Binding and Circular Dichroism Data on Bilirubin-Albumin in the Presence of Oleate and Salicylate

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The binding of equimolar amounts of bilirubin to human and bovine serum albumin in 0.1 M phosphate buffer, pH 7.4, in the presence and absence of various concentrations of oleate or salicylate was studied by the use of an ultracentrifugal technique. The resultant data showed salicylate to be a poor competitor for the bilirubin binding sites; in the presence of a considerable excess of salicylate, only small amounts of bilirubin were liberated from the proteins. The dissociation of bilirubin from albumin by oleate was very dependent on the oleate concentration. No bilirubin was liberated from the proteins at oleate:albumin molar ratios below 5. All the bilirubin was liberated from the proteins at oleate:albumin molar ratios above 8.

Marked changes in the absorption and circular dichroism spectra of the bilirubin–albumin solutions were observed on the addition of salicylate or oleate even under conditions in which little or no bilirubin was liberated from the proteins. While the binding characteristics and absorption spectra of the human and bovine albumin–bilirubin complexes in the presence and absence of oleate or salicylate were very similar, the Cotton effects generated by the addition of bilirubin to the human albumin were very different from those obtained with the bovine protein.

The binding of unconjugated bilirubin by serum albumin has received considerable attention, in large part due to its clinical significance (1–9). Free bilirubin can produce brain stem damage (kernicterus) in the susceptible brain of the newborn, possibly as the result of its action as a mitochondrial toxin (9–13). Such action is not observed in the presence of albumin-bound bilirubin (9, 12, 13). Furthermore, it has been suggested that the presence in plasma of certain small organic molecules, such as salicylate, sulfonamides, or fatty acid, which can displace bilirubin from albumin (4–8, 14, 16) may elevate free bilirubin concentrations to dangerous levels in pathological states involving high plasma bilirubin concentrations.

The determination of bilirubin–albumin

binding data is difficult due to the instability and aggregation of free bilirubin in aqueous solution at physiological pH values. The methods which have been used to investigate this problem have included absorption spectrophotometry (4–6, 16–19), ultracentrifugation (8, 17), ultrafiltration (4, 7), equilibrium dialysis (4, 18), electrophoresis (6, 17, 20), gel filtration (7, 21, 22) and, recently, optical rotatory dispersion (23) and some contradictory data have been reported. The investigations involving salicylate (4, 6, 16) or fatty acid (15, 16) have mostly relied on changes in absorption spectra as indications of changes in bilirubin binding (although some semiquantitative data have been obtained by use of some of the other methods listed above), and the efficacy of these small molecules in removing bilirubin from albumin is uncertain.

In the present investigation it was decided

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to determine the extent of bilirubin-binding by both human and bovine albumin in the presence and absence of varying salicylate or oleate concentrations by use of an ultracentrifugal technique and to semiquantitatively corroborate the ultracentrifugal data by electrophoretic analyses.

Free bilirubin is optically inactive (8), but large extrinsic Cotton effects in the region of the bilirubin absorption maximum have been observed on the addition of bilirubin to BSA (23).[2] Extrinsic Cotton effects of this type have given much valuable information on the binding of various small chromophores by optically active macromolecules (23–30) since the spectra are very sensitive to the environment at the binding site. It thus appeared that this type of analysis was capable of demonstrating subtle changes in the binding of bilirubin by albumin after the addition of salicylate or oleate which might not be detected by other methods. Circular dichroism and absorption spectral analyses were therefore performed in parallel with the ultracentrifugal analyses.

MATERIALS AND METHODS

Fisher Certified bilirubin ($\epsilon_{450m\mu}$ = 59,000–62,000 in chloroform) was obtained from Fisher Scientific Company. Analysis of standard solutions of two lots of this bilirubin gave molar absorbances of between 59,000 and 60,000. All other chemicals were reagent grade. Bovine plasma Fraction V (Lot No. C34301) was obtained from Armour Pharmaceutical Company, and a reworked human plasma Fraction V was obtained from the Michigan Department of Health, Division of Laboratories, Lansing, Mich., through the courtesy of Dr. James Sgouris. The Fraction V preparations were obtained in preference to crystalline albumin preparations since the latter generally contain decanol which might alter the binding characteristics of the albumin and which is difficult to remove from the protein.

Human and bovine albumin preparations were obtained from the Fraction V powders by exhaustive dialysis of 8% solutions against distilled water followed by passage of the dialyzed solutions through mixed-bed deionizing columns (31) to remove most of the globulins present in the Fraction V preparations. The deionized solutions were

Millipore-filtered, freeze-dried, and the dry powders stored at 2°. Electrophoretic analysis of the albumins gave only one band on Sepraphore III strips. Ultracentrifugal analysis showed both human and bovine preparations to contain about 5% dimer. The fatty acid content of the BSA was 0.4 moles/mole while that of the HSA was 1.1 moles/mole as determined by the method of Dole (32).

The protein concentration of the stock albumin solutions was determined by absorbance measurements using the following previously determined absorbances (E $_{1cm, 279 m\mu}^{1\%}$): HSA, 5.34; BSA, 6.67. The molecular weight of albumin was taken as 66,000.

Protein determinations on the bilirubin–albumin solutions were performed using the biruet reagent (33). Salicylate, present in many of the solutions, interfered with this technique and was removed by precipitating and twice rewashing the protein with 3% trichloroacetic acid. The final precipitate was redissolved in 1.5 ml water, mixed with 4.5 ml biuret reagent, and the absorbance of the resultant solution measured after 20 min against a reagent blank at 550 mμ in a Coleman spectrophotometer.

Bilirubin determinations were carried out by the method of Jendrassik and Grof as modified by Gambino (34). However, since salicylate also reacted with the diazo reagent, it was necessary to increase the sodium nitrite concentration to 5.0 g/100 ml and add 2 ml of this solution to the sulfanilic acid solution. The salicylate reaction product had an absorption maximum at 450 mμ but even in high concentration exhibited negligible absorption above 580 mμ. Since the bilirubin reaction product was read at its absorption maximum of 600 mμ, the presence of even large excesses of salicylate did not interfere with the estimation of bilirubin concentration. For uniformity, this modification was adopted in all bilirubin determinations including those on the standard solutions.

