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The kinetics of interactions of bilirubin with lipid bilayers and with serum albumin

Noa Noy, Maurice Leonard and David Zakim

Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, NY 10021 (USA)

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Abstract

Rate constants for the hydration of bilirubin bound to unilamellar bilayers of dioleoylphosphatidylcholine and albumin were measured by stopped-flow methods. Rate constants for association of bilirubin with these vesicles and albumin were calculated from measured rate constants for dissociation and the equilibrium binding constants of bilirubin and lipids or albumin. Rate constants for hydration (dissociation) for bilirubin bound to dioleoylphosphatidylcholine and albumin were 71 s^{-1} and 1.8 s^{-1} respectively. Rate constants for association were 4.0 10^7 s^{-1} and 1.1 10^9 M^{-1} s^{-1}, respectively. Both rates for interactions of bilirubin with bilayers were essentially independent of temperature in the range 0–40°C, indicating that barriers to entry and exit of bilirubin from bilayers were entropic. Rates of transbilayer movement of bilirubin in dioleoylphosphatidylcholine were too fast to resolve by measuring rates of hydration of bilirubin. Rate constants for hydration of bilirubin bound to bilayers with less avidity for bilirubin as compared with dioleoylphosphatidylcholine also were too fast to measure with stopped-flow methods. In addition to providing details of the energetic basis for interactions between bilirubin and membranes, the data allow for calculating the maximal rates at which bilirubin could transfer spontaneously from sites on albumin in blood to the interior of cells. The data show, in this regard, that this rate is 10–50 fold faster than measured rates of uptake of bilirubin by intact liver.

Keywords: Bilirubin; Dioleoylphosphatidylcholine; Binding kinetics; Hydration rate constant; Unilamellar vesicles; Stopped flow fluorescence

1. Introduction

The mechanism by which small molecules, e.g. the size of phospholipids enter and traverse synthetic lipid membranes or biological membranes depends on the hydrophobicity of the molecule. Molecules that are readily hydrated by water, for example, will not partition directly into membranes. Entry and transmembrane movement depend on facilitated mechanisms. By contrast, molecules with limited solubility in water generally will be solvated directly by the polymethylene interior of membrane bilayers and hence will enter membranes spontaneously. although this is well-known, there are few detailed studies of the

Correspondence to: Dr. N. Noy, Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, NY 10021 (USA).

energetics for solvation of hydrophobic substances by lipid bilayers or biological membranes.

The general treatment of the partitioning of water insoluble molecules into lipid bilayers and biological membranes usually is to regard this event as analogous to partitioning of substances between bulk phases. The interior of a membrane is not like a bulk phase, however, because its properties are not isotropic [1,2]. It is quite likely that solvation of water-insoluble compounds by membranes depends on the size and shape of the molecule as well as the detailed structure of the polymethylene interior of the membrane [2–5]. Membranes of different composition could solvate a given compound with selectivity not expected for partitioning between bulk phases [2].

Bilirubin is a physiologic compound of interest in the context of solvation of small molecules by lipid bilayers. It is the catabolic product of heme metabolism; and when present in pathologic amounts, it is toxic [6]. Although bilirubin has several polar groups, bilirubin IXa, the physiologic isomer of bilirubin, behaves like a non-polar compound and has limited solubility in water at physiological pH [7] because the polar groups of bilirubin IXa are internally H-bonded [7,8]. Interestingly, bilirubin IXa is also poorly soluble in many organic solvents, e.g. ether, hexane and ethanol [7]. These observations, together with reported results from studies on the interactions of bilirubin IXa with membranes [9–12] have cast doubt on the idea that the interactions of bilirubin with membranes arise mainly from the solvation of the molecule within the apolar region of membranes. The thermodynamics for partitioning of bilirubin between water and lipid bilayers suggest, however, that bilirubin IXa partitions into the apolar region of model membranes over a range of concentrations from normal to pathologic [13]. High pressure infrared spectroscopic studies have confirmed that bilirubin IXa interacts with the polymethylene chain region of lipid bilayers and have established the orientation of the molecule in bilayers [14]. These conclusions fit with prior data showing that bilirubin freely crosses lipid bilayers, indicating that the molecule dissolves readily in bilayers, diffuses rapidly through them, and dissociates rapidly from lipid bilayers into an aqueous medium [15].

There are no quantitative measurements of the rates of reactions between bilirubin and bilayers. We report in this paper measurements of the rate constants for reactions that determine the solvation of bilirubin IXa in membranes, e.g., rates of hydration of bilirubin bound to lipid bilayers, rates of binding of bilirubin to these structures, and upper limits for the rate of transmembrane movement. In addition, the rate of binding of bilirubin IXa to albumin, which is the carrier protein for bilirubin in blood, and rate of hydration of bilirubin bound to albumin have been measured. These data provide details of the energetics for the associations between bilirubin and membranes or albumin. The data also may have physiologic utility because they give the spontaneous rates for all reactions in the pathway for transfer of bilirubin from sites on albumin in blood to the inside of cells.

