each of which may bind UCB with different avidity. Examples are: albumin in plasma; lipids and transport proteins in membranes; ligandin and Z-protein in the cytosol; and bile salts, mixed micelles and vesicles in bile. The picture is even more complicated, as each of the three species of UCB (H_2B, HB^-, B^{2-}) may have different affinities for each individual binder. As the three unbound species are in rapid equilibrium with each other, preferential binding of any UCB species will decrease the overall concentration of unbound UCB, but not alter the proportions of unbound H_2B, HB^- and B^{2-}, which are determined by the pH in the bulk aqueous phase.

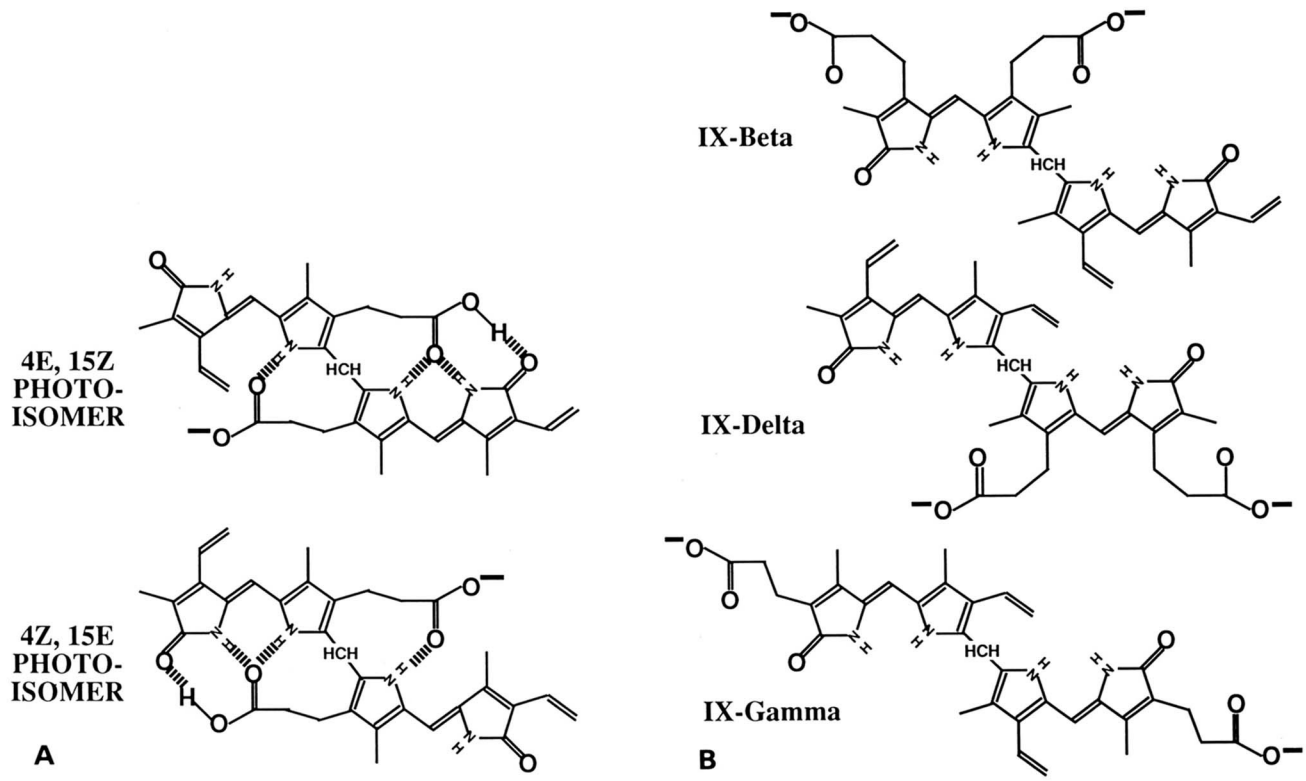

Fig. 5. Structures of conformational isomers of unconjugated bilirubin IXα-Z,Z that are more polar and water-soluble due to partial interruption of hydrogen-bonding. A: Photobilirubins formed by geometrical (Z to E) photo-chemically induced isomerization of the Δ4,5 and/or Δ15,16 methene bridges in unconjugated bilirubin. B: Sidechain isomers of bilirubin IX-Z,Z, formed, respectively, by oxidative removal of the β, δ, or γ>CH groups of heme. (A is adapted, with permission, from Stoll, M. S. Phototherapy of jaundice. *In* Bile Pigments and Jaundice; Molecular, Metabolic and Medical Aspects. J. D. Ostrow, editor. 551–580. Copyright 1986 by Marcel Dekker, New York (271). B is based on Blanckaert, N. et al. 1976 (66)).

In modeling such complex systems, the unbound (free) UCB species in the bulk aqueous phase may be assumed to behave identically with UCB in simple aqueous systems at the same temperature and ionic strength. Therefore, the pK'a values and solubilities of UCB, derived from simple aqueous systems, may be applied to the unbound (free) UCB species in the complex systems, as shown in **Fig. 6.** Each UCB species (H_2B, HB^- and B^{2-}, respectively) is distributed between the free and bound states, with bound/free ratios (D_0, D_1, and D_2) determined by the pH-independent affinity constants of each species for each binder (k_0, k_1 and k_2). In the complex system, the overall solubility (bound + unbound) of UCB is increased by the bound species. The proportion of each UCB species in the bound phase is determined by its intrinsic affinity constant (k) and the proportion of that species in the unbound phase at the ambient pH. At any pH, UCB species with higher affinities will be preferentially represented in the bound phase. This will necessarily change the ratios of total H_2B, HB^- and B^{2-} from the ratios in the unbound phase. The *overall apparent* pK′a value of UCB in the system will, therefore, differ from the true pK'a values of the unbound species. As the proportions of unbound H_2B, HB^- and B^{2-} vary with pH (3) (Fig. 3), the proportion of total UCB that is bound to a given ligand must also vary with pH if there are differences among the binding affinities of the three UCB species. For example, if the affinity of H_2B is greatest, then overall UCB binding must increase as pH decreases, and the overall apparent pK'a values must increase. By contrast, if B^{2-} is preferentially bound, the overall apparent

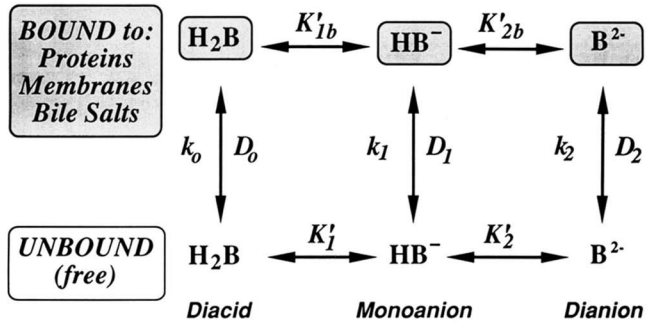

Fig. 6. Schematic of the equilibria between unbound (free) and bound species (shaded area) of unconjugated bilirubin. K'_1 and K'_2 are the apparent ionization constants of the two carboxylic groups of bilirubin; k_0, k_1 and k_2 are the association (formation) constants for binding of H_2B, HB^-, and B^{2-}, respectively. D_0, D_1 and D_2 are the distribution ratios (bound/free) for H_2B, HB^-, and B^{2-}, respectively.

pK'a values of UCB will be lower than the pK'a values in the unbound phase, and overall UCB binding must increase as pH increases.

Experimentally, the total UCB concentration and ambient pH of any system can be measured. From the relationship between these two variables over a wide pH range (3), the overall apparent pK'a values can be determined. Using the known pK'a values of the free UCB species (3), the proportion of each unbound UCB species can be calculated at all UCB concentrations at or below saturation. If UCB is at saturation, the concentrations of the free species will be known. Alternatively, the unbound UCB concentration can be assessed from: *a)* the measured UCB concentration in the organic phase of a solvent partition system (3); *b)* ultrafiltration; or *c)* equilibrium dialysis, although the instability of UCB renders it difficult to perform long-term equilibrium dialysis. The bound species is then calculated as the difference between the total and free species. The distribution ratio (D) between bound and free states, and the affinity of binding (k), can then be derived for each UCB species. Note that this analysis does not apply if total UCB concentrations exceed solubility limits and effects of supersaturation are significant.

