After binding of bilirubin to human serum albumin (1:1), a train of relaxational changes of conformation takes place. The late part of these processes, occurring in the time interval 1–500 s, has been studied by recording the changes of light absorption. Similar processes have been demonstrated after binding of fatty acid anion to the bilirubin-albumin complex as well as after a pH-jump from 6 to 9. Solvent perturbation spectra obtained on the addition of 20% sucrose have failed to demonstrate exposure of the bilirubin chromophores in the complex to the surrounding medium. Xanthobilirubinate which has a single dipyrrolic chromophore compared to the two of bilirubin is bound to albumin in competition with bilirubin, as concluded from co-binding studies with monoacetyldiaminodiphenylsulfone and diazepam, probing two different binding functions of the albumin molecule. Late conformational changes were absent after binding of xanthobilirubinate. Binding of fatty acid to the complex and a pH-jump did not affect the spectrum of xanthobilirubinate-human serum albumin. The findings can be explained by a model, previously proposed, in which the late spectral changes are affected by rotation of one half-domain of albumin, binding one bilirubin chromophore, relative to another half-domain to which the second bilirubin chromophore is bound, whereby a change of exciton splitting occurs. Such changes are not seen with the complex of xanthobilirubinate and albumin, since only a single chromophore is present.

Bilirubin dianions combine reversibly with human albumin in neutral or alkaline solutions (1). At least two molecules of the ligand can be bound to one of the protein (2). Kinetics of this process in both directions, and the final equilibrium, has been studied by several groups (3–7). The molecular structure of one form of bilirubin acid has been elucidated by x-ray crystallography (8) while the structures of albumin and its complexes with bilirubin remain unknown. Spectroscopic studies of the bilirubin-albumin (1:1) complex have indicated that the two dipyrrolic chromophores of the bilirubin molecule in the complex are located at an angle (9). Changes of pH of the medium or co-binding of fatty acids to the albumin causes alterations of the light absorption spectrum of the complex, presumably due to rotation of one chromophore relative to the other so that the angle is changed. Each of the chromophores is visualized as attached to a separate binding unit in the protein molecule. A model accounting for such allosteric effects on the bilirubin binding site has been proposed (10).

A tentative model of the binding structures of albumin, containing six trough-shaped units, each consisting of three parallel helices, has been presented (11) on the basis of the known amino acid sequence (12, 13) and probabilities of α-helical structures (14). Two such troughs could conceivably bind a bilirubin molecule, each holding one bilirubin chromophore and being capable of rotating to one another on changes of pH or on binding of fatty acids elsewhere in the albumin molecule. Affinity labeling (15, 16) and studies of bilirubin binding to proteolytic fragments (17) and to albumin derivatives (18) have further shown that bilirubin in the 1:1 complex is predominantly bound in a region containing amino acids 186–248, suggesting that the second and third of the six binding units, counting from the NH₂-terminal, are involved. Since hydrodynamic properties of the albumin molecule demand the presence of three globular parts (19, 20), it is tempting to suggest that two binding units form one such part, binding the first molecule of bilirubin, and that the remaining four troughs, two by two, constitute two globular parts, capable of binding a second bilirubin, fatty acids, and other ligand molecules (1). The present paper describes further studies of binding kinetics and spectroscopic properties of bilirubin-albumin, compared with observations on binding of xanthobilirubinic acid. The latter substance contains a dipyrrolic structure, while the bilirubin molecule contains two such moieties (Fig. 1). Studies of the kinetics of binding of bilirubin and xanthobilirubinic acid, and of spectral changes of the complexes with varying pH and fatty acid content, are reported. The observations open the possibility of discerning which of the complex features of bilirubin-albumin interaction are related to the presence of two dipyrrolic chromophores and provide a basis for further testing of the previously proposed model of the binding structures of the albumin molecule.