2. Materials and methods

Bilirubin was purchased from Fluka and its purity determined by TLC [16]. Lipids were from Avanti Polar Lipids, fatty acid-free bovine serum albumin was from Calbiochem, and 2-(3-[diphenylhexatrienyl]) propanoyl phosphatidylcholine (DPH-PC) was from Molecular Probes. All other chemicals were from Sigma Chemical Co. Male Wistar rats were from Charles River Laboratories.

2.1 Membranes

Unilamellar vesicles (ULVs) were prepared by sonication. Lipids were co-mixed in hexane and the organic solvent was evaporated under nitrogen. After addition of buffer containing 100 m*M* KCl, 10 m*M* tris and 1 m*M* ascorbate at pH 7.4, the suspension was sonicated to clarification. Vesicles were centrifuged at 100,000 *g* for 15 min in order to pellet traces of titanium from the sonicator probe and multilamellar lipids. Plasma membranes from rat liver were isolated on den-

sity gradients according to [18]. Concentrations of phospholipids in vesicles and membranes were determined after hydrolysis by analyzing inorganic phosphate content [17].

2.2 Transfer of bilirubin between unilamellar vesicles or between vesicles and albumin

Bilirubin was freshly prepared in 10 m*M* NaOH and discarded after 2 h. Bilirubin solutions and reaction mixtures were kept in the dark to minimize decomposition, and work was carried out in dim light. Transfer of bilirubin was monitored by following fluorescence changes in a Durum stopped-flow apparatus that is connected to an OLIS computer (Georgia). Data collection and analysis were done using OLIS software. Vesicles of dioleoylphosphatidylcholine (5–12 μmol/ml) containing DPH-PC at a molar ratio of 1% were prepared by co-sonication. The absorption spectrum of bilirubin which shows maximal absorption at 454 nm extensively overlaps with the emission spectrum of DPH which has a maximum at 430 nm. Transfer of bilirubin to bilayers containing DPH results therefore, in extensive quenching of probe fluorescence. Quenching was found to be linearly related to bilirubin concentration up to a bilirubin/DPH molar ratio of 0.5. All experiments were performed in this range. Bilirubin was mixed with dioleoylphosphatidylcholine (DOPC) vesicles. This population of vesicles was mixed in the stopped-flow apparatus with vesicles containing DPH-PC and transfer of bilirubin to the later was followed by the decrease in fluorescence. Excitation was at 363 nm and emission was detected through a 450 nm wide-pass filter. To determine whether the inclusion of DPH-PC in the vesicles affected the rate of transfer of bilirubin between vesicles, transfer was studied in the reverse direction. Bilirubin was added to vesicles containing DPH-PC and these vesicles were mixed with "empty" DOPC vesicles. Transfer in this case was monitored by the time-dependent increase in fluorescence as bilirubin moved away from probe-containing vesicles. To test whether quenching of DPH-PC fluorescence resulted from movement of the probe out of acceptor vesicles, transfer of DPH-PC from vesicles to rat liver microsomes was studied. Vesicles containing DPH-PC were mixed with rat liver microsomes and the mixture incubated for 1 h at room temperature. The mixture was then centrifuged at 100,000 *g* for 1 h in order to pellet the microsomes. The fluorescence of the supernatant, which contained the vesicles, was measured and compared to intensity of fluorescence of the vesicles before the addition of microsomes. It was found that in 1 h, 4% and 20% of the fluorophore transferred out of vesicles that were incubated with microsomes at mole ratios, respectively, of 10/1 and 1/1, vesicles lipid/microsomal lipids. It was concluded from these observations that no significant transfer would occur within the experimental time frame required for the transfer reaction to reach equilibrium, which was less than 1 s. In addition, the stopped flow experiments were set so that 50–95% of bilirubin used transferred from donor to acceptor vesicles at equilibrium. The observed change in fluorescence in the stopped flow experiments were appropriate for this extent of transfer, which is further evidence that quenching of the fluorophore observed upon mixing of donor and acceptor vesicles was due to transfer of bilirubin to the acceptor vesicles and not to movement of the probe out of them.

Transfer of bilirubin from albumin to vesicles was similarly studied by monitoring the decrease in fluorescence after mixing a solution containing albumin and bilirubin with vesicles containing DPH-PC.