Fatty acid-free HSA was prepared by the charcoal defatting method of Chen (35). Colloidal charcoal remaining in the protein solution at the end of the procedure was removed by passing the solution down a short DEAE-Sephadex A-50 column, 0.2 M phosphate buffer, pH 7.0 at 2°. The resulting preparation contained < 0.01 moles fatty acid/mole HSA.

Stock albumin solutions were prepared by dissolving the albumin powders in 0.1 M phosphate buffer, pH 7.40, determining the albumin concentrations by absorbance measurements at 279 mμ, and adjusting the volume of the albumin solution with buffer such that the final albumin concentration was 68.4 μM (0.451 g/100 ml). An equimolar bilirubin solution was prepared by dissolving 4.00

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[2] Abbreviations used are: HSA, human serum albumin; BSA, bovine serum albumin; CD, circular dichroism; ORD, optical rotatory dispersion.

mg bilirubin in a few drops of 0.2 M NaOH and diluting to 100 ml with the pH 7.4 phosphate buffer. In all experiments except those in which bilirubin concentration was a variable, equal volumes of the albumin and bilirubin solutions were mixed to give a 34.2 μM solution of 1:1 bilirubin–albumin. All bilirubin-containing solutions were protected from light by wrapping the containers in aluminum foil. After initial mixing, the absorbance of the bilirubin–albumin solutions in the region of the bilirubin–albumin absorption maximum increased with time until a stable plateau value was reached about 4 hr after mixing. On further standing, a very slow decrease in the magnitude of the absorption occurred. This greater stability of the albumin-bound bilirubin permitted the use of the lengthy ultracentrifugation procedure to determine albumin-bound bilirubin concentrations without serious error due to oxidation of the bilirubin. The absorption and CD spectra of the 1:1 bilirubin–albumin solutions after ultracentrifugation were not significantly different from those obtained 4 hr after mixing of the constituent solutions. All absorption and CD spectra recorded in this paper were obtained 4–12 hr after mixing the bilirubin and albumin solutions. To minimize variables, no ascorbic acid was added to the solutions to stabilize bilirubin against oxidation.

Various dilutions of a standard ethanolic solution of oleic acid were added to the 1:1 bilirubin–albumin solutions (0.2 ml oleic acid solution : 10 ml bilirubin–albumin solution) to give the desired oleate:albumin molar ratio. A standard stock salicylate solution was prepared in the pH 7.4 phosphate buffer, and serial dilutions of this solution were used to obtain the desired salicylate concentrations. Due to the relatively high salicylate concentrations which were present in some of the bilirubin–albumin solutions, it was necessary to add somewhat large volumes of salicylate to the bilirubin–albumin solutions, and the salicylate–bilirubin–albumin solutions were prepared by mixing 1 vol of salicylate solution with 4 vol of the 34.2 μM 1:1 bilirubin–albumin solutions. The oleic acid and salicylate were added to the bilirubin–albumin solutions immediately after the preparation of the latter, and the well-mixed solutions were allowed to stand in the dark for 4 hr before use.

Absorption spectra were obtained using a Cary Model 15 recording spectrophotometer, and the absorbances of the albumin solutions at 279 mμ were measured on a Zeiss PMQ II spectrophotometer. Circular dichroism measurements were performed on a Jasco ORD/UV/CD-5 spectropolarimeter at 25°. The optical lengths of the quartz cells were 4.5 and 9.0 mm. The absorbances

of the solutions did not exceed 0.6 above 300 mμ. The slit width was never greater than 0.2 mm above 300 mμ wavelength and the scale setting was 0.005 or 0.002. Circular dichroic absorption coefficients ($\Delta\epsilon$) were calculated using the relationship

$$\Delta\epsilon = \epsilon_1 - \epsilon_r = \Delta k \cdot s/cl,$$

where ϵ_1 and ϵ_r are the absorbances of the left and right circularly polarized light, Δk is the experimentally measured difference in absorbance of left and right circularly polarized light, s the scale setting, c the molar concentration of bilirubin, and l the optical path length in centimeters. No corrections for refractive index dispersion were made.

An ultracentrifugal technique was used to determine the albumin-bound bilirubin concentration on the basis of whether or not the bilirubin sedimented with the albumin. The bilirubin–albumin solutions were placed in 12-ml plastic centrifuge tubes in the No. 40 rotor of a Spinco Model L ultracentrifuge and centrifuged for 36 hr at 39,000 rpm at 15°. At the end of this time the albumin and bound bilirubin had sedimented to the bottom 2 ml of the centrifuge tube, whereas any free colloidal bilirubin had sedimented to the bottom of the tube where it packed as an orange pellet. The top 10 ml of the centrifuge tube contained only traces of bilirubin or albumin and was removed by use of a tube-cutter (36). The remaining bottom 2 ml of the bilirubin–albumin solution was carefully removed from the bilirubin pellet and analyzed for protein and bilirubin.

Electrophoretic studies were performed on Sepraphore III strips in a Gelman Rapid Electrophoresis chamber in 0.05 M phosphate buffer, pH 7.4, at 200 V and a current of approximately 3 mA per strip for 85 min at 2°, and 20 μl of the bilirubin–albumin solution was applied to each strip. Bilirubin-containing bands were observed visually while the location of the albumin was determined after staining with amido black 10B.