**MATERIALS AND METHODS**

Human serum albumin was obtained from Kabi, Sweden. It contained about 0.5 mol of fatty acid/mol of protein. In some of the experiments, albumin was defatted with charcoal in acid solution (21). This treatment probably causes certain irreversible changes in the albumin molecule (22, 23). Since the aim of the present studies was to investigate low energy conformational changes, we preferred to use non-defatted albumin, except in experiments designed to test the effect of fatty acids, when we used the defatted protein. In addition, kinetic experiments with binding of bilirubin to albumin and with a pH-jump 6–9 of the bilirubin-albumin complex were carried out with defatted as well as with non-defatted albumin.

Crystalline bilirubin was obtained from Sigma and was used without further purification. Xanthobilirubic acid was received as a gift from Professor David Lightner, University of Nevada, Reno. Meso-bilirubin was from Porphyrin Products, Logan, UT.
Albin-Bilirubin Binding Mechanism

Fig. 1. Chemical structure of bilirubin, mesobilirubin, and xanthobilirubinic acid.

Light absorption spectra were recorded on an ACTA M VI spectrophotometer from Beckman Instruments, Inc.

The kinetic experiments were performed in an Aminco-Morrow stopped flow cell attached to a modified Beckman DU spectrophotometer. Equal volumes of two reactant solutions were mixed and the change in absorbance was registered with a Tektronix storage oscilloscope. Slow changes were monitored on a Beckman recorder. Circulating water from a thermostated bath maintained constant temperature in the mixing cell. The temperature was measured in the effluent tube by inserting a needle with a thermocouple. The dead time for the stopped flow apparatus was 5 ms.

Bilirubin was dissolved in 0.1 M NaOH. Mixtures with albumin were prepared by addition of bilirubin at pH above 8.2 in order to avoid formation of colloidal bilirubin. All operations involving bilirubin were performed in dimmed light.

No change in spectral characteristics of a solution of xanthobilirubinate with variation of concentration could be observed in the range from 10–200 μM, indicating that xanthobilirubinate is present as a monomer in distinction to bilirubinate (25).

Xanthobilirubinate binding equilibrium with human serum albumin was analyzed by a spectrophotometric method based on the difference in light absorption spectra between the unbound and bound xanthobilirubinate. Maximum of the former was 406 nm and of the latter, 425 nm. It was found that all spectra obtained with constant concentration of xanthobilirubinate and varying albumin showed an isosbestic point at 406 nm and could be composed of a spectrum of free xanthobilirubinate and a spectrum of the bound form. All spectra conformed with a model in which the spectrum of bound xanthobilirubinate is unchanged with varying numbers of the ligand bound per albumin molecule. Ratios of free to bound ligand could thus be calculated from the spectra. Light absorption ratios, $A_{254}/A_{425}$, were measured with varying compositions of the equilibrium mixtures and a binding isotherm was established (Fig. 2).

Binding equilibria of mesobilirubin to human serum albumin were studied by measuring oxidation rates with hydrogen peroxide and peroxidase, as previously done for the binding of bilirubin (25).

Reserve albumin equivalent for binding of monoacetylidiamino-diphenylsulfone and diazepam was determined by measuring dialysis rates of these test ligands, isotopically labeled, and added in low concentration, as previously described (26).

RESULTS

Xanthobilirubinate Binding Equilibria—The binding isotherm for xanthobilirubinic acid, Fig. 2, shows that at least four molecules are bound to albumin. Curve fitting in terms of independent binding indicates the presence of at least two binding classes with two ligand molecules in each and binding constants $2.8 \times 10^4$ and $3.6 \times 10^3$ M$^{-1}$ under the same conditions as for bilirubin. The affinities were independent of pH in the range from 6–10. This demonstrates that the anion rather than the acid is bound, since the pK of xanthobilirubinic acid probably is around 5.

Identical binding isotherms were obtained with non-defatted and defatted albumin.

Binding Sites for Bilirubin and Xanthobilirubinate—The following experiments were designed to elucidate whether bilirubin and xanthobilirubinate are bound competitively to albumin or to different sites. Competition of either ligand with two test substances, each probing a distinct binding function of the albumin molecule, was investigated. The two test ligands were monoacetylidiaminodiphenylsulfone and diazepam. Previous work has shown that the former binds competitively with bilirubin and independendely of diazepam, whereas diazepam is bound independently of both bilirubin and xanthobilirubinate.