The fully bonded ridge-tile structure of UCB differs from that of bile salts (68), as there is no hydrophobic or hydrophilic face. Rather, each planar dipyrrinone of UCB has a hydrophobic sector, formed by a chain of 10 or 11 nonpolar groups, which is exposed at the margin of the folded biplane (Fig. 2). These two hydrophobic regions, connected by the central methylene bridge, are arranged diametrically in the biplane. Adjacent to these hydrophobic sectors are two clusters of hydrogen-bonded polar groups, which are also situated diametrically in the biplane. As these clusters lack hydrogen-bound donors, they are best described as "semipolar." These semipolar sectors in the biplanar H_2B molecule may hinder the immersion of the adjacent hydrophobic sectors within a strongly hydrophobic environment, such as the interior of a micelle or phospholipid bilayer (8), as compared to sites near the surface of these lipid assemblies (69–72). It must be emphasized that, in aqueous systems, any binding interactions should optimally shield the hydrophobic sectors of the UCB molecule from the water phase.

With these principles in mind, we will now examine binding of UCB to plasma albumin, cytosolic binding proteins, phospholipids and membranes, and bile salts.

Plasma albumin

Albumin is the major binder of UCB in plasma (73–77), but both the nature of the interactions and the affinity constants remain uncertain. There is general agreement that there is a high-affinity site on albumin for binding one mole of UCB, and one or more lower-affinity sites (78–83). Many amphipathic organic anions (33, 39, 78, 79, 84–90), including medium-chain fatty acids and fatty acyl CoA esters (91) compete for UCB binding at the primary site. Heme (79, 92, 93), bile salts (88, 94, 95), long-chain fatty acids (30, 33, 36, 85, 88, 96–101), and many drugs (85, 89, 90) bind to other sites, and may cause allosteric effects on the affinity of the primary binding site for UCB. The most widely accepted association constants for the UCB binding sites are those obtained with selective peroxidation of unbound UCB by horseradish peroxidase at pH 7.4, with a reported $K_1 = 5.5$ to $6.8 \times 10^7\ M^{-1}$, and $K_2 = 4.4$ to $5.0 \times 10^6\ M^{-1}$ (39, 42, 96, 102–105). Human fetal albumin (106, 107) and albumin from other mammalian species (37, 101, 108–111) generally show lower binding affinities.

Competitive binding studies with other amphiphilic organic anions suggest that ionic interactions are most important (33, 39, 78, 79, 84, 86–90). Studies with proteolytic fragments (112), affinity labelling (113–116), or chemical modifications of human serum albumin (93, 117–119) or bilirubin (28), indicate ionic binding of the $-COO^-$ groups on UCB anions to the terminal $-NH_2$ group of specific lysine residues of albumin, with disruption of all the internal hydrogen bonds of UCB (38). This is supported by the finding that UCB binds to chiral aromatic amines (26) and polypeptides (120), as well as to beads with covalently linked lysine substituents (121–124). The interactive lysines are, however, buried within the albumin molecule (125–127), so that the bound UCB is fully internalized (128). Hydrogen-bonding to a tyrosine residue in each trough is involved also (111, 119, 129, 130) and accounts for the large change in fluorescence that occurs when UCB binds to albumin.

Studies with UCB analogues, including dipyrroles and spin-labeled derivatives, have suggested that each dipyrrinone half of UCB is bound in a separate trough in the 2A,B–1C domains of the albumin molecule, with two facing troughs forming the binding cavity for the internalized tetrapyrrole, like a pair of cupped hands (78, 84, 131–133). Though hydrophobic interactions have been deemed unimportant (78, 84, 105), there are domains of hydrophobic residues within the troughs that could interact with the hydrophobic domains of UCB, and virtually all the competitive binders are amphiphiles which contain largely hydrophobic planar aromatic groups (84, 85). The marked bisignate circular dichroism generated by interaction of UCB with albumin is believed to result mainly from preferential binding of the UCB enantiomer with right-handed chirality (enantioselection) (28–37, 120). Conformational widening of the angle (θ, Fig. 1) between the dipyrromethenone domains of the bound UCB (78, 120, 131), deformation of the synperiplanarity of the bound dipyrrinones (111, 120), and asymmetry of the microenvironment may also contribute to the shifts in the visible and CD spectra. The conformational relationships of the troughs in albumin undergo a transition as the pH

passes through the neutral range (134), further modifying the visible spectra independent of changes in UCB ionization.

Studies with the peroxidase method (78, 82, 84, 85, 103) and paper electrophoresis (73, 75) have detected no change in the affinity or capacity for UCB binding to albumin in the pH range from 6.0 to 10.0. By contrast, absorption spectroscopy (83, 135), circular dichroism (29), fluorescence (33, 136), competitive dye binding (101, 137, 138), and gel filtration (139) have indicated decreased UCB binding affinity as pH is decreased. These disagreements may be related to differences among studies in: *a)* the purity of the UCB and albumin used; *b)* the ratios of UCB to albumin; and, as noted earlier, *c)* the degree of supersaturation of the unbound UCB. Though, with moderate metastable supersaturation of UCB, the excess pigment may behave like stably dissolved UCB (140), precipitation of H_2B aggregates below pH 8.3 (8, 47, 63, 141) may have affected results in some studies (73, 139).

The peroxidase method also has intrinsic problems (39, 103, 140, 142), such as: *a)* the need to determine reference peroxidation rates for unbound UCB at concentrations vastly above saturation, with self-association and aggregation of the pigment; *b)* the steep decrease in peroxidase activity at pH values on either side of the optimum at pH 8.2, which is corrected with a reference compound, pyrogallol, which may not behave like bilirubin; and *c)* the assumption that dissociation of UCB from albumin is sufficiently rapid to maintain the unbound UCB concentration constant as it is peroxidized, so that zero-order kinetics are applicable (39, 103). More likely, the very slow off-rates for dissociation of UCB from albumin (143–148) render this step rate-limiting, as might be expected from the complete sequestration of UCB within the binding pockets in albumin (128). Thus, the peroxidase method may not be valid to study albumin-binding of UCB and the usually quoted binding affinities of 5.5 to 6.8×10^7 M^{-1} derived by this method (39, 42, 96, 102–105) may be in error. Though Sephadex gel filtration (104) and fluorescence quenching (36, 111) have yielded similar affinity constants, much higher values have been obtained by stopped-flow UV-visble spectroscopy (148), while other methods have yielded much lower values (33, 99, 112, 149). Even some peroxidase studies have given considerably higher (142) or lower (82, 112) values.

A good fit of binding data from peroxidase studies is obtained with models which assume that B^{2-} is the only unbound UCB species present at pH values above 6.5 (39, 78, 84, 103, 142). As shown in Fig. 3, however, this assumption is incorrect, as HB^- and H_2B are in fact the dominant unbound UCB species (3, 150). These conflicting concepts could be reconciled if the B^{2-} species has a vastly higher affinity for albumin than the other two species, so that its bound state constitutes a "sink" for B^{2-}, constantly shifting the ionization equilibrium toward this species. This concept is consistent with the finding that, as UCB binds to albumin, the two -COOH groups of UCB ionize, releasing two protons (39, 58). With a sufficiently high proportion of B^{2-} in the bound state, this species would dominate the overall binding equilibria in Fig. 6 ($k_2 >>> k_1 > k_0$), and yield the false impression that B^{2-} was the only species present, as the contributions of HB^- and H_2B to binding would be marginal. Preferential binding of B^{2-} might be expected from two structural considerations: *a)* the demonstrated importance of anionic binding to lysine residues in albumin; and *b)* separate troughs for binding each dipyrrolic half of UCB (78, 84), which could require rupture of all the internal hydrogen bonds of UCB; thus, the fully ionized B^{2-} species, with only four internal hydrogen bonds to rupture, would be preferentially bound. Resonance Raman spectroscopy of the albumin-UCB complex (38) indicates that all the hydrogen bonds of UCB are indeed ruptured and that both $-COO^-$ groups are ionized.