RESULTS

Binding Determinations

Ultracentrifugal studies. Protein and bilirubin determinations were performed on the albumin-containing fraction of the 1:1 bilirubin–albumin solutions in the presence and absence of oleate or salicylate after ultracentrifugation of the solutions. The amount of bilirubin which remained bound to the albumin in the presence of various concentrations of oleate is shown in Fig. 1. No orange bilirubin pellet could be detected at the bottom of the tubes which contained no, or low concentrations of, oleate. At
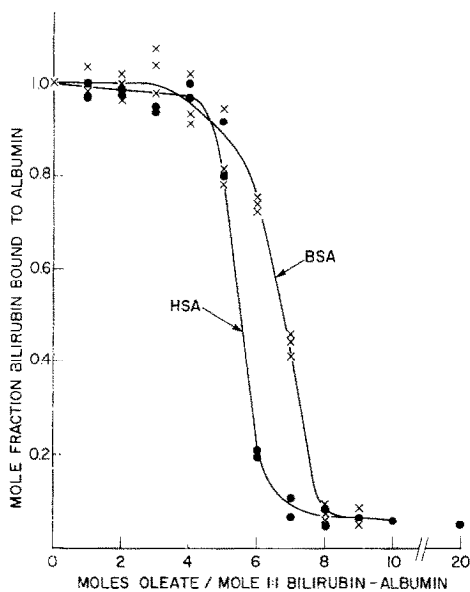
FIG. 1. Dissociation of bilirubin from 1:1 bilirubin–albumin by oleate. Initial bilirubin–albumin concentration = 33.5 μM. ●, bilirubin–HSA (two experiments); ×, bilirubin–BSA (three experiments).

oleate concentrations between 4 and 8 moles/mole bilirubin–albumin there was a marked increase in the size of the bilirubin pellet as the oleate concentration increased. The small but positive bilirubin values which were obtained in the presence of an 8-fold or greater excess of oleate were probably due to inaccuracies in the bilirubin assay at these low bilirubin levels and to the presence of traces of unsedimented free bilirubin in the ultracentrifuged solutions. In all other instances duplicate bilirubin and protein determinations agreed to within 2%.

The effect of salicylate on the binding of bilirubin by albumin in the presence and absence of low oleate concentrations is shown in Figs. 2 and 3. The effect of salicylate was much less marked than that of oleate, and a slow but steady loss of bilirubin was observed as the salicylate concentration increased. The loss of BSA-bound bilirubin (Fig. 3) was somewhat greater than that of HSA-bound bilirubin (Fig. 2), especially at the lower salicylate concentrations. The addition of low concentrations of oleate to the salicylate–bilirubin–albumin solutions had little effect on the extent of bilirubin
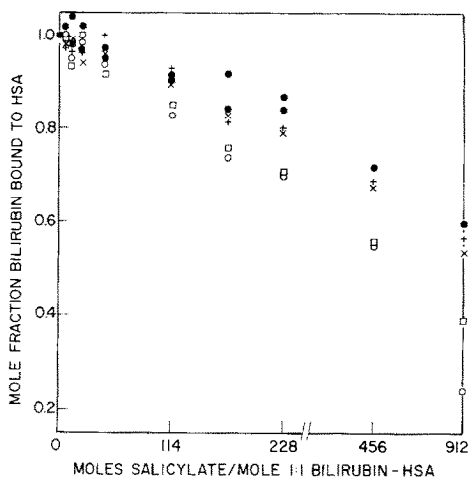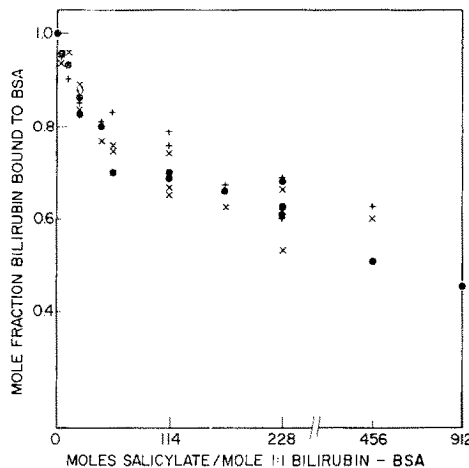

FIG. 2. Dissociation of bilirubin from 1:1 bilirubin–HSA by salicylate in the presence and absence of low concentrations of oleate. Initial HSA concentration = 27.4 μM. ●, no oleate; +, 1 mole oleate; ×, 2 moles oleate; □, 3 moles oleate; ○, 4 moles oleate/mole bilirubin–HSA.

FIG. 3. Dissociation of bilirubin from 1:1 bilirubin–BSA by salicylate in the presence and absence of low concentrations of oleate. Initial BSA concentration = 27.4 μM. ●, no oleate; +, 1 mole oleate; ×, 2 moles oleate/mole bilirubin–BSA.

binding although the data obtained with HSA did show some increase in the loss of bilirubin following the addition of oleate.

Electrophoretic studies. Protein and bilirubin determinations were not performed on the electrophoresed bilirubin–albumin solu-

tions. The bilirubin and protein bands were estimated visually before and after staining with amido black 10B. Free bilirubin did not migrate in the electric field at pH 7.4 and could be observed as a yellow band at the origin. Bound bilirubin migrated with the albumin to the anode as a yellow band which became blue after staining with the amido black. The 1:1 bilirubin–albumin solutions and those which contained less than a 5 M excess of oleate showed no yellow band at the origin and a strong yellow bilirubin–albumin band. As the oleate concentration was increased above 5 moles/mole bilirubin–albumin, bilirubin could be seen at the origin in increasing amounts while the yellow color associated with the albumin band decreased with increasing oleate concentration. When an 8–9-fold excess of oleate was present, no yellow color could be discerned at the position of the albumin band. When electrophoresis was performed on solutions which contained salicylate, a gradual increase in the intensity of the bilirubin band at the origin was observed as the salicylate concentration was increased.

Spectral Studies

Since it has been shown that the optical properties of bilirubin–albumin solutions can depend on the order of mixing the constituents, on the nature and concentration of the added electrolyte, and on the pH (23), it should be reemphasized here that all the data reported in this section of the paper were obtained by mixing 0.1 M, pH 7.4, phosphate buffer solutions of the bilirubin and albumin, adding oleate or salicylate when required, and allowing the solutions to stand for 4–12 hr in the dark before use. The pH of the final solutions was 7.40 ± 0.05. A few absorption and CD spectra were obtained on solutions in which most of the phosphate had been replaced by NaCl. The spectra were indistinguishable from those obtained with phosphate alone.

Absorption spectra. The spectra of bilirubin–HSA and bilirubin–BSA solutions in the presence of 0.5–4.0 moles bilirubin/mole albumin showed a steady increase in the intensity of the absorbance at 450–460 mμ as the bilirubin concentration increased. This

red shift in the wavelength of the bilirubin absorption maximum (free bilirubin has an absorption maximum at 435–440 mμ) was most pronounced at the lower bilirubin concentrations and was accompanied by a distinct shoulder to the absorption curves in the 420–430 mμ region of the spectra.