Fig. 2. Binding isotherm for xanthobilirubinate to human serum albumin in 60 mM sodium phosphate buffer, pH 7.4, 37°C. The curve was obtained by least squares fitting of the data to a model of four independent sites in two classes with two sites in each, $K_1 = 2.8 \times 10^4$ and $K_2 = 3.6 \times 10^3$ M$^{-1}$. Abscissa, concentration of free xanthobilirubinate; ordinates, average number of bound ligand molecules per molecule of albumin.
Albumin-Bilirubin Binding Mechanism

FIG. 3 (left). Competition of binding of bilirubin dianion with monoacetyldiaminodiphenylsulfone, respectively diazepam, to human serum albumin. X, the concentration of available albumin for binding of monoacetyldiaminodiphenylsulfone (ordinates) determined with varying concentrations of bilirubin (abscissa) as previously described (26). O, the concentration of available albumin for binding of diazepam, similarly measured using 3H-labeled diazepam. Sodium phosphate buffer, pH 7.4, 37°C. It is seen that bilirubin, which is tightly bound to albumin, occupies an equimolar amount of the albumin binding capacity for monoacetyldiaminodiphenylsulfone but does not interfere with binding of diazepam.

FIG. 4 (right). Competition of binding of xanthobilirubinate with monoacetyldiaminodiphenylsulfone (X) and diazepam (O) studied by the same technique as in Fig. 3. Under the conditions of this experiment, xanthobilirubinate is 80% bound to albumin. The slope of the line is -0.37 and it is concluded that binding of 1 mol of xanthobilirubinate occupies 0.37/0.80 = 0.47 mol of the binding capacity for monoacetyldiaminodiphenylsulfone but does not interfere with binding of diazepam. Phosphate buffer, pH 7.4, 37°C.

and monoacetyldiaminodiphenylsulfone (26). Two binding functions of the albumin molecule can thus be distinguished, one interacting with bilirubin and monoacetyldiaminodiphenylsulfone and another binding diazepam. Results of these studies are expressed in terms of reserve albumin-equivalent concentrations, as previously defined (26) for monoacetyldiaminodiphenylsulfone, respectively diazepam, with varying amounts of added bilirubin or xanthobilirubinate (Figs. 3 and 4). It is seen that addition of bilirubin in a certain concentration causes an equimolar reduction of the albumin reserve for the binding of monoacetyldiaminodiphenylsulfone (Fig. 3). Bilirubin is almost totally bound under the conditions of this experiment which then indicates that binding of monoacetyldiaminodiphenylsulfone and bilirubin is competitive so that one molecule of monoacetyldiaminodiphenylsulfone may replace one of bilirubin and vice versa. Xanthobilirubinate is bound less firmly. At the albumin concentration used, 300 μM, 80% of xanthobilirubinate is bound when the concentration of the ligand is low. The decrease of albumin reserve for binding of monoacetyldiaminodiphenylsulfone is 0.47 times the concentration of bound xanthobilirubinate, as seen in Fig. 4. It is concluded that two molecules of xanthobilirubinate competitively occupy an amount of albumin which binds one molecule of monoacetyldiaminodiphenylsulfone or one molecule of bilirubin. Figs. 3 and 4 show further that binding of diazepam is largely independent of binding of bilirubin and xanthobilirubinate.

Co-binding of Xanthobilirubinate and Laurate—The effect of binding of fatty acid anion, laurate, to xanthobilirubinate-albumin in equilibrium mixtures was studied by spectroscopy. Mixtures of xanthobilirubinate and albumin showed spectra, as exemplified in Fig. 5. The spectra contain sizable contributions from free and bound xanthobilirubinate, one spectrum...
in Fig. 5 showing predominantly the bound ligand, the other a larger fraction of free pigment. Both spectra were sensitive to changes of binding equilibrium. Addition of laurate to these mixtures, up to 2 mol/mol of albumin, caused no measurable change of the spectra. Laurate is tightly bound to albumin under these conditions. The spectral findings indicate that two molecules of laurate and two of xanthobilirubinate can be bound independently to one albumin molecule and further that the spectrum of bound xanthobilirubinate remains unchanged by co-binding of 2 mol of laurate.

