Self-Association of Unconjugated Bilirubin-IX α in Aqueous Solution at pH 10.0 and Physical-Chemical Interactions with Bile Salt Monomers and Micelles

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(Received 16 October 1978)

Spectrophotometric measurements of bilirubin-IX α in water and in aqueous/organic solvent mixtures at pH 10.0 as a function of bilirubin-IX α concentration (approx. 0.6–400 μ M) are consistent with the formation of dimers ($K_D = 1.5 \mu$ M) in dilute (<10 μ M) aqueous solution and further self-aggregation to multimers at higher concentrations. Added urea (to 10M) and increases in temperature (to 62°C) obliterate the dimer–multimer transition at 10 μ M, but added NaCl (to 0.30M) promotes strong aggregation of dimers over a narrow concentration range, suggesting a ‘micellization’ phenomenon. Concentrations of dioxan or ethanol greater than 60% (v/v) in water were required to obtain the absorption spectrum of bilirubin-IX α monomers, suggesting that both hydrophobic and electrostatic (π -orbital) interactions are involved in stabilizing the dimeric state in water. Micellar concentrations of sodium dodecyl sulphate induced spectrophotometric shifts in the dimer absorption spectrum of bilirubin-IX α consistent with progressive partitioning of bilirubin-IX α monomers into a relatively non-polar region of the micelles and allowed a deduction of the apparent critical micellar concentration that closely approximated the literature values. The pattern of bilirubin IX α association with bile salts is complex, since the absorption spectrum shifts hypsochromically below and bathochromically above the critical micellar concentration of the bile salts. Consistent with these observations, bilirubin IX α appears to bind to the polar face of bile salt monomers and to the polar perimeter of small bile salt micelles. At higher bile salt concentrations some bilirubin-IX α monomers partition into the hydrophobic interior of the bile salt micelles. Our results suggest that under physiological conditions the natural conjugates of bilirubin-IX α may exhibit similar physical chemical properties in bile, in that dimers, highly aggregated multimers and bile salt-associated monomers may co-exist.

Bilirubin-IX α found in normal bile is predominantly conjugated with glucuronic acid (Fevry *et al.*, 1972; Gordon *et al.*, 1977; Blumenthal *et al.*, 1977). Unconjugated bilirubin-IX α (bilirubin-IX α), a major component of gallstones (Toyoda, 1966; Trotman *et al.*, 1977), represents only a minor component (1–5% of total bilirubin) of bile (Blumenthal *et al.*, 1977; Boonyapisit *et al.*, 1978; Goresky *et al.*, 1978), but constitutes a major component of total bilirubin pigments in patients with hepatic UDP-glucuronyltransferase deficiency (Fevry *et al.*, 1977) and in jaundiced neonatal patients during phototherapy (Lund & Jacobsen, 1972). In spite of a vast body of literature on the chemistry, biochemistry and metabolism of bile pigments (see recent review by McDonagh, 1979), relatively little is known about the physicochemical state of bilirubin-IX α or its

conjugates in aqueous systems. Near physiological pH, bilirubin-IX α is extremely insoluble in water (Overbeek *et al.*, 1955; Brodersen & Theilgaard, 1969; Nakama, 1976; Kolosov & Shapovalenko, 1977) and only slightly soluble in bile salt and in bile salt/phosphatidylcholine micellar systems (Nakayama & Nishimura, 1969; Nakama, 1976; Ostrow *et al.*, 1977). The poor aqueous solubility of bilirubin-IX α was considered for many years on the basis of spectroscopic, model building and general chemical considerations to reflect a molecular conformation in which all the hydrophilic regions of the molecule were involved in intramolecular hydrogen-bonding, with the result that the polar groups were enclosed in a hydrophobic shell (Kuenzle *et al.*, 1973; Mannito *et al.*, 1974; Knell *et al.*, 1975). The precise conformation of crystalline bilirubin-IX α was solved by X-ray analysis (Bonnett *et al.*, 1976, 1978) and this prediction was confirmed. Each carboxylic acid group of bilirubin-IX α forms three intramolecular hydrogen bonds, involving the pyrrole imino hydrogen atoms and terminal lactam

Abbreviation used: SDS, sodium dodecyl sulphate.

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systems of the opposite pyrromethenone skeleton (Bonnett *et al.*, 1976, 1978). This secondary structure stabilizes a chiral conformation in which the molecule adopts the form of a 'ridge tile' (Bonnett *et al.*, 1976, 1978), and thus bilirubin-IX α is rendered a non-polar lipid. The dramatic change in conformation and in aqueous solubility on protonation explains why the physical state and ionization constants of bilirubin-IX α under physiological conditions have eluded precise measurement by conventional methods (Lucassen, 1961; Brodersen, 1966; Matheson *et al.*, 1974; Krasner & Yaffe, 1973).

For these reasons we decided to first investigate the solution properties of bilirubin-IX α at a pH (10.0) where the carboxylic acid groups are fully ionized and the compound is moderately water-soluble. Our spectrophotometric measurements suggest that soluble bilirubin-IX α self-associates in a manner reminiscent of that of many water-soluble dyes (Hillson & McKay, 1965), porphyrins (Phillips, 1963) and certain steroid amphiphiles (Carey & Small, 1970; Carey *et al.*, 1975), in that dimers are formed in dilute solution and these in turn self-associate to form multimers as the concentration is increased. Comparing the effects of anionic detergents of different molecular structures, we found that bilirubin-IX α interacts with both monomers and micelles of bile salts, but only with micelles of SDS, a typical anionic detergent, suggesting a complex pattern of both polar and hydrophobic interactions that may have analogies in native bile.

Experimental

Materials

Commercial crystalline bilirubin IX α (BDH) was obtained from Gallard-Schlesinger Corp. (Carle Place, NY, U.S.A.). By silica-gel H t.l.c. [250 μ g application; solvent system chloroform/acetic acid (49:1, v/v)] a slight base-line residue and minor amounts of the two structural isomers, bilirubin-III α and-XIII α , were found in addition to the natural IX α isomer (McDonagh & Assisi, 1971). The material was purified by the procedure of McDonagh & Assisi (1972). In brief, the crude material was dissolved in Spectroanalyzed chloroform, washed thrice at room temperature with 0.1M-NaHCO₃, dried over anhydrous Na₂SO₄, filtered and recrystallized at 4°C from a small volume of chloroform to which a few millilitres of methanol was added to induce incipient turbidity at 60°C. The precipitate was washed with cold chloroform/methanol (1:1, v/v) and dried overnight in a vacuum desiccator at 40°C. The purified material had a molar absorption coefficient (ϵ) in chloroform (23°C) of $6.19 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (McDonagh, 1979), and produced a single spot on repeat t.l.c. with authentic standards (kindly