The absorbance coefficient of the 1:1 bilirubin–HSA ($\epsilon_{mM, 456 m\mu}$) was 45 ± 2, in good agreement with the value of 46 (460 mμ) reported by Odell (4), while that of the bilirubin–BSA ($\epsilon_{mM, 460 m\mu}$) was 54 ± 2. This latter value is somewhat lower than the value of 63.5 reported by Blauer and King (23) for bilirubin–BSA at pH 7.5. Furthermore, these authors observed the wavelength of the absorption maximum to be 471–472 mμ, a wavelength considerably longer than the 460-mμ absorption maximum observed in the present investigations.

Salicylate, in concentrations up to 1000-fold excess, was added to 1:1 bilirubin–BSA solutions. As the salicylate concentration was increased, there was a progressive decrease in the intensity of the absorption and a blue shift in the wavelength of the absorption maximum towards that of free bilirubin. In the presence of 100-fold excess of salicylate the absorbance coefficient at the absorption maximum (450 mμ) was 46 mM^{-1} cm^{-1}. The spectra obtained with bilirubin–HSA showed similar but less marked changes as the salicylate concentration increased and were in general agreement with the spectra reported by Odell (4, 16) and Watson (6).

More complex results were obtained when oleate rather than salicylate was added to the bilirubin–albumin solutions (Figs. 4 and 5). At low oleate concentrations (up to 3–4 moles oleate/mole bilirubin–albumin) there was an increase in the intensity of the absorption with a slight red shift in the absorption maximum. As the oleate concentration was increased above these values, this peak decreased in intensity and the spectra exhibited a broad peak with a maximum at 450 and a pronounced shoulder at 490 mμ. These spectra (7–8 moles oleate/mole bilirubin–BSA and 6 moles oleate/mole bilirubin–HSA) were strikingly similar to those observed by Mustafa and King (13) and on
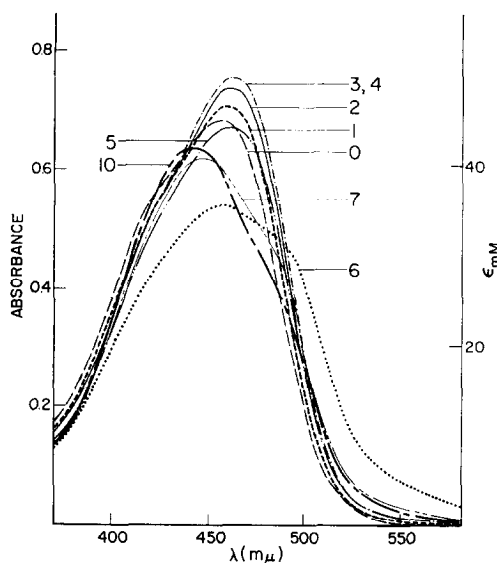
FIG. 4. Absorption spectra of 1:1 bilirubin-HSA (33.5 μM) in the presence of 0, 1, 2, 3, 4, 5, 6, 7, and 10 moles oleate/mole bilirubin-HSA. Optical path length = 4.5 mm. Left ordinate, absorbance of solutions and right ordinate, millimolar absorbance coefficient (mM^{-1} cm^{-1}).

the addition of mitochondria or mitochondrial lipid to bilirubin.

Spectral measurements were also conducted on some 1:1 bilirubin–albumin solutions to which both oleate and salicylate had been added. At low oleate concentrations (below 4 moles oleate/mole bilirubin–albumin) the spectra were composites of those obtained with oleate or salicylate alone. At higher oleate concentrations the effect of the oleate almost completely overwhelmed that of the salicylate except at very high salicylate concentrations (above 500 moles salicylate/mole bilirubin–albumin). The effect of salicylate on the oleate–bilirubin–HSA solutions was somewhat less pronounced than that observed with the bovine protein.

Circular dichroism. Free bilirubin exhibits no circular dichroism in either aqueous[3] or chloroform solution. At the concentrations studied, the albumin solutions displayed no circular dichroism at wavelengths above 300

[3] Unlike the observations of Blauer and King (23) at low salt concentrations, no optical activity was detected in the free bilirubin solutions which contained 0.1 M phosphate, pH 7.4.
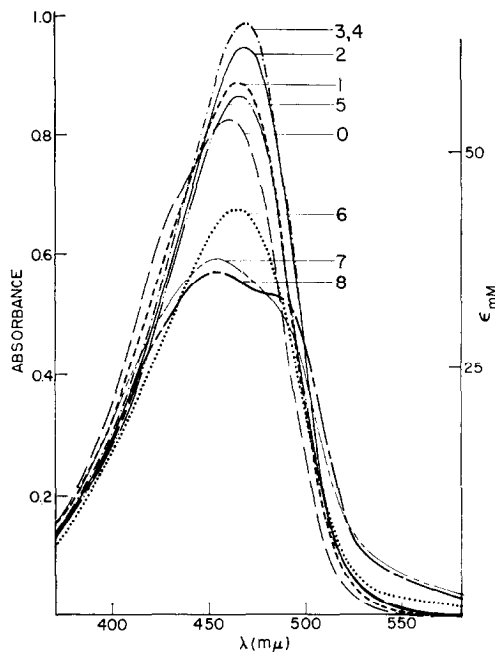

FIG. 5. Absorption spectra of 1:1 bilirubin-BSA (33.5 μM) in the presence of 0, 1, 2, 3, 4, 5, 6, 7, and 8 moles oleate/mole bilirubin–BSA. Optical path length = 4.5 mm. Left ordinate, absorbance of solutions and right ordinate, millimolar absorbance coefficient (mM^{-1} cm^{-1}).
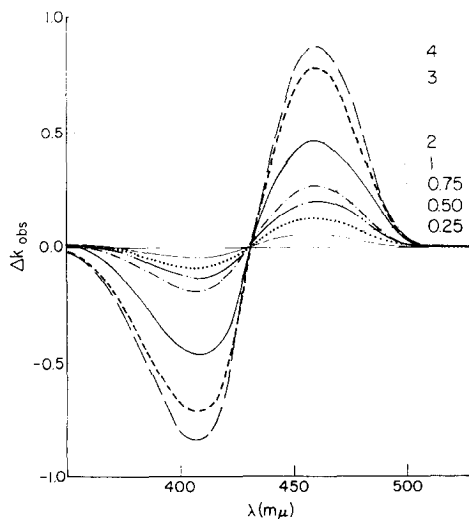