Hepatocytic cytosolic binding proteins

Ligandin (Y-protein), and aminoazo-dye-binding protein A (Z-protein) are two proteins found in the cytosol of hepatocytes (151), as well as renal tubular epithelial cells and enterocytes (152), which have high affinity for bilirubin and other organic anions. Ligandin, which is identical with glutathione-S-transferase B (153), is a 47 kD heterodimer, in which the smaller subunit lacks 25 to 30 amino acids that are present in the C-terminus of the larger subunit (154–156). Subunit A (M_r = 22 kD) contains a single high-affinity binding domain for bilirubin (157, 158), with an association constant estimated as 5×10^7 M^{-1} (159) to 7×10^6 M^{-1} (160) at neutral pH and 1×10^6 M^{-1} (160) at pH 8.2, suggesting that H_2B is the bound species. UCB binding is associated with marked, bisignate circular dichroism that is a mirror image of the ellipticity seen with UCB-albumin complexes (152, 159), indicating binding of the S-enantiomer of UCB. The fluorescence and visible spectra, however, are similar to those of UCB-albumin complexes (159, 160). Porphyrins (161), hematin (158), BSP and other amphipathic organic anions (151, 154, 159, 160), fatty acids (159), steroid hormones (154, 160), and some bile salts (159) all competitively bind to the same site, albeit with lower affinity than UCB. This, and the finding that porphyrins bind in proportion to their hydrophobicity (161), suggest that hydrophobic interactions are very important, although ionic interactions contribute. Lithocholate, glutathione, and BSP-glutathione conjugates, by contrast, do not bind at this site but bind to the catalytic site on the 25 kD B-subunit, which also serves as a much weaker, secondary binding site for the other ligands (157, 159, 162) (K_a for UCB about 3×10^5 M^{-1}). It has been reported that binding of porphyrins (161), but not of UCB (162), to the

primary site on subunit A, decreases the catalytic activity of subunit B.

Z-protein differs from ligandin in having an M_r of only 17 kD and more β-sheet than α-helix structure (163). It has no glutathione-S-transferase activity, but seems to play a major role in the hepatic clearance and esterification of long-chain fatty acids (164). It has only one primary ligand binding site, shared by the same compounds that bind to ligandin (151, 163). The affinity for UCB is 2×10^7 M^{-1} at pH 7.0 (160); variations in binding with pH have not been studied. Fluorescence, visible, and CD spectra strongly resemble those of the UCB-albumin complex, but, unlike ligandin, the ellipticity of bound UCB has the same R-chirality as UCB bound to albumin (160, 163, 165).

Membranes and phospholipid vesicles

The plasma membrane, which separates the external and internal domains of each cell, is a lipid bilayer composed of various classes of phospholipids, into which are inserted cholesterol and many integral proteins (166). The varied enzymatic and transport activities of these proteins maintain the intracellular environment quite constant, despite heavy traffic of multiple compounds across the membrane.

The association of UCB with liposomes or phospholipid dispersions, used as models of membranes, varies among classes of phospholipids. Thus, at pH 7.4–7.5 at which H_2B constitutes at least 75% of the unbound UCB species (Fig. 3), affinity constants for UCB with phospholipids are in the range of 10^5 to 10^6 M^{-1}, in the order sphingomyelin > lecithin > > cephalin > phosphatidylserine > phosphatidylethanolamine > cardiolipin > lysolecithin (141, 167, 168); binding affinities for natural brain and skin lipids are in the same range (141, 168). Among lecithins (phosphatidylcholines = PCs), binding affinity is in the order: egg PC (mainly 1-palmitoyl,2-oleyl PC) > dipalmitoyl PC > dimyristoyl PC > dioleyl PC (167, 169). UCB binding has been found to be enhanced by addition of a small percent of cholesterol to PC or phosphatidylethanolamine vesicles (141, 168), but to be diminished by treatment with neuraminidase or phospholipases (170).

Binding of UCB to lecithins varies among ionic species of UCB (8). Studies that report dissociation of bound B^{2-} as pH is decreased from the alkaline range (141, 171) are, however, confounded by precipitation of H_2B as the pH decreased. All other reports demonstrate that, as pH decreases, there is increased distribution of UCB into membranes of erythrocytes (170, 172–175), mitochondria (174, 176–178) and tissue culture cells (82, 179–182), as well as phospholipid vesicles (141, 171, 183, 184). These findings are compatible with binding of UCB to phospholipids and membranes by a mechanism akin to partition of H_2B into chloroform (3, 167), involving distribution of the soluble H_2B species from aqueous solutions to membranes, without formation of precipitates. Indeed, if it is assumed that H_2B is the species that is preferentially bound, application of the most reliable pK'a values for UCB (3) closely predicts the reported marked increase in UCB binding to egg PC vesicles as pH decreases (Fig. 10 in ref. 183).

It has been proposed that HB^- is the form that diffuses across membranes, principally from a postulated pool of HB^- concentrated at the aqueous/lipid interface (174), but the decreasing proportion of unbound HB^- with decreasing pH (Fig. 3) seems incompatible with this hypothesis. It is agreed that the $-COO^-$ groups of B^{2-} exhibit hydrogen bonding (171) or ionic bonding (141) to the polar headgroups of phospholipids in the outer leaflet of the bilayer, where B^{2-} is bound.

Thus, studies performed with highly purified UCB and phospholipids, without supersaturation of the unbound UCB, strongly imply preferential equilibrium binding of H_2B. Infrared (185) and ^{1}H-NMR (171) spectroscopy, as well as calorimetric studies (171) of UCB-phospholipid vesicles at pH 7.0–7.4 have indicated that H_2B may be buried among the alkyl (polymethylene) chains of PC. No UCB interaction was detected with water at the surface, polar carboxyl or -C-N- groups, or the midplane of the bilayer (171, 185), but the NMR study found interaction with the terminal-CH_3 groups of the alkane chains (171), whereas the infrared study did not (185). It was proposed that UCB solubility in the bilayer is determined mainly by the physical space available among the alkyl chains, which could be expanded by addition of rhodopsin, a protein that does not bind UCB (185). Insertion of H_2B, with its semipolar sectors (Fig. 2), deep into the hydrophobic environment of the bilayer, however, might be an unstable condition. Indeed, studies with fluorescence-quenching (141), gel exclusion chromatography (171), and calorimetry (171) indicate that the H_2B molecule gradually migrates to the exterior surface of unilamellar PC vesicles when the temperature is raised above the gel-to-liquid crystal transition point. This suggests that the true equilibrium condition in vivo is binding of H_2B to the surface or hydrophobic-polar interface of vesicles and biological membranes (186), which, because of their content of unsaturated fatty acyl groups, have transition temperatures below 37°C and are therefore in a liquid crystalline state at mammalian body temperature (187, 188). These findings are consistent with models of solubilization of many slightly polar compounds in lipid assemblies, such as micelles (69–72).

The exact structural relationships of H_2B with membranes need to be determined at physiologic pH values and undersaturation with H_2B. More studies with advanced spectroscopic techniques are needed, using vesicles that contain cholesterol as well as phospholipids, and evaluating the effects of added membrane proteins on the binding. It should be noted that, in studies of distribution

of UCB between albumin and cell membranes, integral membrane proteins render the binding process more complex, and may account in part for the effects of pH and differences among natural membranes (180).