2.3 Measurement of equilibrium constants

The partition constants of bilirubin (K_{eq}) between plasma membranes from rat liver and an aqueous phase, between plasma membranes and vesicles, and between plasma membranes and albumin were determined as follows. The designated phases were mixed in 1 ml of buffer containing 10 m*M* Tris, pH 7.4, 100 m*M* KCl, and 1 m*M* ascorbate. Bilirubin in 10 m*M* NaOH was added. The molar ratio of bilirubin to lipids never exceeded 0.07. The suspension was incubated for 30 min. and plasma membranes were separated from other phases (vesicles, albumin, or aqueous

phase) by centrifugation in a No. 40 Beckman rotor at 39,000 rpm for 30 min. This was sufficient to precipitate the plasma membranes, as was validated by measurement of the phospholipid content of the supernatant. Bilirubin, in the supernatant and in the pellet, was measured by extraction into chloroform/methanol (2/1, v/v) followed by determination of absorbance at 454 nm. The molar extinction coefficient of bilirubin was 59,700. As reported previously [13], the partition coefficients of bilirubin between membrane populations are constant within the concentration range of bilirubin used (2.5–25 μM), indicating that bilirubin does not form dimers in this concentration range. The experiments in which partition constants were measured, were set so that the amount of bilirubin in the supernatant was high enough to be accurately measured. To do so, the ratio of lipids in vesicles vs. lipids in plasma membranes were varied. For example, since the affinity of plasma membranes for bilirubin is about 50 fold higher than the affinity of pure lipid vesicles ([13] and see below), the reaction mixtures contained 200 nmol of plasma membrane lipids and 2200 nmol of vesicle lipids. This resulted in retention of about 15% of bilirubin in the supernatant, which could be accurately measured.

All measurements were performed at 23°C unless indicated otherwise.

3. Results

3.1 Transfer of bilirubin between unilamellar vesicles

This process was studied in vesicles comprised of DOPC. As detailed in Section 2, the fluorescent lipid DPH-PC was incorporated into DOPC

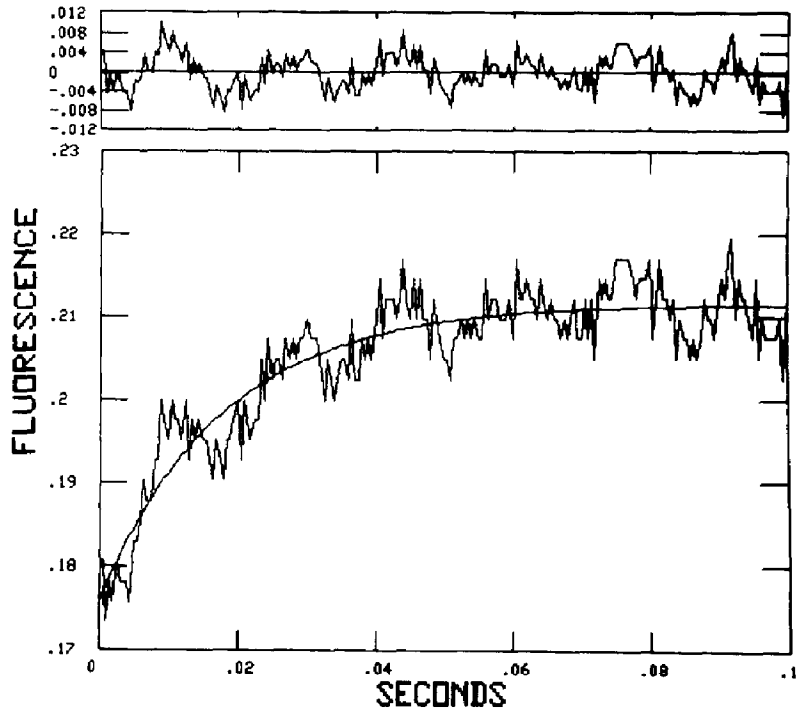

Fig. 1. Transfer of bilirubin between unilamellar vesicles of DOPC. Donor vesicles contained 2 mol% DPH-PC. Bilirubin was added to the donor vesicles from a 1 mM solution in NaOH (see Section 2). Acceptor vesicles consisted of ULVs of DOPC. Donor and acceptor vesicles were mixed in the stopped-flow spectrofluorometer. Final concentrations were 100 nmol/ml of phospholipids in each of the vesicle populations and 0.83 nmol/ml bilirubin. Buffer was 50 mM phosphate, pH 7.4. Transfer of bilirubin from donor to acceptor was monitored by the time-dependent increase in fluorescence. Excitation wavelength was 363 nm, emission was detected through a wide-band 450 nm filter. Upper panel shows the residuals calculated for the computer fitted first order reaction. A representative trace is shown.

by sonication. Acceptor vesicles of DOPC plus DPH-PC were mixed in a stopped-flow apparatus with donor vesicles of DOPC containing bilirubin. The transfer of bilirubin from donor to acceptor vesicles was monitored by quenching of fluorescence following the transfer of bilirubin from donor to acceptor vesicles (Fig. 1). Transfer also was followed in the reverse direction. Donor vesicles, in this case, were constituted of DOPC, DPH-PC, and bilirubin; acceptor vesicles were comprised of DOPC. Movement of bilirubin was followed by the time-dependent increase in fluorescence. Transfer in both directions fit a single first order reaction with a rate constant $k = 71 \pm 3.2\ s^{-1}$, $t_{1/2} = 10$ ms ($n = 10$). This rate is at the limit of resolution by the stopped-flow method; and in fact a considerable proportion of the reaction was completed during the dead-time of the instrument. Nevertheless, data presented later show that this rate constant for transfer of bilirubin between vesicles of DOPC gives a good estimate of the partition constant for the distribution of bilirubin between ULVs of DOPC and albumin. The rate constant for transfer was highly replicable (standard error of measurement was 10% of the value, $n = 6$) and was the same for transfer of bilirubin from ULVs of DOPC to ULVs of DOPC plus DPH-PC and vice versa. The last finding indicates that including DPH-PC in vesicles of DOPC did not affect the rate of transfer of bilirubin between vesicles at the probe/lipid (molar) ratio used in the experiment.