FIG. 6. CD spectra of HSA (11.4 μM) in the presence of 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, and 4.0 moles bilirubin/mole HSA. Optical path length = 4.5 mm, scale setting = 0.002. Left ordinate, CD absorption of solutions and right ordinate, molar CD absorption coefficient (M^{-1} cm^{-1}).
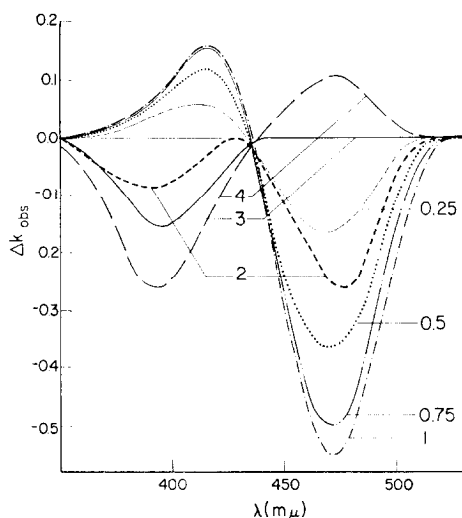
FIG. 7. CD spectra of BSA (11.4 μM) in the presence of 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, and 4.0 moles bilirubin/mole BSA. Optical path length = 4.5 mm, scale setting = 0.002. Left ordinate, CD absorption of solutions and right ordinate, molar CD absorption coefficient (M^{-1} cm^{-1}).

mμ and showed only a shoulder between 250 and 300 mμ before the strong absorption at lower wavelengths. The CD spectra of bilirubin–HSA and bilirubin–BSA in the presence of various amounts of bilirubin are shown in Figs. 6 and 7. Both proteins gave biphasic spectra in the region between 300 and 500 mμ. As the bilirubin concentration increased, the spectra obtained with the HSA showed a steady increase in the intensity of a positive Cotton effect at about 460 mμ and a negative Cotton effect at about 410 mμ and had a crossover point at 431 mμ. The spectra obtained with BSA were more complex. At bilirubin concentrations up to 1 mole/mole BSA there was a steady increase in the intensity of two CD bands at 472 and 416 mμ as the bilirubin concentration increased. Above equimolar bilirbuin concentrations the two bands decreased in intensity and eventually changed sign such that in the presence of a 4 M excess of bilirubin the spectra showed a small positive band at 473 mμ and a somewhat stronger negative band at 392 mμ.

Replicate measurements on 34.2 μM 1:1 bilirubin–HSA solutions gave a circular dichroic absorption coefficient ($\Delta\epsilon_M$, $_{456m\mu}$) of

37 $\pm$ 1. The coefficient obtained with the BSA solutions ($\Delta\epsilon_M$, $_{472m\mu}$) was 72 $\pm$ 1, approximately twice that of the human protein. Blauer and King (23) have reported the wavelength of the inflection point of the ORD Cotton effect to be 472 mμ, a wavelength identical to that observed for the minimum of the major bilirubin–BSA CD band. This wavelength identity is in sharp contrast to the comparable absorption spectra maxima which were at 471–472 (Blauer and King) and 460 mμ (this paper). These differing results could be due to differing ionic strength (Blauer and King's studies were conducted in approximately 1 mμ NaCl) or to the use of differing protein preparations. In this regard, one experiment was performed in which crystalline BSA (Armour Pharmaceutical Co., Lot No. E71503) which contained decanol and 0.66 moles fatty acid/mole BSA was substituted for the BSA prepared from Fraction V. The CD spectra of this 1:1 bilirubin–BSA preparation showed a similar CD band at 472 mμ but no CD band at 416 mμ.

The CD spectra were obtained after the addition of salicylate to the 1:1 bilirubin–albumin solutions. Progressive addition of salicylate to the 1:1 bilirubin–HSA resulted in a progressive decrease in the intensity of both CD bands although the trough at 408 mμ was more affected by low salicylate concentrations than was the peak at 456 mμ. The changes produced in the bilirubin–BSA spectra were greater and more complex (Fig. 8). Relatively low concentrations of salicylate (11 moles/mole bilirubin–BSA) reduced the magnitude of the trough at 472 mμ to about half of its original value and almost completely extinguished the smaller peak at 416 mμ.

The addition of oleate to 1:1 bilirubin–HSA (Fig. 9) produced a steady, marked decrease in the magnitude of the peak at 456 mμ with increasing oleate concentration. The trough at 408 mμ, however, did not decrease in intensity until 3 moles oleate/mole bilirubin–HSA had been added to the solution. When a 5- or 6-fold excess of oleate was present, the spectra changed in character and new CD bands of low intensity were observed. Spectra obtained after the
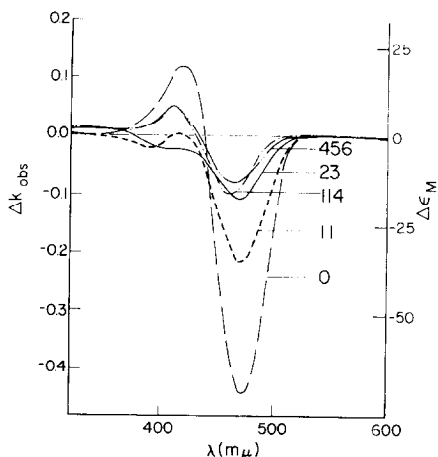
FIG. 8. CD spectra of 1:1 bilirubin–BSA (27.4 μM) in the presence of 0, 11, 23, 114, and 456 moles salicylate/mole bilirubin–BSA. Optical path length = 4.5 mm, scale setting = 0.002. Left ordinate, CD absorption of solutions and right ordinate, molar CD absorption coefficient (M^{-1} cm^{-1}).

addition of 8 moles oleate/mole bilirubin–HSA exhibited essentially no circular dichroism above 300 mμ.