supplied by Dr. A. P. McDonagh, University of California, San Francisco, CA, U.S.A.) with no fluorescent impurities (McDonagh & Assisi, 1971). On scanning of a 100 μ M solution in chloroform through the visible spectrum the absence of an absorption band in the 620–680 nm range confirmed that biliverdin-IX α was not a contaminant (With, 1968). As needed, bilirubin-IX α was purified in 0.5 g batches at a yield of approx. 50% and stored dry under O₂-free argon at –20°C. The sodium salts of taurocholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholanoyltaurine), taurodeoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholanoyltaurine) and taurodehydrocholic acid (3 α ,7 α ,12 α -trioxo-5 β -cholanoyltaurine) were purchased from Calbiochem (San Diego, CA, U.S.A.) and purified as described (Pope, 1967; Carey & Small, 1969). The bile salts after purification gave a single spot by t.l.c. (200 μ g application) and a correct titration curve in water (1 g/dl titrated with 2M-HCl) (Small, 1971). SDS was obtained from BDH Chemicals, Poole, Dorset, U.K. The sample was free of dodecanol as checked by t.l.c. [200 μ g application; chloroform/methanol/water (65:35:4, by vol.)] and contained 0.5% (w/w) of sodium tetradecyl sulphate as shown by g.l.c. of an acid hydrolysate (kindly carried out by Mr. Paul Missel, M.I.T., Cambridge, MA, U.S.A.), and was used after recrystallization from ethanol/water mixtures. Organic solvents and chemicals were reagent grade. NaCl was roasted at 600°C to oxidize and remove organic impurities. Argon Matheson, East Rutherford, NJ, U.S.A.) was 99.998% pure, and water was filtered, deionized and glass-distilled (Corning Automatic System, Corning, NY, U.S.A.). All glassware was sequentially washed (24–48 h) in 1M-HNO₃ and ethanol/2M-KOH (1:1, v/v), rinsed in running distilled water, wrapped in aluminium foil and oven-dried.

Methods

Solutions. Solutions of bilirubin-IX α in aqueous electrolyte solvents containing 0.02–0.30M-Na⁺ ranged in concentration from approx. 0.6 to 400 μ M and were made by dilution of stock solutions. Stock solutions were prepared by weighing the appropriate amount of recrystallized bilirubin-IX α , adding to the solid crystals a few drops of 1M-NaOH to aid dissolution and making up to the appropriate volume with a 0.01M-carbonate/bicarbonate buffer solution (Gomori, 1955) to which was added the desired Na⁺ ion concentration as NaCl. The pH of all bilirubin IX α solutions was 10.0 ± 0.2 (Research pH-meter no. 65; Radiometer, Copenhagen, Denmark) at the experimental temperatures (5–62°C). Bilirubin-IX α solutions in organic solvents or organic solvent/water mixtures were made by dilution of bilirubin-IX α from a stock solution with appropriate volumes of carbonate/bicarbonate buffer (0.01M, pH10.0) and

solvent or by the dissolution of crystalline bilirubin-IXα in the pure solvent. Solutions of bilirubin-IXα and bile salts or SDS were made from stock solutions of bilirubin-IXα in 100mM-detergent (pH 10.0 ± 0.02) at the desired Na⁺ ion concentration and dilution with a bilirubin-IXα stock solution in carbonate/bicarbonate buffer at the same Na⁺ ion concentration and pH to give various concentrations of detergent at a constant bilirubin IXα concentration.

Absorption spectra. A Cary 118C recording UV-Vis spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.) was used. Depending on the bilirubin-IXα concentration, matched glass cuvettes with path lengths ranging from 1 to 50mm were used in order to keep the absorbance readings between 0.05 and 2 units. The spectral band-width of the light used was 10–13nm per mm of slit width. The latter was variable but approximated to 0.05mm during most measurements. The experimental cuvettes and appropriate blanks were fitted to thermostatically controlled jackets in the spectrophotometer, and the temperature was controlled ± 0.05°C by recirculating water from a calibrated Haake model FE heating water bath (Haake, Saddle Brook, NJ, U.S.A.) to which was coupled a Neslab PBC-4 bath cooler (Neslab Instruments, Portsmouth, NH, U.S.A.). At temperatures other than room temperature (23°C), solutions were left to equilibrate for 4min in pre-warmed or pre-cooled cuvettes before the scanning. To minimize chemical decomposition of the bilirubin-IXα solutions, all solvents and buffers were purged with pure argon for 10min before use to remove O₂,

Fig. 1. Dependence of the Soret-band absorption maximum (λ_{max}) on total bilirubin-IXα concentration expressed as $8 + \log\{[bilirubin-IX\alpha](M)\}$

Spectra were obtained at 23°C, in three concentrations of NaCl/carbonate/bicarbonate buffer (●, 0.02M; △, 0.05M; ○, 0.25M) at pH 10.0, in an argon atmosphere within 1–2 min of mixing. The light-pathlength of the cuvettes was 1–50mm depending on the bilirubin-IXα concentration. Note that the integer (8) is used to shift the scale of the abscissa to consecutive positive values (see also Fig. 2).

Table 1. Absorption maxima (λ_{max} , nm) of unconjugated bilirubin-IXα in various solvents (pH 10.0)
The samples were constituted in glass cuvettes (1–50mm) as described in the text and λ_{max} was recorded at 23°C within 1–2min of mixing.

Bilirubin concn. (μM) ...	λ_{max} , (nm)											
	0.6	1.0	1.6	3	7	10	25	50	100	200	400	
8 + log{[bilirubin-IXα]} (M) ...	1.78	2.00	2.20	2.48	2.85	3.00	3.40	3.70	4.00	4.30	4.60	
0.02M-Na ⁺	439.9	439.9	439.9	439.9	439.9	437.8	438.5	437	436.6	435.0	434.6	
0.05M-Na ⁺	439	439	439	439.4	438	—	437.5	436	435	434.4	—	
0.10M-Na ⁺	441	441	442.2	440.6	439.1	437.5	437.5	435.3	432.8	—	429.7	
0.15M-Na ⁺	440	440	438	438	348	437	433	432.5	—	428	425.6	
0.25M-Na ⁺	437	438	437	437	436.8	437.6	435.0	435.0	432.1	430.7	423.2	
0.30M-Na ⁺	435.4	434.4	—	435.6	—	436.5	435.2	—	—	430.3	425.3	
0.15M-Na ⁺ + 2M-urea	439.0	440.2	439.6	440.0	439.6	439.0	438.8	437.5	436.5	434.8	432.8	
0.15M-Na ⁺ + 4M-urea	440.6	440.7	440.7	440.6	440.7	440.3	439.0	437.2	438.0	436.5	434.7	
0.15M-Na ⁺ + 6M-urea	442.8	443.3	442.7	443.6	443.4	443.6	442.9	443.0	442.3	442.0	441.5	
0.15M-Na ⁺ + 10M-urea	444.6	444.8	445.4	444.4	444.6	444.6	444.6	444.4	444.6	444.7	444.2	
90% Ethanol	—	456.8	—	—	—	457.4	—	—	456.9	—	—	
90% Methanol	—	450.3	—	—	—	451	—	—	—	451.5	—	
95% Dioxan	451.9	—	—	—	452.1	—	—	—	452.9	—	—	
95% Butan-1-ol	—	450.0	—	—	—	458	—	—	—	458.4	—	
95% Phenol	—	460.7	—	—	—	—	461.9	—	—	462.9	—	
Pyridine	—	—	—	450.7	—	452.1	—	—	—	455.3	—	
Chloroform	—	451	—	—	—	451.9	—	—	450.9	—	—	