Bile components: bile salts, micelles, and mixed micelles

UCB interactions in native bile have not been well studied, as ultracentrifugation or gel exclusion chromatography of bile (189–191) effectively assess only the dominant bilirubin conjugates. Solubility of UCB has been studied in model buffered aqueous systems, with or without selected bile salts ± added lecithin and/or cholesterol. Incubations using either dissolution of crystalline H_2B (1, 1, 2, 8, 39, 41, 42, 46–48), or rapid acidification of dissolved Na_2B (1, 2, 8, 43–45) in buffered aqueous systems, have generally shown that increases in pH values and/or bile salt concentrations enhance the overall solubility of UCB. There is, however, wide disagreement concerning the absolute solubilities of UCB in these systems at various pH values (2). These discrepancies are in large part due to spuriously low solubilities from inadequate incubation times for equilibration of crystals, and spuriously high solubilities from metastability when dissolved Na_2B is acidified rapidly (3). When incubations are performed for 72 h or more, solubilities of UCB crystals in 50 mM taurocholate (TC) (2) agree closely with values obtained by gradual precipitation of UCB from alkaline solution of Na_2B upon very slow titration with HCl (8, 47). Addition of lecithin to bile salt solutions decreases the equilibrium solubility of UCB by at least 50% (8, 41, 47, 192), but cholesterol has no additional effect on UCB solubility in mixed lecithin-bile salt micelles (8, 47, 192). By contrast, metastable solubilization by bile salts of acidified Na_2B is little affected (43–45), or modestly increased (192) by added lecithin but decreased by added cholesterol (43–45).

The above data, however, are not ideal, due to the aforementioned problems inherent with studies involving dissolution of H_2B crystals or acidification of Na_2B: *a)* degradation of pigment during the prolonged incubations required for equilibration; *b)* variations in solubility with crystal size and orderliness; and *c)* metastability (2, 3). The improvements afforded by solvent partition, including brief equilibration periods and avoidance of the vagaries of crystal effects (193), yielded quite different results (194). On partition of UCB from chloroform into buffered aqueous solutions of 50 mM TC, UCB solubilities were 2–10% of those obtained by crystal dissolution (2) (Fig. 4), and, up to pH 7.9, were below the maximum UCB concentrations of 10 μM in normal human hepatic bile and 35 μM in normal human gallbladder bile (195). Using the rationale discussed earlier (Fig. 6), it was calculated that, in 50 mM TC, the ratio of bound to unbound species at any pH was 717 for B^{2-}, 13 for HB^-, and 1.4 for H_2B (194). B^{2-} was virtually completely bound, and was the dominant overall species above pH 7.0. HB^- was bound much less avidly, and was therefore the dominant unbound anion throughout the pH range of bile. H_2B was least well bound and was the dominant unbound species in the pH range from 6.0 to 8.0, and the dominant overall species below pH 7.0. Similar results have been obtained using micellar electrokinetic capillary chromatography (196), although the differences in binding among the UCB species were less striking and the apparent pK'a values were lower. This is probably due to limitations of this technique (196), including a pH gradient which did not include the derived pK'a values, and the likelihood that the system was oversaturated with unbound UCB.

Only limited studies are available of binding to other bile salts. Binding of UCB to 50 mM taurodehydrocholate (TDHC, mostly monomers and dimers) and taurochenodeoxycholate (TCDC, 96% micellar) was less than half that with TC (80% micellar), as determined by solvent partition (194), by peroxidation of unbound UCB at pH 8.2 (197), or by micellar electrokinetic capillary chromatography (196). Preliminary data from solvent partition of [^{14}C]UCB into 50 mM bile salt solutions at pH 9.0 (198) confirmed these findings and showed also that glycine-amidated bile salts were modestly more effective than taurine amidates, and that dihydroxy bile salts, which have lower apparent critical micellar concentrations (CMCs), were about 30–50% as effective as trihydroxy bile salts. Tauroursodeoxycholate, which is quite hydrophilic, was almost as effective in binding UCB as the very hydrophobic TCDC and taurodeoxycholate (TDC). By contrast, at bile salt concentrations below their CMC, both peroxidation (197) and partition studies (198) revealed similar binding affinities of UCB for all the bile salts studied, with partition ratios less than one-tenth those in 50 mM bile salts, but ten times higher than in the absence of bile salts (3, 199).

Mechanism of UCB binding with bile salts

There are four key features of the above results: *a)* the progressive increase in binding of UCB with increased ionization, despite the increased electrostatic repulsion expected between the negatively charged HB^- and B^{2-} species and the anionic bile salts; *b)* the significant binding with premicellar bile salts (monomers and oligomers), but greater overall binding at bile salt concentrations above the CMC; *c)* the poor correlation between UCB binding and bile salt hydrophobicity; and *d)* the greater binding in the micellar range to bile salts that have higher CMCs and, therefore, a greater proportion of oligomers and monomers.

A variety of methods to evaluate the modes of interaction of UCB with bile salts have yielded conflicting interpretations as to whether the binding is primarily by hydrophobic or polar interactions, and whether the inter-

nal hydrogen bonds are disrupted or preserved in the bound pigment. Some of the conflict derives from studies with systems vastly supersaturated with UCB, which will not be considered further. The common findings of the acceptable reports are that the environment and/or conformation of the bound UCB is affected by bile salt concentration (below or above the CMC) and structure (di- vs. trihydroxy), and by the pH (the ionization state of the UCB), as described below.

The visible absorption spectra of complexes of UCB with a variety of conjugated and unconjugated bile salts, at pH values ranging from 7.9 to 10.8, reveal striking shifts in λ_{max} and increases in absorbance as the bile salt passes from premicellar to predominantly micellar concentrations below and above the apparent CMC respectively (16, 200, 201). By contrast, only a single, gradually changing spectral pattern is observed with concentrations of TDHC (16) or DHC (202) up to 60 mM, well below their apparent CMCs (203). Fluorescence spectra of trihydroxy bile salts show a similar pattern (200–202).

Binding of UCB to bile salts also produces striking bisignate Cotton effects, both below and above the CMC, suggesting that one of the UCB enantiomers is bound preferentially. In aqueous solutions of 5–34 μM UCB with premicellar concentrations of TDC, DC, cholate, and UDC, a weak R-chirality (ellipticity positive at 455–470 nm, negative at 400–415 nm) is uniformly present if the pH is $\geq$8.0 (34, 202, 204–207); the ellipticity decreases as pH increases above 8.0 (more B^{2-}). If the pH is below 7.5, the same R-chirality is present at 5 μM UCB, but a more intense, reversed S-chirality appears at 24–25 μM UCB (204, 205). These studies at pH below 7.5, however, are difficult to interpret, because the systems are at least 10-fold supersaturated with respect to UCB (150, 194, 198, 208). In methanol, in which unaggregated, protonated bile salts and H_2B are likely the only species present, complexes of UCB (25 μM) with the major unconjugated bile salts (12 mM) exhibit R-chirality (207), except with UDC and DHC, which show no chirality. The R-ellipticity in methanol increases with increasing bile acid hydrophobicity and, except for CDC, is greater for a bile acid than its methyl ester or corresponding bile alcohol (207).

Complexes of UCB with both conjugated and unconjugated dihydroxy-bile salts at micellar concentrations of 50–75 mM reveal a strong S-chirality at pH below 8.7 to 8.9, which increases as pH decreases and as UCB concentration increases (34, 204–207, 209). Interestingly, at pH 8.0, the chirality of UCB-DC complexes well above the CMC inverts from S- to R- when the ionic strength is drastically reduced (206, 207). At higher pH, by contrast, there is weak R-chirality with little change in intensity as pH increases up to 10.9 (205, 206). As there is essentially complete dissociation of B^{2-} dimers and multimers on binding to bile salts (150), the R- versus S-chirality pattern is unaffected by UCB concentration (204), and B^{2-} dimerization cannot contribute to changes in the direction or intensity of chirality at high pH. Trihydroxy bile salts show a similar pattern, but the ellipticity is much reduced and the transition from S- to R-chirality occurs at a lower pH (about 8.0) (202, 204). At pH 10.5–10.8, the ellipticities of B^{2-} in unconjugated bile salt micelles decline as hydrophobicity increases (cholate > CDC > DC) (202, 207). At micellar concentrations of DC, binding of the IVα isomer of mesobilirubin, which cannot form internal hydrogen bonds, shows a uniformly weak R-chirality over the pH range from 7.4 to 10.0, at which this isomer is fully ionized (34).