The complex spectra obtained on the addition of oleate to 1:1 bilirubin–BSA are shown in Fig. 10. A complete loss of the peak at 416 mμ and a considerable decrease in the magnitude of the trough at 472 mμ were observed on the addition of 1 mole oleate/mole bilirubin–BSA. Further increases in oleate concentration further decreased the trough at 472 mμ and a new trough at 425 mμ appeared. As the oleate concentration was increased above 4 moles/mole bilirubin–BSA, the CD bands progressively decreased in intensity until in the presence of 8 or more moles oleate/mole bilirubin–BSA the Cotton effect had been completely abolished. In view of the great differences in the CD spectra of the HSA and BSA complexes it is interesting to note that the spectra of both proteins displayed a small positive band at about 500 mμ after the addition of a 6-fold excess of oleate.

Investigation of the CD spectra of bilirubin–albumin solutions containing both oleate and salicylate produced results similar to those observed with the absorption spectra. The less pronounced effect of salicylate on the absorption and CD spectra of
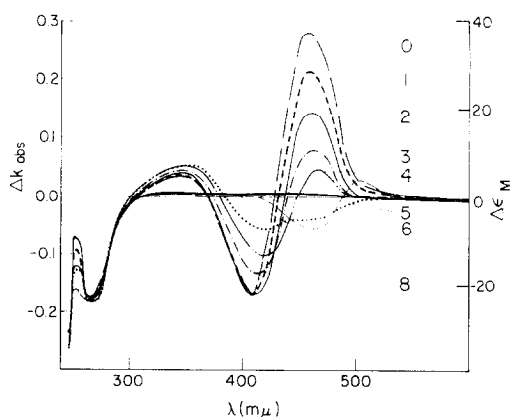

FIG. 9. CD spectra of 1:1 bilirubin–HSA (33.5 μM) in the presence of 0, 1, 2, 3, 4, 5, 6, and 8 moles oleate/mole bilirubin–HSA. Optical path length = 4.5 mm, scale setting = 0.002. Left ordinate, CD absorption of solutions and right ordinate, molar CD absorption coefficient (M^{-1} cm^{-1}).
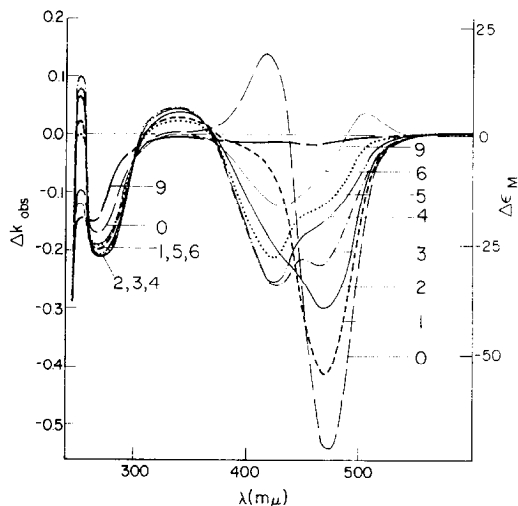

FIG. 10. CD spectra of 1:1 bilirubin–BSA (33.5 μM) in the presence of 0, 1, 2, 3, 4, 5, 6, and 9 moles oleate/mole bilirubin–BSA. Optical path length = 4.5 mm, scale setting = 0.002. Left ordinate, CD absorption of solutions and right ordinate, molar CD absorption coefficient (M^{-1} cm^{-1}).

the bilirubin–HSA complex, especially at the lower salicylate concentrations, paralleled the binding data in which the loss of bilirubin from the bilirubin–HSA was not as great as that observed with the bovine protein.

Hematin and Evan's blue, both of which

are tightly bound by albumin (37), were added to BSA solutions in equimolar amounts in pH 7.4 0.1 M phosphate buffer. No extrinsic Cotton effects were observed with either solution nor did the addition of equimolar amounts of these compounds to 1:1 bilirubin–BSA alter the CD spectrum of that complex. Rosenfeld and Surgenor (37) have reported that the addition of bilirubin did not affect the binding of hematin by albumin and have suggested that the bilirubin and hematin binding sites are different and independent of one another, a situation which would be in accord with the CD data.

Since the HSA preparation contained over 1 mole fatty acid/mole HSA, it was decided to defat the protein and examine the CD spectra of the defatted BSA after the addition of equimolar amounts of bilirubin. The spectra obtained with the 1:1 bilirubin-defatted HSA solutions in the presence and absence of low oleate concentrations were very similar to those obtained with the unde-fatted protein although some enhancement in the intensity of the CD band at 456 mμ was observed in the absence of oleate.

DISCUSSION

The instability, aggregation, and insolubility of free bilirubin in aqueous solution at physiological pH values presents serious technical problems in investigations involving this compound. While various and sometimes contradictory values have been reported for the solubility of bilirubin, the most complete investigation of this problem is that of Burnstine and Schmid (38). They found the solubility of bilirubin in 0.1 M phosphate, pH 7.4, to be about 7 mg/100 ml. However, interpolation of their data to pH 7.4 ionic strength 0.1 phosphate buffer gives a bilirubin solubility that is about 30 times greater than the value of 0.1 mg/100 ml reported by Martin (18) at the same pH and ionic strength. Equilibrium dialysis (4) and ultrafiltration (4, 7) data involving bilirubin concentrations of 1 mg/100 ml or greater have been reported, but we were unable to even approach equilibrium bilirubin concentrations on dialysis in cellulose dialysis tubing of 34.2 μM (2 mg/100 ml) bilirubin solutions in 0.1 M phosphate, pH 7.4.

Furthermore, ultrafiltration of the bilirubin solution through a Diaflo PM-10 membrane (exclusion mol wt approx. 10,000) resulted in a filtrate which contained only traces of bilirubin.