and all stock solutions were bubbled with argon throughout each experiment. Both stock and experimental solutions were prepared and handled under subdued diffuse light in a windowless room, and stock solutions were wrapped in aluminium foil to further exclude light. Individual experimental solutions were studied within a few minutes of mixing and were not re-used. For each bilirubin-IX α solution, the Soret region of the absorption spectrum, from 380 to 520nm, was rapidly (approx. 25s) scanned. The curves were reproducible when run in this fashion; however, if solutions were left for longer than 10min a steady decrease in absorbance resulted owing to chemical decomposition (Lightner *et al.*, 1976; Lightner, 1977; McDonagh & Assisi, 1971). Two types of spectra were obtained: conventional spectra with a reference cuvette containing solvent without bilirubin-IX α , and difference spectra with equal concentrations of bilirubin-IX α in both sample and reference cuvettes but with added detergent (SDS or the sodium salts of taurocholic acid, taurodeoxycholic acid or taurodehydrocholic acid) in the sample cuvette.

Fig. 2. Dependence of the Soret-band absorption maximum ($\lambda_{\max}$) on total bilirubin-IX α concentration expressed as $8 + \log \{[\text{bilirubin-IX}\alpha] (\text{M})\}$ in the presence of added urea. Spectra were obtained within 1–2min of mixing at 23°C in 0.15M-NaCl/carbonate/bicarbonate buffer to which was added urea to achieve final molar concentrations: (■, 0M; △, 2M; ▲, 4M; ○, 6M; ●, 10M). The light-pathlength of the cuvettes was 1–50mm depending on the bilirubin-IX α concentration.

Results

Influence of bilirubin-IX α concentration, ionic strength (Na^+), solvent and added urea

Mean values for $\lambda_{\max}$ of bilirubin-IX α solutions (0.6–400 μM) plotted as $8 + \log \{[\text{bilirubin-IX}\alpha] (\text{M})\}$

Fig. 3. Beer's-Law plot of observed (----) and theoretical (—) absorbances versus total bilirubin-IX α concentration. Absorbances are normalized to 1 cm light-pathlength. Bilirubin-IX α concentration varied from 0.6 to 350 μM . Spectra were obtained within 1–2min of mixing at 23°C in 0.02M-NaCl/carbonate/bicarbonate buffer, pH 10.0. The inset shows an expanded Beer's-Law plot of absorbances of dilute bilirubin-IX α solutions (<8 μM) demonstrating agreement between theory and experiment. The molar absorption coefficient at 460nm ($\epsilon_{460} = 4.71 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was derived from the mean slope of the inset data.

Fig. 4. Temperature-dependence of the Soret-band absorption maximum ($\lambda_{\max}$) of bilirubin-IX α solutions. Spectra of three bilirubin-IX α solutions (●, 1 μM ; ▲, 12 μM ; ■, 290 μM) were obtained in 0.15M-NaCl/carbonate/bicarbonate buffer, pH 10.0, exactly 4 min after equilibration at each temperature. The light-pathlength of the cuvettes was 5–50mm.

Fig. 5. Soret-band absorption spectra of bilirubin-IX α in ethanol/water (a) and dioxan/water (b) mixtures

The concentration of ethanol or dioxan (% v/v) in 0.01 M-Na⁺/carbonate/bicarbonate buffer, pH 10.0, was increased in 10% increments from 0% (curves 1) to 90% (curves 10). The final bilirubin-IX α concentration in all mixtures was 13 μ M. The spectra were obtained at 23°C in 1 cm-light-pathlength cells within 1 min of mixing. Curves are numbered consecutively, with curve 1 having the lowest absorption maximum and curve 10 the highest. An isosbestic point appears at approx. 430 nm in the bilirubin-IX α /ethanol/water spectra.

(1.78–4.6) as a function of ionic strength (0.02–0.30 M-Na⁺) added urea (2–10 M) and various solvents are summarized in Table 1. Selected values to illustrate the effects of added Na⁺ and urea on the concentration-dependence of $\lambda_{\max}$ are shown in Figs. 1 and 2 respectively. In aqueous buffer the $\lambda_{\max}$ is hypsochromically shifted relative to values in organic solvents and remains constant (plateau region in Fig. 1) up to a concentration of approx. 10 μ M ($8 + \log\{[\text{bilirubin-IX}\alpha](\text{M})\} = 3$). Above this concentration a sharp hypsochromic shift occurs with increases in the bilirubin-IX α concentration. Added ionic strength shifts the plateau region to shorter wavelengths and accentuates the hypsochromic shifts that occur at higher (>10 μ M) bilirubin-IX α concentrations (Fig. 1), with maximum shifts of 10–15 nm (Table 1). In pure organic solvents and in organic solvents containing 5–10% of aqueous buffer $\lambda_{\max}$ is shifted bathochromically compared with water, but no dependence on bilirubin-IX α concentration is

observed (Table 1). With progressive additions of urea (bilirubin-IX α solutions in 0.15 M-Na⁺) all $\lambda_{\max}$ values are shifted bathochromically and the concentration-dependence of $\lambda_{\max}$ above 10 μ M-bilirubin-IX α is progressively abolished (Table 1 and Fig. 2). Plots of the absorbance of bilirubin-IX α (normalized for a 1 cm path length) at 460 nm against its aqueous concentration obey Beer's Law up to 10 μ M (Fig. 3). At bilirubin-IX α concentrations greater than this the points deviate from Beer's Law (Fig. 3) and the extent of deviation is proportional to the ionic strength (not plotted). The points that obey Beer's Law (Fig. 3, inset) give an ϵ value of $4.71 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in 0.02 M-Na⁺. The mean ϵ value from all Beer's-Law plots was $4.42 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The ϵ values from Beer's-Law plots for dilute bilirubin-IX α solution (<10 μ M) in different concentrations of added urea all fall on a straight line that extrapolates to a value of $4.37 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at zero urea concentration.