Field shifts in individual protons (202, 204, 205, 209, 210) and nuclear Overhauser effects (210) on ^{1}H-NMR spectra of both micellar and premicellar bile salt-B^{2-} complexes have indicated: *a)* hydrophobic interactions of the C18 and C19 methyl groups, on the nonpolar β-face of the bile acid molecule, with the nonpolar methyl and vinyl side-chains of UCB; and *b)* maintenance of the internally bonded, ridge-tile conformation of B^{2-} in the micelle. Changes in visible spectra of B^{2-} with added bile salts were likewise interpreted as indicating that interactions with bile salts above their CMCs were predominantly hydrophobic, but that the internal hydrogen bonds of B^{2-} were disrupted in favor of intermolecular hydrogen bonds that stabilized the complex (16, 201). Interactions of B^{2-} with TC, TDC, and TDHC oligomers below their CMCs were felt to be predominantly polar and involve hydrogen bonding (16, 207). As with albumin (131), studies of binding of the dipyrrinone, xanthobilirubic acid, suggested that each half of the UCB molecule interacted independently with a bile salt monomer (201). Gel exclusion chromatography with cholate-UCB mixtures at pH 10.5 (202), and double-reciprocal plots of UCB absorbance at 480 nm versus TC concentration at pH 7.9 (200), both suggested that the complex above the CMC contained four bile salts per UCB molecule. Comparable analyses have not been performed with dihydroxy-bile salts, which form much larger micelles (68).

The key features of all the above studies can be reconciled if both hydrophobic and hydrophilic interactions are involved, for example in the hypothetical schema shown in **Fig. 7.** The planar, nonpolar domains of each dipyrrinone of UCB could interact by hydrophobic stacking interactions with the hydrophobic β-face of the bile salt. In this schema, the anionic sidechains of UCB and the bile salt are maximally distant from each other, minimizing the charge repulsion between B^{2-} and bile salt anions. In addition, the pyrrole > NH groups of B^{2-} would be unlikely to interact with the α-OH groups of the bile salts since the latter would be directed away from the B^{2-} molecule bound to the β-face of the bile salts. By contrast, the long and flexible $-CH_2-CH_2-COO^-$ groups of UCB could hydrogen-bond with the 3α-hydroxyl groups of the

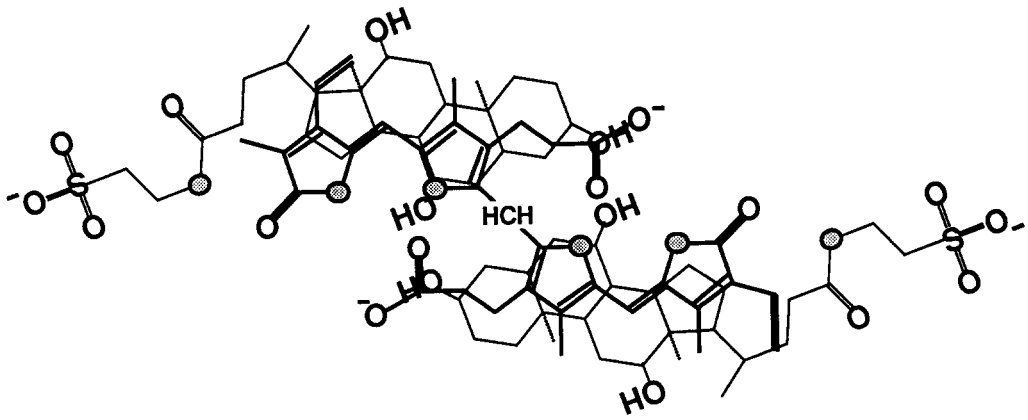

Fig. 7. Proposed model of the structure of the complex formed by hydrophobic binding of two molecules of a bile salt, taurocholate (light lines), to one molecule of bilirubin dianion (heavy lines). The filled circles are pyrrole ring >NH groups. The hydrophobic β-face of the steroid nucleus of each taurocholate molecule faces the hydrophobic domain of one of the dipyrromethenone chromophores of the unfolded, planar bilirubin dianion, which has broken all its hydrogen bonds (see Fig. 2). The C18 and C19 methyl groups of the bile salts (····) interact with methyl and vinyl sidechains of the dipyrrinones, in keeping with the findings by ^{1}H-NMR spectroscopy. The anionic sulfonate side-chains of the taurocholate molecules are maximally separated from each other and from the anionic $-COO^-$ groups of the bilirubin, minimizing electrostatic repulsion. Each $-COO^-$ group of B^{2-} hydrogen bonds with the 3α-hydroxy group, and the ring nitrogen of each central pyrrole ring overlaps the 7α-hydroxy group, of one taurocholate molecule, secondarily stabilizing the complex. Another taurocholate molecule could bind similarly to the unoccupied opposite face of each dipyrromethenone, yielding the limiting complex of four bile salts per bilirubin.

bile salts (8), but this would require breaking the internal hydrogen bonds and unfolding of UCB (16).

Such unfolding would likely be necessary if more than three bile salts were bound to each UCB molecule, because access of more than one large, rigid molecule, such as a bile salt, to the concave face of the ridge-tile form of UCB (Fig. 1), would be sterically inhibited. In the unbonded, unfolded biplanar intermediate conformation, by contrast, the UCB molecule (6, 21, 23–25), like its unbonded dimethyl ester (211), is highly flexible and both surfaces of the nonpolar regions of the UCB molecule are comparably exposed for hydrophobic interactions with bile salts (Fig. 2). Comparisons of the CD, resonance Raman, and ^{1}H-NMR spectra of UCB in a variety of solvents with those of modified bilirubins in which one enantiomer is stabilized and/or one or more hydrogen bonds are interrupted (6, 11, 14, 15, 17, 21, 25, 28, 211) suggest, however, that the internal hydrogen-bonding and folded conformation are essentially intact in the free B^{2-} species in aqueous solution at pH 9 to 11 (5, 8). On the other hand, bile salts, like albumin (see above), have preferential affinity for the unfolded UCB conformation, as they bind each dipyrrinone moiety separately (201), and the relative affinities of $B^{2-} >> HB^- > H_2B$ species for 50 mM TC (194, 196) also suggest that the unfolded conformation predominates for bound B^{2-}. The fact that the pK'a values of UCB dissolved in bile salts are lower than the pK'a values of UCB in free solution (150) indicate that the unfolded conformation is still of some importance for bound HB^- but of little importance for bound H_2B. This schema could explain the changes observed in visible spectra and ellipticity of UCB with increasing bile salt concentrations and pH, as the exciton coupling of the dipyrrinone chromophores would be diminished and the flat conformation of UCB would have little optical activity.

Although most studies of UCB-bile salt interactions have been interpreted in terms of binding of UCB monomers to preformed micelles, it is equally plausible that the B^{2-} species interacts with multiple bile salt monomers to produce hetero-aggregates that have a different structure from bile salts self-associated in simple micelles. Similar arguments have been presented for formation of aggregates between bile salts and a fluorescent planar dye, TNS (70, 212). Mathematical modelling of changes in binding of B^{2-} as TC concentration increases (208), assessed by partition from $CHCl_3$, suggests that fewer TC molecules are involved in the mixed (TC_nB^{2-}) complex than the five or six molecules that compose the simple TC_n micelle (68), and that there is successive binding of TC (or cholate) monomers to the B^{2-} molecule, forming mixed complexes (TC B^{2-}), (TC_2B^{2-}) and (TC_4B^{2-}), as proposed by others (210). The limiting complex has the same molar stoichiometry (4 TC:1 UCB) deduced from other studies (200, 202). Similar patterns of changes are observed in visible (16, 200, 201), CD (34, 202, 206, 207), and fluorescence spectra (201, 202) of UCB as concentrations of bile salts are increased above their CMC. In those studies, however, as increasing concentrations of bile salts are added to a fixed concentration of UCB, the activity of the unbound UCB progressively declines, which may contribute to the decreased increments in spectral intensity seen at high bile salt concentrations. In the partition

method, by contrast, the activity of unbound UCB is maintained essentially constant at all bile salt concentrations.