Co-binding of Bilirubin and Laurate—Similarly, the spectral effects of binding laurate to bilirubin-albumin equilibrium mixtures were studied (Fig. 6). A complex of one molecule of bilirubin and one of defatted albumin (a) shows a considerable change of the spectrum, with a red-shift of light absorption and disappearance of the shoulder, on addition of 1 mol of laurate/mol of albumin. A 2nd mol of laurate causes further changes whereas the effect of a third molecule of fatty acid anion is slight. An isosbestic point is seen at 447 nm.

Spectral Effects of pH—Solutions of xanthobilirubinate and albumin showed unchanged spectra with variation of pH within a wide range, from 6-10 (Fig. 7a). An isosbestic point is not seen in this case.

**Bilirubin Binding Kinetics**—Binding of one molecule of bilirubin dianion to human serum albumin was studied in a stopped flow apparatus by the light absorbance at 472 nm. The change of absorbance, relative to the total change, is plotted against time in Fig. 8. The bimolecular combination of bilirubin and albumin is fast and completed in less than 10 ms under the circumstances used in Fig. 8. It is followed by a train of rapid conformational changes within the first seconds, resulting in an increase and thereafter a slight decrease in absorbance. These changes account for about 1/3 of the total spectral change. A slow increase of the light absorption (from 0.271 in the actual case) follows, finally resulting in an absorbance of 0.344, reached in about 500 s.

The above experiment was repeated, using defatted albumin. A similar course was observed with only minor, quantitative differences.

The spectral changes on binding of bilirubin to albumin have been further investigated by measuring the absorbance from 1.2 s to several minutes in a series of experiments conducted at varying wavelengths. Light absorption spectra of the reaction mixture at different points of time were thus obtained, as shown in Fig. 9 where the spectrum of free bilirubin dianion is shown as well. The faster part of the process, occurring before 1 s, results in a red-shift of light absorption maximum from 438 to 460 nm. During the slow relaxations, after 1 s, the maximum remains at 460 nm while absorbances increase at wavelengths around the maximum and decrease at shorter wavelengths where the spectrum has a shoulder. An isosbestic point for the slow processes is seen at 439 nm.

**Laurate Co-binding Kinetics**—The kinetic course of the light absorption changes of bilirubin-albumin taking place on addition of laurate was investigated in the stopped flow apparatus. The processes were found to consist of fast reactions, completed before 1 s, and a considerably slower component, rather similar to the slow part of the bilirubin-albumin binding process (Fig. 8).

**Kinetics of pH-Jump**—The course of spectral changes taking place in the bilirubin-albumin 1:1 complex subsequent to a pH-jump from 6 to 9 was likewise studied by the stopped flow technique and was again found to consist of fast reactions, completed in less than 1 s, and a considerably slower increase of light absorption at 472 nm, similar to the slow part of the bilirubin-albumin binding process, as seen in Fig. 8.

The pH-jump experiment was repeated, using defatted albumin. The course observed was similar to that seen with
Albumin-Bilirubin Binding Mechanism

A 'h

FIG. 9. Light absorption spectra of a solution of bilirubin, 15 μM, and albumin, 30 μM, obtained at varying times after mixing the components in the stopped flow apparatus. The curve designated 0 sec is the spectrum of free bilirubin dianion. Tris/Pipes buffer, pH 8.8, 25 °C. It is seen that the spectrum of bilirubin within about 1 s undergoes a red-shift. Thereafter, a slow spectral change is observed with an increase of absorbance at 460 nm and a decrease of the shoulder around 420 nm. An isosbestic point is observed at 439 nm.

albumin containing the natural fatty acids except for minor, quantitative differences.