Influence of variations in temperature on $\lambda_{\max.}$ of aqueous bilirubin IX α solutions

The influence of increases in temperature (5–60°C) on $\lambda_{\max.}$ of three concentrations of bilirubin-IX α in 0.15M-Na⁺ is shown in Fig. 4. The increase in temperature produces a monotonic bathochromic shift in $\lambda_{\max.}$ of the most dilute (1 μ M and 12 μ M) solutions, whereas solutions containing 290 μ M-bilirubin-IX α display an initial hypsochromic shift between 5 and 23°C followed by a continuous bathochromic shift above 30°C. At 60°C the $\lambda_{\max.}$ of all bilirubin-IX α concentrations shifts to values similar to those for <10 μ M-bilirubin-IX α solutions at 23°C (Table 1).

Influence of variations in solvent polarity on $\lambda_{\max.}$ and ϵ of bilirubin IX α solutions

Mixtures of various proportions of ethanol or dioxan and 0.01M-Na⁺ aqueous solvent at three bilirubin-IX α concentrations (1.2, 13 and 140 μ M) were studied and the resulting spectra for the middle

concentration are shown in Fig. 5. As either the amount of ethanol or dioxan is increased, there is a progressive bathochromic shift in $\lambda_{\max.}$ and an increase in absorbance. An isosbestic point (approx. 430nm) occurs at the lower percentage of ethanol (10–30%), indicating an equilibrium between two chromophoric species. With a 140 μ M-bilirubin-IX α concentration, the colour of the solutions changed from red-orange to bright yellow in 20–30% (v/v) ethanol/water or dioxan/water mixtures.

To investigate further whether the results obtained in ethanol were primarily solvent effects or a result of a change in the physical (i.e. self-aggregated) state of bilirubin-IX α , plots of $\lambda_{\max.}$ and ϵ_{460} versus the *Z* scale (kJ/mol) of solvent polarity (Kosower, 1958*a,b*) were constructed (Figs. 6*a* and 6*b*). The *Z* scale is an empirical description of solvent polarity and the values are derived by measuring the u.v.-visible transition energy (from $\lambda_{\max.}$) of a non-aggregating reference molecule in solvents of various polarities (Kosower, 1958*a*). As it is well established (Rabinowitch & Epstein, 1941) that concentrations of ethanol in excess of approx. 60% (v/v) are adequate

Fig. 6. Dependence of (a) Soret-band absorption maximum ($\lambda_{\max.}$) and (b) molar extinction coefficient (ϵ_{460}) on ethanol/buffer polarity (*Z*)

Data derived from absorption spectra of three concentrations of bilirubin-IX α (Δ , 1.2 μ M; $\circ$, 13 μ M; $\bullet$, 140 μ M) obtained at 23°C within 1 min of mixing in 0–90% (v/v) ethanol in 0.01M-Na⁺/carbonate/bicarbonate buffer, pH 10.0. The light-pathlength of the cuvettes was 1–50mm. The *Z* values (kJ/mol) were obtained from Kosower (1958*a,b*) for various ethanol/water mixtures. In (a) the vertical broken line represents the *Z* value for water and the continuous lines connecting the upper four points represent extrapolations of $\lambda_{\max.}$ in >60% ethanol in the buffer (*Z* < 21 kJ/mol) to the *Z* value for pure water. In (b) the vertical broken line represents the *Z* value for water and the continuous lines connecting the upper four points represent extrapolations of ϵ_{460} in >60% ethanol in the buffer (*Z* < 21 kJ/mol) to that of pure water (see the text for further details).

to disrupt non-polar molecular interactions, the linear portions of the curves at high ethanol concentrations ($Z < 21$ kJ/mol) represent principally solvent effects on the absorption spectrum of bilirubin-IX α monomers (Gallagher & Elliott, 1973). Deviation from this behaviour, as shown by the sharp breaks in Figs. 6(a) and 6(b), can be attributed to bilirubin-IX α self-aggregation in the dilute ethanol/water mixtures. Therefore an extrapolation of the linear portion of the plots in Figs. 6(a) and 6(b) to the Z value of pure water (22.6 kJ/mol) gives values of 456.7 nm for $\lambda_{\max}$, and $6.91 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ϵ_{460} for monomeric bilirubin-IX α in water. As these values are very different from our observed $\lambda_{\max}$, (435–445 nm) and ϵ_{460} ($4.71 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) values for the most dilute bilirubin-IX α solutions in pure aqueous solutions, and as bilirubin-IX α in pure organic solvents such as chloroform give similar $\lambda_{\max}$ values (Table 1), we will assume for the purposes of the present discussion that in pure aqueous electrolyte solvents at concentrations below $10 \mu\text{M}$ bilirubin-IX α aggregation is restricted to dimerization.

By using the above derived value for the ϵ_{460} of monomeric bilirubin-IX α and averaged values of ϵ_{460} for bilirubin-IX α dimers (from Beer's-Law plots), a crude estimate of K_D , the dimerization constant, can be obtained as shown in the equilibrium:

where B_1 is the bilirubin-IX α monomer and B_2 is the dimer. As solutions of bilirubin-IX α in 10–40% ethanol can be construed as constituting a variable equilibrium of monomers and dimers, since an isosbestic point occurs in the spectra (Fig. 5a), the absorbance (A) may be described by the following equation:

$$A = \epsilon_{460 \text{ dimer}} C_{\text{dimer}} l + \epsilon_{460 \text{ monomer}} C_{\text{monomer}} l \quad (2)$$

where C_{dimer} is the concentration of the dimer (M), C_{monomer} is the concentration of the monomer (M) and l is the pathlength of the cuvettes (cm). By using the values $6.91 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for $\epsilon_{\text{monomer}}$ and $4.42 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ϵ_{dimer} , the values of C_{dimer} and C_{monomer} could be calculated since:

$$C_{\text{total}} = C_{\text{monomer}} + C_{\text{dimer}} \quad (3)$$

Then for each concentration (10–40%, v/v) of ethanol K_D is calculated by using the equation:

$$K_D = \frac{[\text{B}_1]^2}{[\text{B}_2]} = \frac{C_{\text{monomer}}^2}{C_{\text{dimer}}} \quad (4)$$

The results fall on a straight line, which when extrapolated to the Z value of water gives $K_D = 1.5 \mu\text{M}$. The small value of K_D obtained is thus consistent with the assumption that at the lowest concentrations studied ($< 10 \mu\text{M}$) bilirubin-IX α exists predominantly as a dimer in water and thus gives constant values of

$\lambda_{\max}$. The most probable explanation for the strong shift in $\lambda_{\max}$, that occurs at concentrations above $10 \mu\text{M}$ (Table 1 and Fig. 1) and the deviations from Beer's Law at higher concentration (Fig. 3) indicates further self-aggregation of bilirubin-IX α dimers to form multimers.