This scheme could also account for the comparable hydrophobic binding of B^{2-} to monomers of the six common bile salts, but less avid binding of UDC and DHC and their conjugates (197, 198, 207), due to incursion of the β-OH and keto groups, respectively, on the hydrophobic face of these latter bile salts. Above the CMC, the higher binding affinities of UCB for tri- versus dihydroxy- and tauro- versus glyco-amidated bile salts (198) would result from the greater availability of bile salt monomers when CMCs are higher. The system is thus viewed as competition for bile salt monomers between self-aggregation into simple micelles and heteroaggregation with B^{2-}.

Our schema differs from that proposed by others (16, 204, 205, 207, 209) in which the convex face of the folded UCB ridge-tile lies athwart the concave face of bile salt monomer, with their interactions stabilized by van der Waals forces and by hydrogen-bonding between the -COOH groups of UCB and the -OH groups on the polar α-face of the bile salt. The interaction of UCB with bile salt micelles is then visualized as hydrophobic binding of the concave face of the rigid, folded UCB molecule to the hydrophobic β-face of a bile salt exposed on the surface of helical micelles. We feel, however, that this alternative schema is unsuitable to explain the binding of B^{2-} as compared with H_2B; *a)* it does not account for the markedly stronger binding of B^{2-}, as hydrogen-bonding by ionized $-COO^-$ groups should be less than protonated -COOH groups; *b)* it does not account for the similar R-chirality of complexes of B^{2-} with bile salts at both premicellar and micellar concentrations; *c)* it does not account for the striking decrease in enantioselectivity (chirality) of B^{2-} at high pH as compared with H_2B at neutral pH; *d)* it does not minimize electrostatic repulsion between the anionic sidechains of the B^{2-} and bile salts by maximizing their spatial separation; *e)* it does not minimize exposure of the hydrophobic domains of B^{2-} or the bile salts to the aqueous environment; *f)* it does not account for the evidence, from ^{1}H-NMR studies, that the intermolecular interactions at premicellar as well as micellar bile salt concentrations are dominated by hydrophobic binding of the C18 and C19 methyl groups on the bile salts with methyl and vinyl sidechains on B^{2-}

Resolution of the many questions concerning interactions of UCB with biliary components await additional studies of: *a)* a variety of bile salts of varied hydrophobicity and structure, at premicellar as well as micellar concentrations; *b)* UCB analogues with modified -COOH and lactam groups that alter hydrogen-bonding and conformation; *c)* mixed micelles and vesicles containing lecithins with various fatty acyl substituents; and *d)* biliary proteins. Experiments in aqueous solutions, at physiologic ionic strength and pH values, might render the experimental data more applicable to the in vivo situation.

FUNCTION

General considerations

Apart from its probable role as an antioxidant (213), UCB has no known beneficial function (214). Its harmful effects, however, are well appreciated (215), especially bilirubin encephalopathy in the neonatal period (215–217), and precipitation of calcium bilirubinates in gallstones (218–220). These clinical phenomena, as well as mechanisms of hepatic uptake, will be addressed in terms of the physico-chemical considerations discussed above.

Bilirubin encephalopathy (kernicterus)

In vivo, even during mild, physiological neonatal hyperbilirubinemia, unbound UCB diffuses across the blood–brain barrier and deposits diffusely in the central nervous system (brain icterus) (215, 216). At higher UCB levels, which might saturate the binding capacity of albumin, deposition of UCB into the central nervous system increases, with precipitation of H_2B within particular neuronal regions (kernicterus), resulting in neurologic deficits (215, 216).

It has been proposed (40) that, if the total UCB concentration is sufficiently high and the pH is sufficiently low, plasma becomes markedly supersaturated with unbound H_2B, which then aggregates and precipitates in the phospholipid membranes of brain neurones (39, 141, 171, 180, 181, 221–223). This hypothesis, however, is based upon outdated data regarding UCB solubility and ionization (39, 42, 84), which concluded that the aqueous solubility of UCB at pH 7.4 was below 10 nM and that B^{2-} was the only bilirubin anion present in physiological systems. Newer data (3) suggest, rather, that maximum aqueous UCB solubility at pH 7.4 is actually closer to 100 nM (Fig. 4) and that there is little unbound B^{2-} present at this pH (Fig. 3). If the dissociation constant for binding to the first site on plasma albumin is 1.5×10^{-8} M (39, 96, 101–103, 142), then, at a normal plasma albumin concentration of 0.6 mM and a normal maximum plasma UCB concentration of about 20 μM (224), the concentration of unbound UCB must be about 0.5 nM, of which over 80% is H_2B at pH 7.4 (Fig. 3). Thus, as already reported (Fig. 50 in ref. 78), normal plasma is under-saturated with unbound UCB, and would become saturated only if the total UCB concentration increased more than 20-fold above normal (to >400 μM), or UCB was extensively displaced from albumin by binding of other molecules.

Development of kernicterus requires excessive tissue binding and/or uptake of unbound UCB, but it is disputed whether UCB enters the brain as H_2B (179, 225), as in-

soluble aggregates of H_2B (39, 40, 63, 141, 222), or as HB^- (174). As noted earlier (see Membrane components), binding of UCB to vesicles (183) and the proportion of unbound H_2B (Fig. 3) (3) increase in parallel as pH declines within the physiological range. This favors the concept that the association of UCB with membranes involves mainly H_2B. Precipitation of H_2B in membranes is unlikely, since, as in the solvent partition system (3), thermodynamic supersaturation of the organic phase (the membrane) cannot occur without supersaturation of the aqueous phase with which it is in equilibrium (plasma), and the latter rarely occurs for bilirubin in vivo (226).

Based on a linear relationship found between reversible transfer of UCB from albumin across plasma membranes of erythrocytes and rat liver mitochondria, it has been proposed that unbound HB^- is the form of UCB that diffuses into cells, principally from a postulated concentrated pool of HB^- at the aqueous-lipid interface at the external surface of the plasma membrane (174). Factors other than ionization and diffusion of H_2B, however, may have affected UCB uptake into the erythrocytes and mitochondria, such as: *a)* changes in binding of UCB to albumin with pH; *b)* the proposed protonation of HB^- as it diffused across the membrane; and *c)* proteins and UCB transporters in those membranes. The known decrease in the proportion of unbound HB^- as pH decreases is evidence against the conclusion of those studies (3). The two viewpoints might be reconciled if uptake of HB^- into a vesicle or cell involves processes different from binding of H_2B to a vesicular membrane. For example, net transport of HB^- would require associated co-transport of cations or counter-transport of anions to maintain electrical neutrality. These conflicts can only be resolved by studies with pure UCB and neuronal membranes prepared from different regions of the brain.

All three possible mechanisms are compatible with clinical (85, 215, 216, 226) and experimental (215–217, 225, 227, 228) evidence indicating that acidosis increases UCB neurotoxicity in vivo. As pH decreases, there is an increased proportion of H_2B (Fig. 3), which may be less well bound to albumin than UCB anions; indeed, except for the peroxidase method (78, 82, 84, 85, 103), experiments indicate decreased overall binding of UCB to albumin as pH decreases (29, 33, 83, 101, 135–139, 177). Among UCB species, H_2B binds best to phospholipid vesicles (183), and there is increased distribution of UCB from albumin into cell membranes in vitro as pH decreases (82, 173, 174, 176, 179–181), though there are two contrary reports (172, 182). Finally, a decrease in external pH would decrease the negative transmembrane (out to in) proton gradient, promoting contransport of protons and HB^-, in spite of a lesser external concentration of HB^-.