3.2 The rate constant for dissociation of bilirubin from bilayers of DOPC

Transfer of bilirubin between vesicles could occur, theoretically, via two mechanisms. Bilirubin could dissociate from the donor bilayer into the aqueous phase, diffuse to an acceptor bilayer and associate with it. Alternatively, bilirubin could be transferred directly between vesicles during collisions. If the later mechanism applied, the rate of transfer would increase as the ratio of [acceptor]/[donor] increased. Varying this ratio between 1:1 to 20:1, by increasing the concentration of the acceptor vesicles, had no effect on the rate of transfer of bilirubin. It can be concluded, therefore, that bilirubin moved between unilamellar vesicles *via* the aqueous phase and that the following scheme represented the transfer process:

$$B - (ULV)_D (\text{in } H_2O) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B - (H_2O)_n \qquad (1)$$

$$B - (H_2O)_n + (ULV)_A \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} B - (ULV)_A \qquad (2)$$

B in reactions (1) and (2) is bilirubin. A and D designate acceptor and donor vesicles respectively. In this particular case, since the donor and acceptor vesicles were identical, $k_1 = k_2$ and $k_{-1} = k_{-2}$.

Partitioning of bilirubin between aqueous phase and lipid bilayers ($K_{eq} = k_{-1}/k_1$) considerably favors the bilayers, i.e. k_{-1} is much larger than k_1. Therefore, it is reasonable to conclude that the rate determining step in reactions (1) and (2) was the hydration of bilirubin present initially in the bilayer. The rate constant for this first order reaction (k_1) can thus be obtained directly from the rate of transfer of bilirubin between the vesicles and was found to be $71\ s^{-1}$.

3.3 The rate of trans-membrane movement of bilirubin

To investigate whether the rate of trans-membrane movement of bilirubin was a rate determining step for transfer of bilirubin between bilayers, transfer was monitored upon mixing ULVs of DOPC plus bilirubin with a 100 fold excess of ULVs of DOPC plus DPH-PC. In this experiment, 99% of bilirubin present initially in the donor vesicles will be transferred to acceptor vesicles at equilibrium. If trans-membrane movement were slower than hydration, then bilirubin embedded in the inner leaflet of the donor bilayer would be transferred to acceptor ULVs at a slower rate then bilirubin in the outer leaflet. The progress curves for the transfer, in this case, would show two distinct rates. We took precautions in the design of these experiments that bilirubin added to vesicles of DOPC was associated initially with the two leaflets of the donor bilayer. Bilirubin was added to preformed vesicles

and the mixture was sonicated for 1 min. The rate of transfer of bilirubin from these vesicles to acceptor vesicles of DOPC was then monitored. The rate constant for transfer in this experiment was 72 s^{-1}. Analysis of the data showed that the complete time course for transfer was fitted by a single first order rate constant. These data indicate, therefore, that trans-membrane movement of bilirubin must have been much faster than solvation (reaction (1)). This conclusion is expected on the basis of the structure of bilirubin (no exposed groups that will form hydrogen-bonds to water) and the location of bilirubin within lipid bilayers (completely within the region of the polymethylene chains [14]).

3.4 Effect of temperature on the rate of transfer of bilirubin between vesicles

To gain further insight into the nature of the interactions between bilirubin and lipid bilayers, the rate of transfer of bilirubin between two populations of DOPC vesicles was measured at various temperatures. Figure 2 shows the temperature dependence of the rate constant for dissociation of bilirubin from vesicles of DOPC in the form of an Arrhenius plot (log k_1 *vs* $1/T$). The energy of activation of the dissociation reaction (E_a) obtained from the data in Fig. 2 was 1.5 kcal/mol. The enthalpy ($\Delta H^{\#}$) and the entropy ($\Delta S^{\#}$) of activation for the dissociation reaction at 20°C were calculated using expressions (I) and (II):

$$\Delta H^{\#} = E_a - RT \qquad \text{(I)}$$

$$\Delta S^{\#} = R \ln (NhX/RT),$$

$$\text{where } X = k_1/e^{-\Delta H/RT} \qquad \text{(II)}$$

T in expressions (I) and (II) is the temperature in K, R, N, and h are the universal gas constant, Avogadro's number, and Planck's constant, respectively. The value of $\Delta H^{\#}$ was 0.94 kcal/mol, and $\Delta S^{\#}$ was 48.2 cal/mol K, so that $T\,\Delta S^{\#}$ was -14.1 kcal/mol. These values indicate that the enthalpy of activation for transfer of bilirubin from the membrane interior to the aqueous phase was quite small, and that the rate of this process was determined almost exclusively by the entropy of activation.