Influence of SDS on the spectral characteristics of bilirubin-IX α solutions

The Soret-band spectra of $29 \mu\text{M}$ -bilirubin IX α in SDS solutions (0–90 mM; 23°C ; 0.01 M-Na^+) are shown in Fig. 7. As the SDS concentration is increased, the $\lambda_{\max}$ of bilirubin-IX α shifts bathochromically (435 to 454 nm), and during this interconversion the absorbance is decreased and a tight isosbestic point is observed at approx. 504 nm. The influence of SDS concentration on $\lambda_{\max}$ of three bilirubin-IX α concentrations is shown in Fig. 8. The sigmoid shape

Fig. 7. Soret-band absorption spectra of bilirubin-IX α in aqueous SDS

Spectra of $29 \mu\text{M}$ -bilirubin-IX α were obtained in aqueous SDS in 0.01 M-Na^+ /carbonate/bicarbonate buffer, pH 10.0, at 23°C . The light-pathlength of the cuvettes was 1 cm. The numbered spectra correspond consecutively to SDS concentrations of 0, 0.9, 1.8, 2.7, 5.4, 9.0, 18.0, 27.0 and 90 mM. A tight isosbestic point is observed in the spectra at approx. 504 nm.

Fig. 8. Dependence of Soret-band absorption maximum ($\lambda_{\max}$) of bilirubin-IX α solutions on the final SDS concentration plotted logarithmically

The inset shows the apparent critical micellar concentrations (CMC) (mM) of SDS obtained from the first break points in the $\lambda_{\max}$ -versus-SDS-concentration curves plotted as a function of the bilirubin IX α concentration expressed as $8 + \log\{[\text{bilirubin-IX}\alpha] (\text{M})\}$. The interpolated critical-micellar-concentration value (Mukerjee & Mysels, 1970) of pure SDS in 0.15M-NaCl at 22.5°C (1.12mM) is indicated by the broken horizontal line. Spectra were obtained at 23°C (pH 10.0) by titrating 100mM-SDS in various bilirubin IX α /0.15M-NaCl/carbonate/bicarbonate buffer solutions with identical concentrations of bilirubin IX α /buffer solution. Results for three concentrations of bilirubin IX α (●, 30 μM ; ○, 82 μM ; ▲, 271 μM) are plotted on the Figure.

of these curves shows that the $\lambda_{\max}$ values are constant up to apparent critical micellar concentration of SDS (Fig. 8), and at higher concentrations $\lambda_{\max}$ increases steeply to reach nearly constant values above 20mM-SDS. The concentrations at the break points at the dilute ends of the series of curves (critical-micellar-concentration values) are plotted against the bilirubin-IX α concentration in the inset in Fig. 8. The apparent critical-micellar-concentration values are constant up to a bilirubin-IX α concentration of 10 μM , above which a linear increase occurs in proportion to the bilirubin-IX α concentration. The broken line in the inset shows the interpolated (0.15M-NaCl, 22.5°C) literature critical-micellar-concentration value (Mukerjee & Mysels, 1970). With bilirubin-IX α concentrations in excess of 10 μM , the colour of the solutions changed from deep red (no SDS) to bright yellow at SDS concentrations in excess of 10mM, in accord with changes in $\lambda_{\max}$ (Fig. 8) and analogous to the effects of progressive additions of ethanol or dioxan to bilirubin-IX α /water mixtures.

Influence of taurine conjugated bile salts on the spectral characteristics of bilirubin-IX α solutions

The absorption spectra of bilirubin-IX α (280 μM) in increasing concentrations of sodium taurodehydro-

cholate are shown in Fig. 9. The $\lambda_{\max}$ of bilirubin-IX α first shifts hypsochromically in dilute sodium taurodehydrocholate solutions followed by a slight bathochromic shoulder (at approx. 460nm) developing at the highest sodium taurodehydrocholate concentrations. A tight isosbestic point occurs at about 490nm. In three separate experiments with bilirubin-IX α concentrations of 9.5–280 μM the curves and isosbestic points are similar, with shifts in $\lambda_{\max}$ to a constant value of 422–423nm and ϵ_{460} values similar to those of bilirubin-IX α dimers in water. The spectra for two bilirubin-IX α concentrations (29 μM and 270 μM) in increasing concentrations of sodium taurocholate are displayed in Fig. 10. With small sub-micellar additions of sodium taurocholate, $\lambda_{\max}$ first shifts hypsochromically, but as the sodium taurocholate concentration is further increased a shoulder forms at approx. 465nm and develops into a peak that increases in parallel with increases in the sodium taurocholate concentration. At the highest sodium taurocholate concentration the peak is well developed and now contains a shoulder at a wavelength similar to the $\lambda_{\max}$ of bilirubin-IX α in the absence of sodium taurocholate. The principal effects of the higher bilirubin-IX α concentration (Fig. 10) over the same range of sodium taurocholate concentrations are that the extent of the initial hypsochromic shift is greater and

Fig. 9. Soret-band absorption spectra of bilirubin-IX α in solutions of sodium taurodehydrocholate

Spectra were obtained at 23°C as for SDS. The bilirubin-IX α concentration was 280 μ M. The range of taurodehydrocholate concentrations corresponding to the numbered spectra were in consecutive order 0, 0.5, 1.25, 2.5, 5.0, 10.0, 30.0 and 100 mM. The buffer was 0.01 M-Na⁺/carbonate/bicarbonate, pH 10.0. An isosbestic point is present in the spectra at approx. 490 nm.

the development of the new $\lambda_{\max}$ at longer wavelengths is less. No isosbestic point occurs in these spectra (Fig. 10).

In order to clarify further these spectral changes, difference spectra were obtained with all three bile salts, and the results for sodium taurocholate are shown in Fig. 11. Two positive peaks are observed at all concentrations of the three bile salts, both below and above their critical-micellar-concentration values. As shown in Figs. 12(a) and 12(b), at a constant bilirubin-IX α concentration (26 μ M) the shifts in both absorption maxima are strikingly dissimilar as the bile salt concentrations are increased. The shorter-wavelength peak (Fig. 12a) centred between 410.5 and 413 nm is constant up to a bile salt concentration equivalent to the individual critical-micellar-concentration values measured by other methods (Carey & Small, 1969; M. C. Carey &

A. P. Koretsky, unpublished observations on taurodehydrocholate). A strong hypsochromic shift is then observed in the case of taurocholate and taurodeoxycholate, but not with taurodehydrocholate. The initial hypsochromic shifts are reversed once the taurodeoxycholate and taurocholate concentrations exceed 3 and 50 mM respectively, whereas with taurodehydrocholate the bathochromic shift occurs monotonically at all concentrations greater than 3 mM. For the longer-wavelength peaks (Fig. 12b) the values for all three bile salts are once again constant up to the individual critical-micellar-concentration values and then increase sharply to longer wavelengths for taurocholate and taurodeoxycholate and monotonically for taurodehydrocholate. Above 3 mM-taurodeoxycholate and 10 mM-taurocholate the absorption maxima become essentially constant. The effects of increases in temperature (23 to 62°C) on the taurocholate-bilirubin-IX α difference spectra are complex (results not shown). In general, increases in temperature obliterate the hypsochromic shifts of the shorter-wavelength absorption maximum and accentuate the bathochromic shifts of the longer-wavelength absorption maximum.