Analysis of the Slow Steps—Kinetic analysis of the slow processes, occurring after 1 s, showed that the curves are complex, significantly different from a first order course. All three reactions, following binding of bilirubin, pH-jump, and binding of fatty acid, could be interpreted as composed of three consecutive first order steps with similar rate constants in all three cases but with different numerical magnitudes of the light absorption changes of each step. After mixing of a solution of albumin, 30 μM, with an equal volume of a bilirubin solution, 15 μM, at pH 8.8 and at 6 °C, we obtained the following first order rate constants for three consecutive steps, 0.53, 0.13, and 0.031 s⁻¹. Identical rate constants were obtained with varying concentrations of the reactants as long as the molar ratio of bilirubin/albumin was less than 0.5 when the final equilibrium solutions predominantly contain the 1:1 bilirubin-albumin complex. The findings are thus consistent with the presumption that the slow changes are monomolecular relaxations. Qualitatively identical processes occur in all three cases, after binding of bilirubin to albumin, after co-binding of laurate to the complex, and after a pH-jump of the bilirubin-albumin solution. However, the analysis in three consecutive, unidirectional steps is not unique and other solutions could not be excluded.

Xanthobilirubinate Binding Kinetics—Binding of the xanthobilirubinate ion to albumin, monitored by the same technique at 440 nm, is also shown in Fig. 8. A continuous rise of absorbance was seen and was completed in about 0.5 s.

Progress curves obtained with varying concentrations of xanthobilirubinate and albumin were analyzed and appeared to consist of a very fast bimolecular binding step, completed in less than 5 ms, followed by at least two relaxational, first order reactions. We could not determine rate constants with any reasonable precision, mainly because we were unable to study the reverse process, dissociation of the complex.

A slow increase of absorbance, as the one observed for bilirubin-albumin in the time interval 1–500 s, was not seen on binding of xanthobilirubinate.

Solvent Perturbation Spectra—The light absorption spectrum of bilirubin dianion in alkaline solution, and of the bilirubin-albumin complex, is perturbed on addition of sucrose (20% by volume) to the medium. Fig. 10a shows a difference spectrum recorded with an alkaline solution of bilirubin alone, in buffer containing 20% sucrose in the sample position, and

![Diagram](http://www.jbc.org/)

FIG. 10. Difference absorption spectra. These were obtained by adding 20% (v/v) sucrose to solutions containing: a, 3.75 μM bilirubin (4-cm cells); b, 7.5 μM xanthobilirubin (4-cm cells); c, 15 μM bilirubin and 30 μM human serum albumin (4-cm cells); and d, 15 μM bilirubin and 6 μM human serum albumin (1-cm cells). Sodium carbonate bicarbonate buffer, pH 10.0, 25 °C.
a similar solution without sucrose in the reference. The main feature of this spectrum is a red-shift, as expected when a $\pi \rightarrow \pi^*$ excitation is perturbed by making the medium less polar (27). A red-shift is also observed in a similar experiment with xanthobilirubin acid (Fig. 10b). It is noted that the spectrum obtained with bilirubin is more complex. It is possible to explain this by two concerted mechanisms, 1) the solution of bilirubin contains monomer and dimer bilirubin dianions in an equilibrium (24), sensitive to changes of the medium, and 2) exciton splitting, due to the presence of two neighboring chromophores, is altered by a change of medium. The xanthobilirubinate anion is monomeric (see “Materials and Methods”) and has a single chromophore.

If one or both bilirubin chromophores in the complex with albumin are exposed to the medium, we will accordingly expect a red-shift of the light absorption spectrum on addition of sucrose to the medium. This was not found experimentally, as seen in Fig. 10c. The spectrum of bilirubin-albumin (1:1) undergoes a blue-shift on addition of 20% sucrose. Exposure of the chromophores to the medium could thus not be demonstrated. The spectral change observed has a similarity to the inverse of the slow relaxation changes seen after binding of bilirubin to albumin as well as after addition of laurate to the complex and shift of pH, as described above. It is possible, therefore, that addition of sucrose to the medium induces a conformational change in the bilirubin-albumin (1:1) complex.