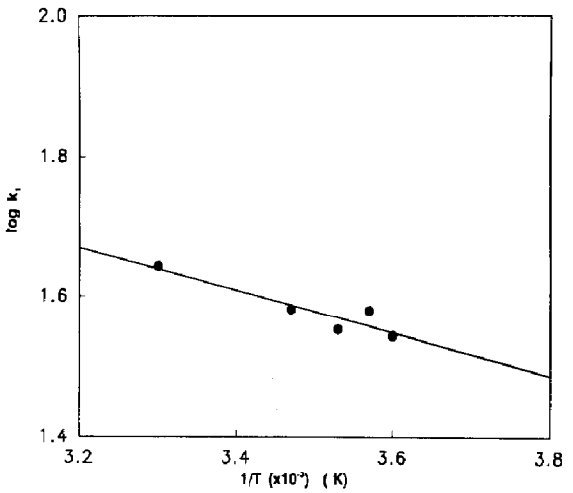

Fig. 2. Temperature dependence of the rate constant of the hydration of bilirubin from vesicles of DOPC. Transfer of bilirubin from vesicles of DOPC containing 2 mol% DPH-PC to donor vesicles were measured is in Fig. 1 at various temperatures. Rate constants were calculated by the on-line computer. Each point represents a mean of 4–6 measurements, standard errors of measurents were 6–20% of the values.

We have shown previously that the partitioning of bilirubin between various lipid bilayers and biological membranes is independent of temperature [13] and thus that the selective affinity of bilirubin for different bilayers is determined predominantly by entropic parameters. The kinetic data presented here show that the rate of dissociation of bilirubin from bilayers also is determined by entropic factors. Since the affinity of a bilayer for a ligand is determined both by the rate of association and by the rate of dissociation, $K_{eq} = k_1/k_{-1}$, these observations, taken together, indicate that k_{-1} will also be independent of temperature. It can be concluded, therefore, that the interactions of bilirubin with lipid bilayers and membranes, as well as its interactions with water are not enthalpic and that the transfer of bilirubin from an aqueous phase to membranes is driven by an increase in entropy.

3.5 Rate constant for association of bilirubin with vesicles of DOPC

This rate constant can be calculated using data obtained for reaction [1]. The partition coefficient

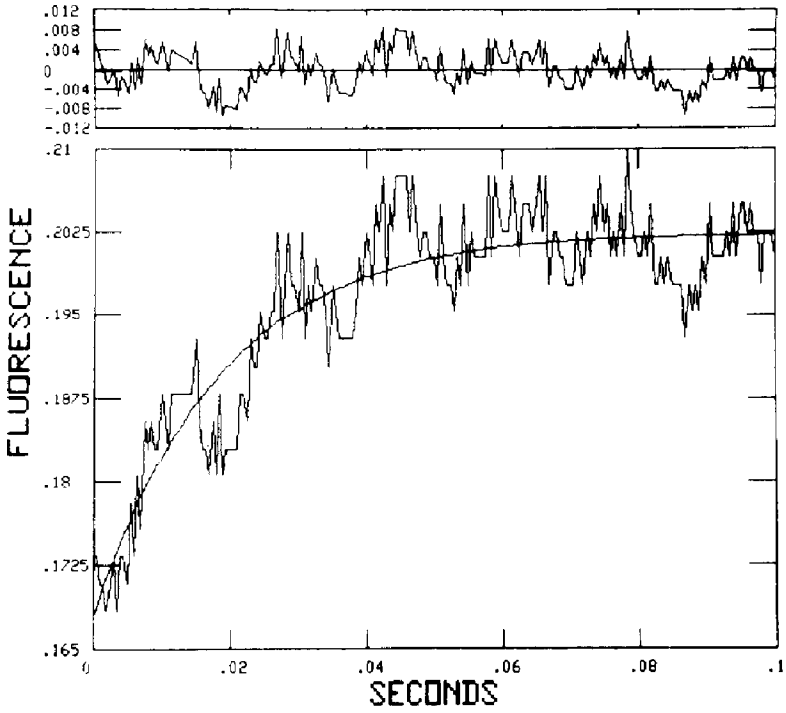

Fig. 3. Transfer of bilirubin from DOPC vesicles to albumin. Bilirubin was added to DOPC vesicles containing 2 mol% DPH-PC. Vesicles were mixed with albumin in the stopped-flow apparatus. Final concentrations: 100 nmol of DOPC, 0.83 nmol bilirubin and 167 nmol albumin. Transfer was followed by the increase in fluorescence as described for the experiment in Fig. 1. A representative trace is shown.