Discussion

Physicochemical properties of bilirubin-IX α in aqueous systems

Like many porphyrins (Phillips, 1963), bilirubin-IX α , an open-chain tetrapyrrole, is not sufficiently soluble in aqueous solution at neutral pH (approx. 10 nM; Kolosov & Shapovalenko, 1977) to facilitate exact physicochemical examination (Overbeek *et al.*, 1955). Thus the aim of the present study was to investigate the solution properties of the disodium salt of pure bilirubin-IX α in aqueous solution at pH 10.0, 1 pH unit higher than where protonation commences and at least 1.5 pH units above its equilibrium precipitation pH values (A. P. Koretsky & M. C. Carey, unpublished work). Even under these conditions, disodium bilirubin-IX α is not very soluble. For example in water and in 0.15 M-NaCl (23°C, pH 10.0) a solution is saturated (M. C. Carey & S. J. Wu, unpublished work) with 50 mM (31.3 mg/ml)- and 36 mM (22.8 mg/ml)-bilirubin-IX α respectively, in agreement with other measurements at the same pH (Kolosov & Shapovalenko, 1977). Although the colligative properties of a system cannot be directly measured by the changes in $\lambda_{\max}$ and ϵ , considerable physicochemical insights may be gained provided that the spectral characteristics of the dye are sensitive to the physicochemical environment. Our results show that both the absolute wavelength of $\lambda_{\max}$ and its response to increases in bilirubin-IX α concentration clearly differ between aqueous electrolyte solutions and organic solvents (Table 1). The

Fig. 10. Soret-band absorption spectra of bilirubin-IX α in solutions of sodium taurocholate. Spectra were obtained at 23°C with bilirubin-IX α concentrations of 29 μ M (a) and 270 μ M (b). The numbered spectra in (a) correspond consecutively to taurocholate concentrations of 0.18, 0.45, 9.0, 27.0, 54.0 and 90 mM. The numbered spectra in (b) correspond consecutively to taurocholate concentrations of 2, 4, 10, 20, 30, 50 and 100 mM. The buffer was 0.01 M-Na⁺/carbonate/bicarbonate, pH 10.0. No isosbestic point was observed in either set of spectra.

Fig. 11. Difference spectra of bilirubin-IX α in solutions of sodium taurocholate

Spectra were obtained at 23°C by using an identical concentration of bilirubin-IX α (26 μ M) in both reference and sample cuvettes (light-pathlength was 1 cm). Taurocholate concentrations corresponding to each numbered curve correspond consecutively to 0, 0.45, 1.8, 9.0, 18.0, 36.0 and 90.0 mM. The buffer was 0.01 M-Na⁺/carbonate/bicarbonate, pH 10.0. Two absorption maxima are observed at all bile salt concentrations.

Fig. 12. Difference-spectra absorption maxima ($\lambda_{\max}$) of bilirubin-IX α versus the final bile salt concentration plotted logarithmically

Spectra were obtained under conditions identical with that in Fig. 11 (0.01 M-Na⁺/carbonate/bicarbonate buffer, 23°C, pH 10, bilirubin-IX α concentration 26 μ M). ●, Sodium taurocholate; ○, sodium taurodeoxycholate; ▲, sodium taurodeoxycholate. (a) Shorter-wavelength absorption maxima; (b) longer-wavelength absorption maxima.

fact that the $\lambda_{\max}$ of bilirubin-IX α is shifted hypsochromically at concentrations below 10 μM compared with organic solvents is itself suggestive of self-aggregation which is most probably dimerization, as occurs with other water-soluble dyes (Vickerstaff, 1950). Further, the steep hypsochromic shifts with bilirubin-IX α concentrations above 10 μM (Table 1 and Fig. 1) are entirely consistent with further self-aggregation of bilirubin dimers to form more extensive aggregates. As the ϵ values for bilirubin-IX α in urea solutions extrapolated to zero urea concentration and in dilute aqueous electrolyte solutions were similar (4.42×10^4 and $4.37 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and as increases in temperature and added urea reverse the shifts in $\lambda_{\max}$ of more concentrated bilirubin-IX α solutions, the logical inference is that these effects indicate splitting of higher aggregates to the dimer form. The bathochromic shifts in $\lambda_{\max}$ with tight isosbestic points with additions of ethanol suggest an equilibrium of monomers and dimers, and only monomers above an ethanol concentration of about 60% (v/v) (Fig. 7). This facilitated an estimate of ϵ for bilirubin-IX α monomers and thereby a crude estimate of the dimerization constant (K_D) by using ϵ values for the bilirubin-IX α dimer in water. This value (1.5 μM) is similar in magnitude to the dimerization constants (approx. 0.5–6 μM) derived for copro-, deuto-, haemato- and proto-porphyrin self-association in water (Gibson, 1964; Brown *et al.*, 1976; Tipping *et al.*, 1978) and to the estimated association constants (0.3–1.0 μM) of bilirubin-IX α binding to whale apomyoglobin (Lind & Møller, 1976), to rat ligandin (Tipping *et al.*, 1976) and to the second binding site of human albumin (Beaven *et al.*, 1973) under mild alkaline conditions (pH 8.2–9.0), where bilirubin-IX α should only be partially protonated (A. P. Koretsky & M. C. Carey, unpublished work).