Of the other methods available for determining the extent of bilirubin binding by albumin [gel filtration (7, 21, 22), electrophoresis (6, 17, 20), the peroxidase method of Jacobsen (39), and ultracentrifugation (8, 17)] the last named was deemed most suitable for large numbers of samples. It also had the advantage that it did not subject the solutions to light or to large volumes of oxygen-containing solvents or solid matrices which could promote oxidation of the bilirubin. There was some concern about the long period of time required for the ultracentrifugation, but preliminary investigations on the absorption and CD spectra of the 1:1 bilirubin–albumin solutions before and after centrifugation showed the complex to be stable under these conditions. A further problem could be encountered in the bilirubin–albumin solutions which contained oleate or salicylate and free bilirubin. Since all but traces ($<$ 5%) of the free bilirubin sedimented to the bottom of the centrifuge tube where it packed as a pellet, true equilibrium between albumin-bound bilirubin and free bilirubin was not achieved. This could introduce an error into the correlation of spectral and ultracentrifugal data if the presence of the oleate or salicylate reduced the association constant of the bilirubin for the albumin to a sufficiently low value resulting in liberation of bound bilirubin during centrifugation. However, the loss of the extrinsic Cotton effects in the CD spectra at the same oleate concentrations as those which resulted in loss of bilirubin binding as determined by the ultracentrfuge experiments (8–9 moles oleate/mole bilirubin–albumin) would indicate that the binding data represent reasonably accurate estimates of the extent of bilirubin binding at the major bilirubin binding sites on the albumin. Furthermore the electrophoretic data provide some corroborative evidence for the validity of the ultracentrifugal data although here again the bilirubin–albumin was removed from the free bilirubin during electrophoresis.

Recent data obtained by Jacobsen (39) have shown that the major bilirubin binding sites on HSA are of two classes, the first containing a single site at which bilirubin is bound with a dissociation constant of 7×10^{-9} M at pH 7.4 in 0.1 M Tris–HCl buffer and the second containing two sites with corresponding constants of 2×10^{-6} M. Thus in a 1:1 bilirubin–HSA solution, the bilirubin binding should occur almost entirely at the primary binding site, and the simplifying assumption can be made that the entire Cotton effect arises from the stereospecific binding of a single bilirubin molecule at a single asymmetric locus on the HSA molecule.

Similar binding data are not available for BSA; however, the ORD titration data of Blauer and King (23) have indicated the presence of one primary bilirubin binding site on BSA with an association constant of 5.7×10^6 M^{-1} at pH 5. Data obtained at pH 7.4 indicated that the association constant at this pH was at least one order of magnitude greater than that determined at pH 5. The existence of one primary bilirubin binding site on BSA is also suggested by the CD spectra obtained in the presence of various bilirubin concentrations as shown in Fig. 7. Progressive addition of bilirubin to BSA up to equimolar bilirubin concentrations produced a steady increase in the intensity of both Cotton effect CD bands as would be expected for the purely additive effect of saturating a single site. As the bilirubin concentration was raised above the 1:1 molar ratio, the CD spectra changed in character as if secondary binding sites producing different Cotton effects were now binding bilirubin. Furthermore, the association constant of the BSA primary bilirubin binding site must be greater than 10^7 M^{-1} in pH 7.4, 0.1 M phosphate buffer since less than 5 % of the bilirubin was not albumin-bound after ultracentrifugation of the 34.2 μM 1:1 bilirubin–albumin solutions.

The molar amplitudes of the major 1:1 bilirubin–albumin CD bands are high. The $\Delta\epsilon_{M,\ 472\ m\mu}$ of the 1:1 bilirubin–BSA is 72, greater than the values reported by Schechter (30) for the covalent binding of 2,7-dibromo-4-hydroxy mercurifluorescein to the sulfhydryl group of BSA ($\Delta\epsilon_{M,\ 540\ m\mu} = 13$

at pH 5 and 5 at pH 7) and the noncovalent binding of equimolar amounts of pyridoxal phosphate to BSA ($\Delta\epsilon_{M,\ 335\ m\mu} = $ slightly over 4) and is the same order of magnitude as those reported for the major CD bands of various species of cytochrome c at about 400 mμ (40). While both 1:1 bilirubin–albumin complexes are very similar in their behavior, the spectral data provide striking evidence for the nonidentity of the primary bilirubin binding sites on the two proteins. Although the magnitudes of the molar absorbances at the absorption maxima are quite similar, the magnitude of the major BSA CD absorption maximum is about twice that of the HSA. Furthermore although the wavelength of maximum absorption is the same for both the absorption and CD spectra in the case of the HSA, the major CD band observed with the bovine protein has a maximum at a wavelength 12 mμ longer than that observed in the absorbance measurements.

Blauer and King (23) have interpreted the large Cotton effect which they obtained with bilirubin–BSA at pH 5 as being associated with a high degree of inherent dissymmetry and dipole–dipole coupling in the bound bilirubin molecule and have proposed that the bilirubin bound to BSA prossesses the sense of a right-handed helix. They have also suggested that a less dissymmetric conformation with a reduced degree of dipole–dipole coupling between the chromophores of the two bilirubin halves exists at more alkaline pH values. Some such couplet could be largely responsible for the Cotton effects observed with the bilirubin–HSA at pH 7.4 (41) where the symmetry of the two CD bands is most pronounced. The complexity of the bilirubin–BSA spectra would suggest that it is a composite of several different types of interaction and that any unique description of the configuration of bilirubin bound to BSA will require further information on the nature of the various transitions which contribute to the Cotton effects. While very little information is available on the bilirubin and albumin groups which are necessary for bilirubin–albumin association, two relevant facts have been published: (1) guanidination of the lysyl residues of HSA results in greatly decreased binding of bili-

rubin (18) and, (2) the methyl ester of bilirubin is bound by albumin as strongly as is bilirubin (42, 43).

While no oleate or salicylate binding measurements were performed on the bilirubin–albumin solutions which contained these anions, previously reported data on the binding of oleate by BSA (44) at pH 7.4 ($n_1 = 3$, $k_1' = 3.96 \times 10^6$; $n_2 = 3$, $k_2' = 1.26 \times 10^5$; $n_3 = 63$, $k_3' = 0.5 \times 10^3$) and by HSA (45) at pH 7.4 ($n_1 = 2$, $k_1' = 1.1 \times 10^8$; $n_2 = 5$, $k_2' = 4.0 \times 10^6$; $n_3 = 20$, $k_3' = 1 \times 10^3$) would predicate almost complete binding of the first few moles of added oleate and substantial binding of the rest of the added oleate. The binding of salicylate by albumin is considerably weaker than that of oleate. Davison and Smith (46) have reported an association constant of about 2 $\times$ 10^4 for the first salicylate binding site on BSA at pH 7.4 and weaker binding at five other binding sites, and a survey of the available data on HSA would suggest that a similar situation exists in the human protein. Even at low salicylate concentrations appreciable amounts of free salicylate will be present in the salicylate-containing solutions.