The Bilirubin-Albumin 2:1 Complex—A final word should be said about the bilirubin-albumin (2:1) complex. Slow changes of light absorption occur after binding of a second bilirubin dianion to the 1:1 complex. Addition of laurate to a solution predominantly containing the 2:1 complex causes little change of the spectrum (Fig. 6b); the magnitude of the change in fact corresponds to the calculated amount of the 1:1 complex present in the equilibrium mixture, which indicates that the spectrum of the 2:1 complex remains unaltered by binding of laurate. Spectral shifts on increase of pH are equally small (Fig. 7b). Sucrose perturbation (Fig. 10d) fails to demonstrate any exposure of the chromophores in the 2:1 complex: a blue-shift is observed, somewhat smaller than with 1:1 complex. These observations indicate that slow relaxation processes occur in the 2:1 complex and that both ligand molecules are shielded from contact with the medium. On the other hand, conformational changes involved seem to be different from those taking place in the 1:1 complex. Mesobilirubin-Albumin Binding—Mesobilirubin (Fig. 1) binds to human serum albumin with high affinity, similar to the affinity for binding of bilirubin. Light absorption spectra of mesobilirubin-albumin show a maximum at 435 nm and vary with pH and with addition of laurate similarly to the spectra of bilirubin-albumin.

**DISCUSSION**

**Bilirubin-Albumin Binding Kinetics**—The course of spectral changes observed on binding of bilirubin to serum albumin has been studied in considerable detail by several groups (3-7). The results indicate that bilirubin combines with albumin in a second order process which is fast and has to be observed at low concentrations of the reactants and at low temperature. The primary binding is followed by a train of relaxations, usually analyzed in terms of consecutive, unidirectional, monomolecular reactions.

Most investigators have terminated the observations before 5 s after mixing when the rates of spectral changes have decreased to an apparent zero. The present studies show that additional, slow changes of light absorbance can be observed during the following 8 min (at 5 °C). These changes are of sizable magnitude and can be observed at pH 7.4 as well as in slightly alkaline solutions and at 5 °C as well as at room temperature. We preferred to do most of our experiments at pH 8.8 since the solubility of bilirubin is very low at pH 7.4 (25). Although bilirubin has a tendency to remain in supersaturated solution, there is a risk that a bilirubin acid colloid may be formed. The colloid suspension appears clear and yellow when observed visually. This risk is eliminated at pH 8.8. The slow changes are further independent of whether the albumin preparation contained its natural fatty acid (about 0.5 mol/mol of albumin in our series) or defatted albumin was used.

**Bilirubin-Albumin Structure**—It has previously been shown that at least two molecules of bilirubin dianion can be bound reversibly to one of albumin (2). In a model of independent binding, the two binding constants are 5.5 × 10^6 and 4.4 × 10^6 M^-1, at 37 °C in a 60 mM sodium phosphate buffer (25). The binding affinity is independent of pH within a range from 6-10, indicating that bilirubin dianion is the ligand.

Blauer and co-workers (9, 28) have studied light absorption and circular dichroism spectra of bilirubin and its complex with albumin and spectral changes with varying pH and co-binding of fatty acids. According to Blauer, the two chromophores of bilirubin in the complex with albumin are fixed at two neighboring molecules (28). Spectral changes on binding are determined by exciton splitting among the chromophores. Changes of pH or co-binding of fatty acids results in conformational changes of the albumin molecule, shifting the dihedral angle and thereby the exciton splitting which explains the observed changes of light absorption and circular dichroism. Each bilirubin chromophore must be bound to a separate part of the protein molecule. This is in good agreement with previous ideas of a flexible albumin structure containing several movable parts (29). A number of models have been proposed in order to account for these and other experimental observations (1, 10, 30). One model, based on the 6-half-domain structure of Brown (11), underlines the possibility that parts of the albumin molecule could combine in various ways, forming sites for binding of different ligands. Fig. 11 shows one possible arrangement of six half-domains, forming two bilirubin binding sites and a third site for fatty acids. In this model, changes of light absorption and circular dichroism caused by co-binding of laurate and by changes of pH are explained by rotation of one half-domain relative to the other of the same site whereby the bilirubin molecule is twisted with a change of the dihedral angle between the chromophores, resulting in a change of exciton splitting.