Many water-soluble dyes tend to dimerize and in certain cases to extensively aggregate with increasing concentration in aqueous solution (Donnan & Harris, 1911; Robinson & Mills, 1931a,b; Alexander & Stacey, 1952; Vickerstaff, 1950). With Methylene Blue as an example (Hillson & McKay, 1965; Braswell, 1968; Mukerjee & Ghosh, 1970), first dimers followed by small multimers (three to five monomers) form near the limit of aqueous solubility (approx. 30 mM), whereas Congo Red displays aggregates composed of 20000 monomers in 3 mM solutions (Hillson & McKay, 1965). Along similar lines, a variety of self-aggregation patterns have been suggested for synthetic and natural porphyrins in the 0.1–100 μM concentration range (Das *et al.*, 1970; Pasternack *et al.*, 1972; Brown *et al.*, 1976; Gallagher & Elliott, 1973; Gibson, 1964; Tipping *et al.*, 1978). The more hydrophilic porphyrins appear to be restricted to dimer formation, but the most hydrophobic ones, such as protoporphyrin-IX, the porphyrin analogue of bilirubin-IX α , aggregate more

extensively. However, since the degree of ionization of self-aggregating molecules profoundly influences the extent of association (Small, 1968), and as many of these studies were carried out near neutral pH where partial protonation of the carboxylic side chains would be expected (Phillips, 1960), the aggregation patterns are not strictly comparable with the present work. Nevertheless Brown *et al.* (1976) observed that at pH 11.0 the absorption spectra of haematoporphyrin shifted sharply hypsochromically (16–25 nm), producing a 'critical concentration' of 30 μM rather similar to the concentration of the break points in our bilirubin-IX α curves at pH 10.0 (approx. 10 μM), suggesting a dimer $\rightarrow$ multimer transition. Further, Brown *et al.* (1976) observed that variations in ionic strength (0.1–0.4 M-NaCl) influenced neither this 'critical concentration' nor the extent of the spectral shifts, but lowering of the pH to 6.98 moved the break point to lower concentrations. The partial protonation of this porphyrin appears to have induced 'mixed multimer' (ionized + protonated) formation with a predictable decrease in the critical concentration of the dimer $\rightarrow$ multimer transition analogous to what is observed when steroid detergents form 'mixed' micelles as the pH is lowered (Carey & Small, 1972).

The fact that the bilirubin-IX α dimerization is disrupted only by decreasing the polarity of the solvent, whereas the multimer form can be split to dimers by increasing the temperature or adding urea, suggests that the binding forces in the dimers are much stronger than that in the multimers. The most likely driving force for the association of monomers to dimers and of dimers to multimers is hydrophobic interactions. It is quite likely that the forces responsible for the stability of the dimer state are mainly due to π - π -electron interactions of the aromatic pyrrole rings of the bilirubin-IX α molecules, since such electrostatic bonds in a hydrophobic micro-environment are unusually strong (Chothia & Janin, 1975). Further, the visible colour change observed when bilirubin-IX α dimers disaggregate to monomers in ethanol/water or SDS solutions is indicative of direct interactions between the delocalized conjugated π -orbital systems of the molecules in the dimer form. If, however, π - π -electron interactions or hydrogen bonds were the main dimerization force, the K_D for such an association should be several orders of magnitude higher (less affinity) than what we observed (Huyskens *et al.*, 1977), and the association should be capable of being disrupted by increasing the temperature or ionic strength (Carey *et al.*, 1976). The mixed nature of the bonding forces in the dimer is further supported by the fact that the 'critical concentration' for the aggregation of dimers is insensitive to variations in ionic strength (Fig. 1). By screening the negative charges Na^+ ions should increase the tendency to aggregation, but electrostatic

π - π -electron attractive forces would be concomitantly weakened. The interactions between dimers to form multimers are promoted by the addition of Na^+ and disrupted by increases of temperature and added urea, and further the visible colour change associated with the monomer-to-dimer transition is not observed, all suggesting that much weaker hydrophobic forces are involved possibly because π - π -electron interactions are not available for their stabilization. The predominant nature of the hydrophobic interactions in the multimers is further supported by the minimum in the curve of the plot of λ_{max} versus temperature (Fig. 4) as a result of the endothermic nature of the hydrophobic effect. Spectral analysis did not allow an absorption coefficient for the multimer form to be determined, as the hypsochromic shifts were open-ended over the concentration range studied; thus it is not possible to give a thermodynamic description of the dimer $\rightleftharpoons$ multimer equilibrium as either a continuous association process of dimers to multimers or a 'micellization' showing a co-operative effect over a narrow concentration range. However, the sharp breaks in the curves of the plots of λ_{max} versus bilirubin-IX α concentration (Figs. 1 and 2) indicate that the dimer $\rightleftharpoons$ multimer equilibrium has some features of micellization similar to that of natural porphyrins (Brown *et al.*, 1976). Brodersen (1966) in a short communication reported dimerization of bilirubin-IX α in the same concentration range as that studied here. Unfortunately he worked with crude bilirubin-IX α and at a pH where the bilirubin-IX α is partially protonated. The possibility that he was observing a 'dimerization' of dimers cannot be ruled out, though we have shown here that what he mistook for the absorption spectrum of bilirubin-IX α monomers was in reality due to bilirubin-IX α dimers.

The possible structures of bilirubin-IX α aggregates cannot be derived from the present work. However, the molecular exciton model (Kasha *et al.*, 1965) for interacting dye molecules predicts that hypsochromic shifts in the Soret absorption peak should occur when the transition dipole moments of the molecules are aligned parallel to one another, i.e. a parallel 'playing-card' type of stacking. A similar type of association has been suggested for the self-aggregation of aromatic planar dye molecules (Sheppard & Geddes, 1944; Booi, 1949; Mukerjee, 1974; Robinson *et al.*, 1973) and porphyrins (Brown *et al.*, 1976).

Physical chemistry of bilirubin-IX α -anionic detergent interactions

When water-soluble dyes and detergents interact, the association often results in a change in the spectral characteristics of the dye when micelles begin to form (Shinoda *et al.*, 1963). Generally, molecules of oppositely charged dyes and detergents

interact to form insoluble ion-pairs below the critical micellar concentration, and the micellar incorporation of this fine dispersion results in a spectral charge detectable at the apparent critical micellar concentration of the system (Tori & Nakagawa, 1963). When dyes and detergents of similar charge interact (Kapoor & Mishra, 1976), the spectral shifts are often more favourable thermodynamically for measuring the critical-micellar-concentration values because saturation of the micelle with an insoluble precipitate is avoided (Mukerjee & Mysels, 1970). Our results with bilirubin-IX α and SDS provide an excellent example of the latter type of interaction, and the observed spectral shifts are similar to that found in other studies for the micellar incorporation of a dye of similar charge (Shinoda *et al.*, 1963). The spectral changes observed show no apparent interaction between SDS monomers and bilirubin-IX α below the critical micellar concentration. Once the SDS concentration reaches 1.5–4.0 mM the spectral shifts indicate that micelles of SDS influence the equilibrium, possibly by inducing a monomeric distribution of bilirubin-IX α within the micelles. With concentrations of bilirubin-IX α where only dimers are expected, the isosbestic points in the spectra (Fig. 7) suggest an equilibrium between intermicellar dimers and micellar monomers only. Further, the extent of the spectral shifts is similar to that of the transition from dimer to monomeric form of bilirubin-IX α in 50–60% ethanol, suggesting not only that bilirubin-IX α is in monomeric form but that it resides at a site in the micelle where water penetrates possibly as far as the first seven carbon atoms of the molecule (Menger *et al.*, 1978). This supposition is strengthened by the fact that at all bilirubin-IX α concentrations the measured critical-micellar-concentration values are higher than the true critical micellar concentration of SDS (Fig. 8) and increase linearly above 10 μM , indicative of augmented charge repulsion at the aqueous/micelle interface. With bilirubin-IX α concentrations above 50 μM tight isosbestic points were not observed in the spectra, indicative of an equilibrium between more than two species in solution, possibly dimers and higher aggregates in the intermicellar environment and monomers within micelles. The possibility that the structure of bilirubin-IX α in the micellar environment is akin to the internally hydrogen-bonded secondary structure of the crystal form (Bonnett *et al.*, 1976) has been suggested (McDonagh, 1979), but can be discounted because the apparent critical micellar concentration of SDS should be lowered by the addition of a 'non-polar' additive and not increased, as was found in this study (Shinoda *et al.*, 1963).