Various mechanisms can be considered for the alterations in the CD spectra following the addition of oleate or salicylate. It is apparent that the spectra do not reflect the simple loss of bound bilirubin with no change in the manner of binding of the bilirubin which remained bound to the albumin. Some dislocation or translocation of the bound bilirubin or change in the conformation of the albumin must have occurred. Since the lack of information on the conformation of albumin in the presence of salicylate and the extensive loss of bilirubin at the higher oleate concentrations make any interpretation of the relevant CD data extremely tenuous, we shall restrict any further discussion of this topic to the CD spectra which were obtained in the presence of low oleate concentrations, conditions under which no bilirubin was liberated from the albumin.

The CD spectral changes which were observed under these conditions could result from (1) binding of oleate at sites distant from the bilirubin binding site with the production of overall conformational changes in

the albumin, (2) displacement of the bilirubin to secondary binding sites, or (3) binding of oleate at, or close to, the bilirubin binding site with some perturbation of that site.

(1) Chen (35) has reported that the ORD of HSA (0.74 moles fatty acid/mole HSA) at pH 7.0 remained unchanged after the protein had been defatted (0.01 moles fatty acid/mole HSA). Zakrzewski and Goch (47) have observed changes in the optical activity of HSA at pH 7.0 following the addition of up to 10 moles dodecanoate/mole HSA. They calculated the Moffit-Yang parameters a_0 and b_0 and found that the ORD changes were reflected only in the a_0 parameter; b_0 remained constant throughout the investigated range of binding ratios. It would therefore appear that although the addition of fatty acid can perturb residues at certain loci on the albumin molecule [the absorption (47) and fluorescence (48) spectral changes that have been observed after the addition of fatty acid offer further evidence of this perturbation], the overall structural conformation of the albumin remains unchanged after the addition of fatty acid at pH 7.0 Furthermore, though not absolutely conclusive, the data of Blauer and King (23) would suggest that no overall conformational changes in the BSA occur on the addition of bilirubin to BSA at neutral pH values. The changes in the CD spectra which were observed on the addition of low oleate concentrations to the 1:1 bilirubin–albumin solutions are thus unlikely to be due to overall conformational changes induced by the oleate binding at loci far removed from and independent of the bilirubin binding site.

(2) Since the association constants of the primary bilirubin–HSA site (1.3×10^8 M^{-1}) and the bilirubin–BSA site ($> 10^7$ M^{-1}) are much the same or somewhat greater than those of the respective primary oleate binding sites and since several primary oleate binding sites are available for oleate binding, one would not expect the addition of 1 or 2 moles of oleate/mole bilirubin–albumin to result in any considerable displacement of the bilirubin from its primary binding site.

(3) With the information at present available, it would appear that the absorption and CD spectral changes could best be

explained in terms of oleate–bilirubin–albumin interaction at the bilirubin binding site with some resultant perturbation of the site.

Kernicterus, the deposition of unconjugated bilirubin in the central nervous system, is a disease of the newborn which especially threatens premature babies and infants with icterus gravis. While a large percentage of newborns have serum bilirubin levels (10 mg/100 ml or more) higher than that of the normal adult (1.0 mg/100 ml), most clinicians agree that the critical serum limit for the occurrence of kernicterus is about 20 mg/100 ml, and most kernicterus cases have bilirubin concentrations higher than this. Most of this excess bilirubin is present in the unconjugated form. Studies on jaundiced patients have shown that almost all the bilirubin is bound to albumin (4, 6, 15) and a serum bilirubin concentration of 20 mg/100 ml (0.342 mм) will result in a bilirubin: albumin molar ratio of about 0.7.

High serum free (i.e., nonesterified) fatty acid levels (0.5–2.0 mм) are characteristic of newborns (49) and essentially all this fatty acid is bound to albumin giving rise to bound fatty acid:albumin molar ratios of between 1 and 4. The free fatty acid level rises markedly following starvation and any such increase in the fatty acid level is generally paralleled by a decrease in the serum bilirubin level (15, 50). Furthermore, *in vitro* studies on kernicterus sera and bilirubin–albumin solutions (16, 51) have shown that the addition of oleate will liberate bilirubin from the albumin.

The binding and CD data described in this paper demonstrate that oleate and bilirubin compete for the primary bilirubin binding site on albumin. While it is hazardous to extrapolate *in vitro* data to the situation *in vivo*, the binding data do indicate that any increase in the fatty acid:albumin ratio above 4 would result in a considerable loss of serum bilirubin to the intracellular fluid and thus aggravate the disease. The data would also suggest that, at high fatty acid levels, serum bilirubin values may underestimate the severity of the disease.

The normal therapeutic serum level of salicylate is about 2 mм resulting in salicylate:albumin molar ratios of about 4. The studies of Schmid *et al.* (52) on hyperbilirubinemic rats and of Watson (6) on human subjects have shown a marked decrease in the serum bilirubin levels following the infusion or ingestion of salicylate, and various *in vitro* investigations (4, 6, 16) have also indicated the loss of albumin-bound bilirubin in the presence of salicylate. The binding data presented in this paper show only a slight decrease in the albumin-bound bilirubin on the addition of a 4 M excess of salicylate, in marked contrast to the large decrease in serum bilirubin observed *in vivo*. The *in vivo* data cannot be directly explained in terms of the *in vitro* binding data. A similar discrepancy has been reported by Odell *et al.* (16). These authors observed that, at bilirubin:albumin molar ratios less than 1, the sera of jaundiced patients showed significant displacement of bilirubin on addition of salicylate whereas comparable bilirubin–HSA solutions showed little, if any, displacement of bilirubin from the albumin.

ACKNOWLEDGMENTS

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