**Xanthobilirubinate Binding**—According to this model, xanthobilirubinate, which has only one dipyrrolic chromophore, could be bound to one half-domain. We would thus expect that xanthobilirubinate would be bound with considerably lower affinity than bilirubin and that four molecules of xanthobilirubinate would compete with two of bilirubin. We would further expect that changes on co-binding of fatty acid and on variation of pH would be absent and finally that the pattern of relaxational steps after binding of xanthobilirubinate would be simpler than that observed after binding of bilirubin since changes related to the exciton splitting among bilirubin chromophores would be absent. The present study was undertaken to verify or negate these predictions.

The xanthobilirubinate binding isotherm, Fig. 2, and the analysis of it presented above, confirm that four molecules of the xanthobilirubinate anion can be bound to human serum albumin with lower affinity than that observed for binding of bilirubin.

It proved experimentally difficult to investigate competition of binding of xanthobilirubinate and bilirubin in systems with
both ligands present, due to the spectral similarities. An indirect method was chosen. It was already known that bilirubin competes with binding of monoacetyldiaminodiphenylsulfone and that both bilirubin and monoacetyldiaminodiphenylsulfone are bound independently of diazepam (26) and the present work has shown that two molecules of xanthobilirubinate compete for a site which binds one molecule of monoacetyldiaminodiphenylsulfone while binding of xanthobilirubinate and diazepam is independent. This indicates that two molecules of xanthobilirubinate are bound to one bilirubin site. The location of binding of the other two xanthobilirubinate molecules remains unknown except that they are not bound to the other main binding function of the albumin molecule, that interacting with diazepam, which leaves the possibility open that the other xanthobilirubinates compete with the second bilirubin molecule.

Spectral changes of xanthobilirubinate-albumin complexes are not observed on co-binding of laurate or on variation with pH. This finding seems to confirm that one xanthobilirubinate anion is in fact bound to one albumin half-domain. The two half-domains which form the first bilirubin binding site in the bilirubin-albumin complex may accordingly be situated at a distance in complexes with xanthobilirubinate and exciton splitting does not take place. This again is in keeping with the simple one-maximum spectrum of xanthobilirubinate-albumin (Fig. 5), as opposed to the maximum and shoulder seen in bilirubin-albumin (Fig. 9).

Slow spectral changes, as those observed later than 1 s after mixing bilirubin and albumin, were totally absent after binding of xanthobilirubinate (Fig. 8).

**Mesobilirubin Binding**—A few experiments were performed with mesobilirubin in order to investigate whether the differences of binding behavior between bilirubin and xanthobilirubinate could be related to the presence of vinyl groups in bilirubin where xanthobilirubinate has an ethyl side chain (Fig. 1). Mesobilirubin, with its two ethyl groups, binds to albumin with high affinity and shows similar spectral shifts as observed for bilirubin. The light absorption spectrum of mesobilirubin-albumin shows a maximum and a shoulder, as seen for bilirubin-albumin, and changes similarly on addition of laurate and variation of pH. It seems safe to conclude that mesobilirubin is bound similarly to bilirubin and shows the same phenomena of exciton splitting. The different behavior observed with xanthobilirubinate can thus be ascribed to the presence of a single dipyrrolic chromophore, rather than to the absence of a vinyl double bond.

**Conclusions**—In terms of the model, Fig. 11, we may presume that the slow changes are due to rotation of one half-domain relative to the other of the same site, resulting in a change of exciton splitting among the bilirubin chromophores. The presence of an isosbestic point for the slow spectral shift (Fig. 9) is in agreement with this single mechanism. The complex nature of the time course, with at least three first order components, can be explained if the rotation of the binding half-domains is caused by elastic forces originating from relaxational movements in several other parts of the albumin molecule. The weak nature of such forces from distant areas of the protein and the large mass of the binding half-domains may account for the low velocities observed.

The slow relaxational steps observed after co-binding of laurate and after a pH-jump can be explained by the same mechanism.

The solvent perturbation spectra of the bilirubin-albumin 1:1 and 2:1 complexes (Fig. 10) failed to demonstrate any exposure of the chromophores to the medium, in good agreement with the model.

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