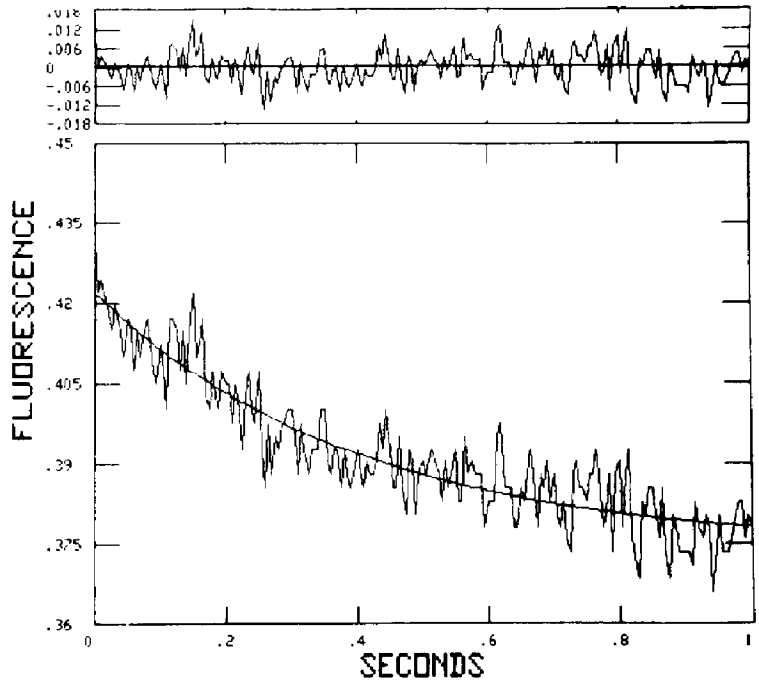

Fig. 4. Transfer of bilirubin from albumin to DOPC vesicles. Bilirubin was added to albumin from a 1 m*M* solution in NaOH. The albumin/bilirubin solution was mixed with DOPC vesicles containing 2 mol% DPH-PC. Final concentrations were as in the experiment in Fig. 2. A representative trace is shown.

for the distribution of bilirubin between membranes and the aqueous phase is $K_{eq} = k_1/k_{-1}$. It is technically difficult, however, to measure K_{eq} for the distribution of bilirubin between pure lipid vesicles and the aqueous phase because of the difficulty in quantitative removal of the vesicles from the aqueous phase. Methods like gel filtration for separating vesicles from water are not suitable for these experiments because they lead to a continuous flux of bilirubin from vesicles to water as the components are separated on the gel bed. The equilibrium constant K_{eq} was measured, therefore, by the following method. First, the partition constant of bilirubin between plasma membranes from rat liver and the aqueous phase was determined as described in detail in Section 2. Bilirubin and plasma membranes were mixed together for 20 min to reach an equilibrium distribution of bilirubin between membranes and the aqueous phase. Plasma membranes then were precipitated by centrifugation, and the amounts of bilirubin in the supernatant and in the pellet were determined as described in Section 2. Measurements of phospholipid in the supernatant confirmed that all plasma membranes precipitated by centrifugation. For the distribution of bilirubin between plasma membranes and the aqueous phase, presented as the ratio of molal fractions of bilirubin in the membrane and in the aqueous phase, K_{eq} was $1.3\ 10^7$ ($n = 3$). To determine K_{eq} for the distribution of bilirubin between the aqueous phase and vesicles of DOPC, bilirubin was equilibrated with vesicles of DOPC and plasma membranes for 20 min. Plasma membranes were precipitated by centrifugation, and the amounts of bilirubin in the pellet (associated with the membranes) and in the supernatant (associated with DOPC vesicles and free in the aqueous phase) were determined. The amount of bilirubin in the aqueous phase was calculated from the amount in the pellet and the partition constant between plasma membranes and the aqueous phase. The amount of bilirubin associated with DOPC vesicles was determined by subtracting the amount in the aqueous phase from total bilirubin found in the supernatant. Concentrations of bilirubin in various phases were expressed as mol fractions in the medium or phospholipids since this avoids assumptions about the volume of the various phospholipid phases. For the distribution of bilirubin between vesicles of DOPC and the aqueous phase K_{eq} was $5.6\ 10^5$ (mean of 3 measurements). The rate constant for transfer of bilirubin from the aqueous phase to bilayers of DOPC could then be calculated to be $k_{-1} = K_{eq}\ k_1 = 4\ 10^7\ s^{-1}$.

3.6 Rate constant for dissociation of bilirubin from different bilayers and natural membrane

Attempts to measure the rate of dissociation of bilirubin from vesicles of egg phosphatidylcholine (PC), which have a lower affinity for bilirubin as compared with vesicles of DOPC (see below), showed that k_1 for bilirubin in unilamellar vesicles (ULVs) of egg PC was too fast to measure by the Stopped-flow technique. This result was expected because bilirubin partitions preferentially into DOPC in a mixture of ULVs comprised either of pure DOPC or egg PC [13]. On the other hand, equilibrium partitioning data are not a good basis for calculating exact rates of hydration of bilirubin bound to different bilayers because we have no independent validation that rates of entry of bilirubin into bilayers do not depend on the composition of bilayers. In fact, as discussed below, there is good reason to believe that values of k_{-1} for bilirubin will depend on the composition of bilayers. All that can be concluded at this time, therefore, is that values of k_1 can be different for different lipid bilayers and that k_1 for the complex DOPC–bilirubin is smaller than for the complex egg PC–bilirubin.