The apparently lower affinity and capacity of fetal albumin for UCB (106, 107) probably contributes to the risk of deposition of UCB in the tissue in jaundiced neonates. Binding of UCB to albumin may be decreased further by co-binding of various drugs (39, 78, 79, 84–86), volatile anesthetics (32), and fatty acids (30, 33, 36, 79, 85, 91, 96–99, 229), through either competitive displacement of UCB or allosteric induction of conformational changes in albumin. These effects would aggravate kernicterus, as is indeed the case (215). The effect of chloroform on albumin conformation (32) invalidates use of this solvent to assess binding of UCB to albumin by partition.

Hepatocellular uptake

Uptake may occur by two types of processes, one due to passive diffusion and the other linked to specific carriers (membrane transport proteins) (230). The driving force for diffusion across the membrane is a gradient in chemical potential for H_2B or electrochemical potential for HB^-, whereas carrier-mediated transport is regulated mainly by the affinity of the substrate for the membrane carrier protein(s) (230). Concepts concerning UCB transport across plasma membranes have been obtained largely by inference, due to difficulties in working with the poorly water-soluble and unstable UCB. Rather, studies have been performed with related amphiphilic organic anions (such as bromosulfonphthalein, BSP), which are believed to enter the liver cell by mechanisms similar to or identical to UCB (9, 10, 231–234). This assumption, however, requires direct confirmation, as, among apparently related organic anions, differences in molecular structure appear to influence hepatic uptake mechanisms (232).

The concept that UCB uptake into any cell (including hepatocytes) may occur via passive diffusion has been supported by data indicating that pure lipid bilayers, composed of protein-free phospholipids of varied fatty acid composition, may internalize UCB (169, 185). As noted earlier, UCB is buried among the alkyl chains of PC, whose packing can be altered by addition of membrane proteins that do not bind UCB (169, 185). To enter the membrane, UCB must be dissociated from plasma albumin (146). When the rates of UCB interactions with DOPC vesicles are studied in the presence of albumin, the rate of movement of UCB across the bilayer is faster than the rate of dissociation of UCB from albumin (143). This indicates that the off-rate of UCB from albumin, rather than the velocity of transmembrane passage, is rate-limiting, in agreement with kinetic data obtained in isolated, perfused rat liver (146).

These results suggest that UCB movement across artificial membranes, driven simply by transmembrane gradients of the unbound ligand (143, 235), is sufficiently rapid to account for the hepatocytic uptake of UCB without invoking specific carrier molecules. Physiological plasma membranes, however, are more complex, as they contain cholesterol, multiple classes of phospholipids, and integral proteins with specialized functions (166). It is, therefore, tenuous to extrapolate the data obtained with

simple PC vesicles to in vivo transport processes. Although the relevant studies were performed at physiological pH values, at which unbound H_2B is dominant, it is not known whether this species, rather than HB^-, is primarily involved in passive transfer across the membrane (174). It is generally assumed that the most hydrophobic species will most readily diffuse across lipid membranes, which favors H_2B as the species involved. If so, the ionization data in Fig. 3 indicate that as extracellular pH decreases the concentration of H_2B, and therefore diffusion of UCB into the cell, should increase.

Several lines of evidence indicate, nonetheless, that saturable, carrier-mediated processes also participate in hepatocellular uptake of UCB (**Fig. 8**) (231). Studies with BSP, which competes with UCB for transport, have indicated the presence of at least three distinct putative carrier proteins in the basolateral domain of the hepatocyte plasma membrane (for details, see refs. 236–238). For BSP (233), as well as for the related organic anions, tetrabromosulfonephthalein (TBS) (234) and thymol blue (239), part of the uptake process is electrogenic. This component appears predominant at low substrate concentrations, whereas, at higher concentrations, an electroneutral transport process becomes dominant (240). Uptake of TBS (234) and thymol blue (239) by basolateral liver plasma membrane vesicles decreases as external pH decreases, indicating that the charged monoanion species is transported. The inverse relationship between uptake and intravesicular pH, on the other hand, indicates that the monoanion is protonated inside the vesicle, maintaining the inwardly directed chemical gradient by consuming (protonating) the transported monoanion (174) and thus overcoming the electrical gradient (negative inside). By analogy, HB^- is probably the UCB species transported by the electrogenic component, and presumably also by the electroneutral component, for which electroneutrality is achieved by countertransport of a negatively charged species, possibly OH^- (232, 241). To confirm these hypotheses, studies are needed of uptake of UCB by liver plasma membrane vesicles or isolated hepatocytes, below saturating concentrations of UCB, over a range of external and internal pH values.

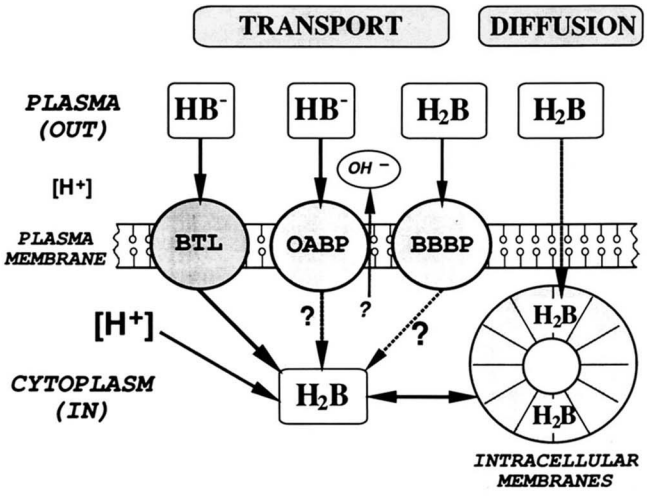

Fig. 8. Schematic of the possible mechanisms of passage of bilirubin from plasma across the basolateral plasma membrane of the hepatocyte into the cytoplasmic compartment, based on analogy from studies with BSP. The membrane-bound organic anion transporters, BTL (bilitranslocase, left) and possibly OABP (organic anion binding protein, center), would preferentially transport the bilirubin monoanion, HB^-. This is in part protonated to H_2B at the lower pH of the cytoplasm, favoring interaction with intracellular membranes, such as the smooth endoplasmic reticulum, where conjugation occurs. H_2B might be transported by BBBP (bilirubin-BSP binding protein,right) and can also diffuse passively across the lipid bilayer of the membrane (far right).

Hepatocellular storage and reflux

The binding of UCB to ligandin and Z-protein in the cytosol has been shown clearly to trap UCB within the hepatocyte, limiting its reflux back into plasma, and thus enhancing net hepatocellular uptake (242, 243). Induction or inhibition of ligandin synthesis with various drugs and hormones is associated with parallel changes in net hepatic uptake of organic anions (244, 245), and low levels of ligandin in the neonatal liver likely contribute to the "physiological" hyperbilirubinemia of the newborn (246–248).

Although HB^- and H_2B seem to be the species transported across the basolateral plasma membrane of the hepatocyte (10), it is not known which UCB species binds preferentially to ligandin and Z-protein. The similarities of binding of UCB to these cytosolic proteins and to albumin (84) have suggested that it might be B^{2-}, but there are also many differences. Most importantly, the increase in UCB binding to ligandin with decreasing pH suggests that either HB^- and/or H_2B are bound in preference to B^{2-} (160). A possibility to be considered is that one protein preferentially binds HB^- and the other binds H_2B. Binding of the poorly soluble H_2B would seem particularly important to prevent its precipitation and toxicity.