The interactions between bilirubin-IX α and bile salts are much more complex, since the difference spectra give two positive peaks at all bile salt concen-

trations, including a concentration one order of magnitude below the critical-micellar-concentration values (Fig. 11), whereas with SDS the difference spectra gave an almost flat line below the critical micellar concentration (results not shown). Thus bilirubin-IX α appears to interact with bile salt monomers and micelles, but only with SDS micelles. Both taurocholate and taurodeoxycholate exhibit temperature-dependent initial self-association (in 0.15M-Na⁺ the critical-micellar-concentration ranges are 2.7–4.7 and 0.8–1.8mM respectively) as detected by spectrophotometric titration with the cationic dye Rhodamine 6G (Carey & Small, 1969). Previous studies suggested that dehydrocholate did not exhibit any evidence of self-aggregation in water until the concentration reached about 100–200mM (Fontell, 1972; Djavanbakht *et al.*, 1977), and at approx. 20mM the solution in 1M-NaCl was found to contain only trimers by ultracentrifugal analysis (Small, 1968). In separate experiments with Rhodamine 6G (M. C. Carey & A. P. Koretsky, unpublished work) we found an initial aggregation of taurodehydrocholate at approx. 3mM in 0.15M-NaCl at 23°C, but the spectral shift was much weaker than with the other bile salts. A possible dimerization of taurodehydrocholate is consistent with the change in the osmotic coefficient of unconjugated dehydrocholate solutions in this concentration range (Fontell, 1971). These critical-micellar-concentration values are confirmed by the spectral shifts of bilirubin-IX α /bile salt mixtures in the present work, where both absorption peaks in the difference spectra begin to shift (Figs. 12a and 12b) at 2.7mM (taurocholate), 1mM (taurodeoxycholate) and 2.8mM (taurodehydrocholate). In view of the fact that the dye and detergent have negative charges and the bilirubin-IX α /bile salt molar ratio at the critical micellar concentration does not exceed 1:100, the concentration of bilirubin-IX α (26 μ M) used would not be expected to influence the critical-micellar-concentration values of the bile salts significantly. Monomeric taurodehydrocholate appears in part to disaggregate the large bilirubin-IX α -aggregates to monomers or dimers by interacting hydrophilically to form taurodehydrocholate-bilirubin-IX α complexes, since the $\lambda_{\max}$ always shifts to 424nm, an isosbestic point is found and the absorption coefficient reaches a plateau at approximately the same value regardless of bilirubin-IX α concentration. At taurodehydrocholate concentrations greater than 30mM the shoulder at 460nm suggests that some incorporation of bilirubin-IX α monomers into taurodehydrocholate micelles occurs. On the other hand, the more complex interactions with the common bile salts are consistent with strong hydrophilic interactions with bile salt monomers and both hydrophilic and hydrophobic interaction with bile salt micelles. It is believed that the spectral Soret band of porphyrins is related to a shifting of ring

electrons to the periphery of the porphyrin ring (Phillips, 1963). The greater the electron density of the ring nitrogen atoms the more energy that is required for the electronic transition, and hence there is a hypsochromic shift in $\lambda_{\max}$. An interaction that would lead to electron density being placed on the bilirubin-IX α nitrogen atoms is hydrogen-bonding between the pyrrole nitrogen atoms of bilirubin-IX α and the hydroxy groups of the bile salts. As micelle formation begins to take place, two sequential interactions with bilirubin-IX α molecules occur. First, as exhibited by the peak shifting further to shorter wavelengths at the critical micellar concentration (Fig. 12a), bilirubin-IX α molecules probably remain hydrogen-bonded to bile salt monomers, but now reside on the polar face of the micelle, an interaction facilitated by the high density of hydroxy groups on the surface. At higher bile salt concentrations there is some incorporation of bilirubin-IX α monomers into the micelle, as exhibited by the longer-wavelength peak (similar to that in non-aqueous solvents) progressively shifting upwards above the critical micellar concentration (Fig. 12b). This shift is probably due to bilirubin-IX α molecules residing in the vicinity of the aqueous/hydrocarbon interface of the bile salt micelles, analogous to our interpretation for SDS-bilirubin IX α interactions. Our results lend support to this sequence because with binding at concentrations near the critical micellar concentration of the bile salts the shorter-wavelength peak is more prominent than the longer-wavelength one. As more and more micelles form, incorporation into the micelle takes precedence and the longer-wavelength peak predominates, and the solutions as observed visually become bright yellow. The findings in another report (Perrin & Wilsey, 1971) that optical activity is induced in bilirubin-IX α solutions in the presence of sodium deoxycholate (pH 8.0) well below its critical-micellar-concentration value lends strong support to our interpretation. Further, our observation that a temperature increase disrupts the short-wavelength peak lends further support to a hydrogen-bonding type of interaction with monomers, whereas a temperature increase does not affect the red-peak interaction, further supporting our hypothesis of hydrophobic interactions between bilirubin-IX α monomers and bile salt micelles at concentrations well above their critical-micellar-concentration values. Finally, the fact that the progressive increase in absorbance of the longer-wavelength peak with equimolar amounts of taurocholate (Fig. 10) is much less in 270 μ M- than in 29 μ M-bilirubin-IX α solutions is consistent with the possibility that quantitatively most of the bilirubin-IX α in the more concentrated solution exists as self-aggregated multimers.

We thank Dr. A. F. McDonagh (Department of Medicine, University of California, San Francisco, CA,

U.S.A.), for his generous advice and gifts of samples at the initiation of the study. The sample of SDS (BDH) was kindly procured by Dr. R. H. Dowling and Dr. G. Murphy, Guy's Hospital Medical School, London, U.K. A. P. K. was supported by an Undergraduate Research Opportunities Program (UROP) grant from Massachusetts Institute of Technology (Cambridge, MA, U.S.A.). Ms. Jean Wu provided excellent technical assistance and Ms. Terri Fox typed the manuscript. The study was supported in part by U.S. Public Health Service Research Grant AM 18559 and Research Career Development Award AM 00195 to M. C. C.

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