3.7 Transfer of bilirubin between albumin and bilayers

Transfer of bilirubin between albumin and lipid vesicles can be described by reactions (3) and (2),

$$\text{B-alb(in water)} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{B} - (\text{H}_2\text{O})_n + \text{alb} \qquad (3)$$

$$\text{B} - (\text{H}_2\text{O})_n + \text{ULV} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{B} - \text{ULV} \qquad (4)$$

in which 'alb' is serum albumin. Since k_2 and k_3 are much smaller then k_{-2} and k_{-3}, the rate determining step for transfer of bilirubin from albumin to vesicles will be the solvation of bilirubin bound to albumin. Transfer can thus be expected to follow first order kinetics characterized by the rate constant k_3. Transfer of bilirubin, initially associated with bilayers, to albumin similarly should follow first order kinetics with the rate constant k_2. Transfer of bilirubin between albumin and vesicles of DOPC plus DPH-PC was followed in a stopped-flow apparatus as described in Section 2. Figures 3 and 4 show, respectively, representative traces obtained when bilirubin, initially associated with bilayers, was mixed with albumin and when bilirubin initially bound to albumin was mixed with ULVs. The time courses for both reactions were fitted best by a single first order rate constant. The rate constant obtained for transfer of bilirubin from ULVs of DOPC to albumin was 69 ± 1.6 s^{-1} ($n = 8$). This value is essentially identical to the rate constant obtained from measurements for transfer of bilirubin between vesicles of DOPC (k_1) validating that hydration of bilirubin off the bilayers is the rate determining step of this reaction and that no direct interactions between donor and acceptor phases are required. The forward rate of reaction (3) k_3 was 1.79 ± 0.11 s^{-1} ($n = 8$), $t_{1/2} = 0.387$ s. This rate constant for the dissociation of the bilirubin–albumin complex is two orders of magnitudes faster than the value reported by Faerch and Jacobsen [22], but in good agreement with the dissociation rate measured by Reed [23]. If the above analysis is correct, i.e. the steps of hydration of bilirubin bound to lipid vesicles and albumin are rate determining for transfer of bilirubin between these two phases, then the partition constant for bilirubin between albumin and ULV of DOPC should be given by k_2/k_3, which is 40 in favor of albumin. The partition coefficient could not be measured directly because of the difficulty in separating albumin from ULVs. It was obtained, therefore, from the ratio of equilibrium constants for the partitioning of bilirubin between ULVs of DOPC and plasma membranes and between albumin and plasma membranes. The calculated K_{eq} for partitioning of bilirubin between ULVs of DOPC and albumin was 50 in favor of albumin. This value is in excellent agreement with the K_{eq} calculated above from the rate constants for dissociation of bilirubin from ULVs of DOPC and albumin. This agreement supports the accuracy of the measured rate constant for dissociation of bilirubin from ULVs of DOPC despite the fact that the measured rate constant was at the limit of resolution of the stopped-flow method.

The rate constant for association of bilirubin with bovine serum albumin (k_{-3}) can be calculated from the rate constant for dissociation (k_3) and the binding constant between albumin and bilirubin (K_b), by using the expression $K_b = k_{-3}/k_3$. The binding constant of bilirubin to albumin was measured by the partitioning of bilirubin between plasma membranes of rat liver, albumin, and the medium, as described above for vesicles of DOPC. It was found that K_b equals 6 10^8 M^{-1} (mean of 3 measurements). The rate constant for association of bilirubin with albumin could then be calculated and was found to be 1.1 10^9 M^{-1} s^{-1}. This value indicates that the association of bilirubin with albumin is a diffusion-limited reaction.

4. Discussion

The results presented above show that the rate constant for the hydration of bilirubin from membranes was only slightly dependent on temperature. The free energy for activation of the hydration reaction (reaction (1)), hence has a small enthalpic component, implying that there are limited interactions between bilirubin and its environment within membranes. Since differential partitioning of bilirubin between different membranes is temperature independent [13], the rate of entry of bilirubin into membranes also must be essentially temperature independent, which implies no enthalpic component for removal of bilirubin from water. These conclusions fit with the idea proposed earlier that bilirubin intercalates into void volumes within membranes [13] and thus has limited interactions with either the aqueous or polymethylene environment [14]

within membranes. Moreover, the idea that bilirubin intercalates into void volumes within membranes that are created by mismatch in packing can account for the apparent disparity in the poor solubility of bilirubin in bulk phase organic solvents [5] versus high solubility in lipid bilayers [13]. Whether or not a molecule of bilirubin will remain solvated within the bilayer or move into water, or vice versa, appears to depend, therefore, on the statistical likelihood that a void volume large enough to allow for diffusive movement of bilirubin appears first in the bilayer or water phase. Differences in the partitioning of bilirubin between various lipid bilayers and between rates of hydration of bilirubin bound to different membranes can be understood on the basis of differences in void volumes in different bilayers. Both k_{-1} and k_1 should depend, therefore, on the lipid composition of bilayers. The data above show this is true for k_{-1}; but as yet we have no direct data for values of k_{off} as a function of membrane composition.