Hepatic conjugation and excretion

Although both conjugated and unconjugated bilirubins are equally well taken up by the hepatocyte (249, 250), conjugated bilirubins, formed from UCB in the endoplasmic reticulum (251), constitute over 99% of bilirubins in bile (195); UCB-IXα-Z,Z is secreted minimally (252). Studies of the excretion of a wide variety of UCB analogues by normal and mutant UDP-glucuronosyltransferase-deficient Gunn rats have revealed the structural requirements for conjugation and biliary secretion of bilirubins (7). For biliary secretion, at least one unbonded, ionized $-COO^-$ group is required, as exists in bilirubin glucuronides. For glucuronidation, by contrast, at least one internally bonded –COOH group is required. Thus, both UCB and bilirubin monoglucuronide (BMG) can be conjugated. As the β and δ side-chain isomers and

Z,E photoisomers retain one internally bonded –COOH and one unbonded –COO^-, they can be conjugated by normal rats as well as secreted without conjugation by glucuronosyltransferase-deficient Gunn rats and neonates (7, 253–255). The latter is the fundamental mechanism of phototherapy of unconjugated hyperbilirubinemia (65, 256, 257).

UCB and BMG are conjugated at comparable rates by hepatic microsomes in vitro (258), even though it might be expected that UCB would be favored due to: *a)* the presence of two internally bonded –COOH groups in UCB, as opposed to only one in BMG; and *b)* better diffusion of the less polar UCB from the cytosolic compartment across the microsomal membrane into the cisternal space where the UDP-glucuronosyltransferase is located (251). By contrast, UCB administered in vivo is more extensively conjugated than BMG (249). This may be related to the finding that, as compared with transfer from ligandin (258), membrane-to-membrane transfer enhances UCB conjugation by microsomes (184, 259), which would favor the more membrane-soluble H_2B as compared with BMG. It is not known which UCB species is conjugated. The pH optimum of the transferase is 7.8 (260), a pH value at which H_2B and HB^- are equally prevalent among unbound UCB species (Fig. 3), but H_2B is dominant at the physiological intracellular pH of 7.0. The enhanced membrane-binding and greater internal hydrogen-bonding of H_2B as compared with HB^- might also favor the former as the substrate for conjugation.

Precipitation of bile pigments in gallstones

Bile pigments are present in cholesterol (220) as well as pigment (219, 261) gallstones, mainly in the form of calcium bilirubinate salts and their polymers rather than UCB. The calcium salts in the stones consist mainly of $Ca(HB)_2$ rather than CaB (220, 262, 263). The data on UCB ionization and solubility (Figs. 3 and 4) (3, 150) can explain these findings. The calcium salts of bilirubin conjugates, like the conjugates themselves, are quite soluble (264, 265). Hydrolysis of the highly soluble bilirubin conjugates that predominate in bile is the major mechanism for the increased concentrations of UCB found in bile of patients with pigmented gallstones (266, 267).

In simple buffered aqueous systems at pH 6 to 8, solubilities of UCB are less than 1% of the maximum concentrations found in normal human gallbladder bile (35 μM) or hepatic bile (10 μM) (195). This indicates that UCB must be solubilized by interaction with other components of bile, principally bile salts (2, 8, 41, 43–47, 150, 197, 198, 219, 220, 261), but it is unclear whether those interactions are sufficient to fully solubilize the UCB in bile. The fact that UCB is found in gallstones only as its calcium salts, and not as H_2B (219, 220, 261–263), suggests that bile is not supersaturated with UCB per se, and/or that antinucleating factors for UCB are present in bile (220). It indicates also that the solubility products of calcium salts of bilirubin anions, and therefore the unbound concentrations of Ca^{2+}, HB^-, and B^{2-}, must be major determinants of the precipitation of pigments in bile (218–220, 261). As the major unbound anion in bile is HB^- and there is little B^{2-} present, one would expect mainly the $Ca(HB^-)_2$ salt to be present in gallstones, as is actually the case (219, 220, 261–263). Clearly, the ion products of both the $Ca(HB)_2$ and CaB salts, and therefore their relative saturation in bile, would increase with increasing bile pH, so that defective acidification of bile in the gallbladder might promote supersaturation and precipitation of calcium bilirubinates, as it does for calcium carbonates (218, 268–270). The actual activities of unbound HB^- and B^{2-} in the presence of bile salts and lecithin, and the solubility products of the calcium bilirubinates, remain to be determined.

CONCLUSIONS AND PERSPECTIVES

The key concepts of this review are that UCB exists as three species with different degrees of ionization (H_2B, HB^-, and B^{2-}) and that their relative concentrations, as determined by pH, play a crucial role in explaining the varied behavior of UCB in different body fluids and tissues. Physiologically, each species exists in two states (Fig. 6): unbound (free), and bound to ligands such as plasma albumin, membrane phospholipids and carrier molecules, cytosolic ligandin and Y-protein, and biliary bile salts, micelles, and vesicles. The unbound (free) species are the intermediaries in the distribution of UCB among the different ligands, as determined by the binding affinity of each UCB species for each ligand.

In this review, these concepts have been applied to explain aspects of the toxicity, transport, and biliary precipi-

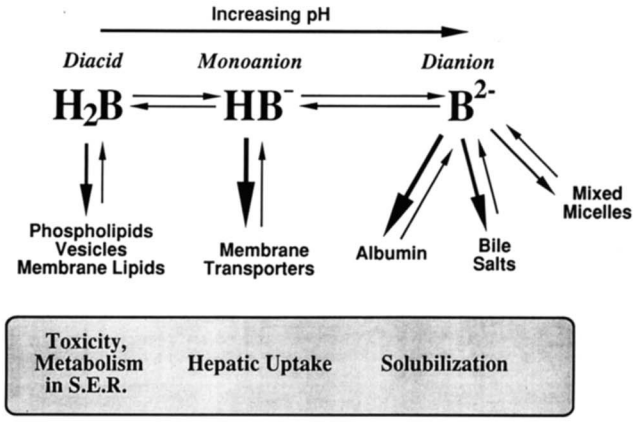

Fig. 9. Schematic of the preferential heteromolecular interactions of the three species of unconjugated bilirubin, showing below the functional role subserved by each interaction. As in Fig. 8, cytosolic binding proteins are not shown as it is not known which UCB species is bound by them.

tation of UCB. As illustrated in **Fig. 9,** H_2B is the species primarily involved in interactions with phospholipid vesicles and membranes. Thus, an increased concentration of H_2B, as a result of a decrease in pH, would favor passive distribution of UCB into membrane lipids. If the cell is not able to bind or metabolize H_2B, the accumulation of excess pigment may damage the cell, as is the case in bilirubin encephalopathy (kernicterus). An increase in pH is associated with increased proportions of HB^- and B^{2-} at the expense of H_2B. HB^- is the predominant unbound ionized species throughout the physiological pH range of 6.0 to 8.0. Based on indirect evidence from related organic anions, HB^- is likely the species most readily transported into the hepatocyte by carrier-mediated mechanisms (Fig. 9). As pH increases above 7.2, B^{2-} appears as a small but increasing fraction of the unbound UCB. Evidence suggests that this species is preferentially bound to albumin in plasma and to bile salts and mixed micelles in bile (Fig. 9), and that the bound B^{2-} may be in a nearly planar configuration, in which the internal hydrogen bonds are ruptured and the ridge-tile has been unfolded. This binding of B^{2-} accounts for the high total concentrations of UCB that can be normally dissolved in plasma and bile without precipitation.

Some of these concepts have been proven experimentally, while others await confirmation. In particular: *a)* it is not known which species preferentially bind to ligandin, Y-protein, and bilirubin glucuronosyl transferase; *b)* the conflicting data regarding the nature of interactions between B^{2-} and bile salts requires resolution; and *c)* studies are needed of the hepatocytic transport of UCB itself, rather than surrogate organic anions like BSP. Many published studies of UCB interactions with other molecules and lipid aggregates, especially below pH 8.0, need to be redone without supersaturation of the system with UCB, to avoid confounding microprecipitation of the pigment.

The "trinity of UCB species" has much more importance than the "trinity of isomers" in explaining many of the physicochemical properties and functions of UCB. This means that variations with pH should be included in any studies dealing with UCB, in order to appreciate which species of the trinity is primarily involved in the observed phenomena.

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