It is interesting to consider, in the context of the entropically driven entry of bilirubin from water into bilayers, the differences between the interactions of membranes with bilirubin and long chain fatty acids. In contrast with bilirubin, long chain fatty acids have a carboxyl group that is free to interact with water. The presence of this group on fatty acids should affect interactions with membranes. For example, fatty acids should enter membranes only in a highly oriented manner, with the methyl terminal tail pointing to the interior of the membrane. Bilirubin, in contrast, is hydrophobic on all surfaces and should enter the membrane with less. constraint of orientation. It can be predicted from these considerations that bilirubin will enter membranes faster than long chain fatty acids. In addition, whereas fatty acids will have limited orientational freedom within the membrane, because the carboxyl group will be at the membrane–water interface, the orientation of bilirubin in membranes will not be limited in this way. One would predict then, that the entropy gain for transfer of a molecule of bilirubin to a membrane, independent of interactions with water should be greater than for the corresponding transfer of a long chain fatty acid. Both of these predictions are verified by the data presented above. For example, the equilibrium constants for binding of bilirubin and fatty acids to albumin are nearly the same [19–23]; but the partition coefficient for distribution of bilirubin between albumin and DOPC is 40/1 in favor of albumin. This distribution for long chain fatty acids is 250/1 [24,25]. This difference can originate either from a slower rate of dissociation of bilirubin from membranes or from a faster rate of association of bilirubin with membranes vs. fatty acids. The rate constant for hydration of bilirubin from membranes is larger for bilirubin than for fatty acids when comparisons are made for the identical membranes (the present work and [24]). It seems then, that the greater avidity of membranes for bilirubin vs. for fatty acids is due to the faster rate at which bilirubin enters membranes. We do not know whether the entry of fatty acids into membranes has an enthalpic component. The rate constants for hydration of fatty acids in membranes, however, are temperature-dependent [24]; so there is an enthalpic component to the activation process for release of fatty acids from membranes. This is reasonable in view of the potential for electrostatic interactions between the carboxyl group of fatty acids and other membrane components and that the fatty acids should have favorable Van der Waals interactions with the acyl chains of the membrane phospholipids.

The data presented above have implications for understanding the physiology of bilirubin transport into cells. The kinetic data allow one to calculate maximal rates at which bilirubin can move spontaneously between albumin in blood and the cytosol of cells. The calculated spontaneous rates for each of the steps in this transfer are shown in Table 1 for a physiologic concentration of bilirubin in blood. The slowest step in the overall transfer is the dissociation of bilirubin from albumin. The observed rate of uptake of bilirubin by liver *in vivo* [6] is about 12–50 fold slower than the rate of dissociation of bilirubin from albumin. Clearly, none of the initial reactions in the process of movement of bilirubin from binding sites on albumin to the interior of cells will limit the rate of uptake of bilirubin by

Table 1

Comparison of maximal spontaneous rates [a] of individual steps of transfer of bilirubin from albumin to membranes with actual rates of uptake by the liver

Compartment	[B] in Compartment (nmol/g liver)	Transfer step	Spontaneous rate (nmol/min·g liver)
Albumin	3.0	$Alb-B+H_2O \rightarrow Alb+H_2O-B$	4.2
Plasma water	$1.8\ 10^{-4}$	$H_2O-B+PM \rightarrow H_2O+PM-B$	9890
Plasma membrane	3.4	flip-flop	> 47120
Observed rate of uptake by liver			0.08–0.36

[a] Spontaneous rates for each step of transfer were calculated from the expression $v = k$ [B]. Values of k's were the rate constants obtained in this work. The rate constant for "flip-flop" of bilirubin across the plasma membranes was assumed to be similar to this step across the bilayer of ULVs of DOPC, which is an upper limit for this value. The concentration of bilirubin [B] bound to albumin and free in plasma and observed rates for uptake of bilirubin from liver are based on data in [6]. [B] in blood was taken as 8.5 μM. The volume of sinusoids/g liver of was 0.35 ml [26]. The binding constant of bilirubin–albumin was $3\ 10^7\ M^{-1}$ (see text). The concentration of bilirubin in plasma membranes (PM) was calculated from K_{eq} for the distribution of bilirubin between albumin and plasma membranes, which was 14.5 (ratio of mol fraction of bilirubin bound to albumin and in plasma membrane phospholipids). Concentrations were normalized to 1 g liver on the basis of 3200 nmol plasma membrane phospholipid/g liver [26].

liver. There appears to be no basis, therefore, for proposing that any of these steps needs to be facilitated in order to account for observed rates of uptake of bilirubin *in vivo*.

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