Mechanism of the Spontaneous Transfer of Unconjugated Bilirubin between Small Unilamellar Phosphatidylcholine Vesicles†

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Received November 4, 1991; Revised Manuscript Received January 10, 1992

ABSTRACT: Unconjugated bilirubin (bilirubin-IXα), the hydrophobic end product of heme degradation, is esterified in the hepatocyte endoplasmic reticulum to water-soluble conjugates prior to excretion in bile. To characterize the process of intracellular bilirubin transport, the kinetic and thermodynamic activation parameters for the spontaneous transfer of bilirubin between small unilamellar egg lecithin vesicles were determined. Bilirubin-IXα was added to donor vesicles labeled with the fluorescent phospholipid probe, (5-(dimethylamino)naphthalene-1-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (dansyl-PE). When bound to the donor vesicles, bilirubin quenches the dansyl probe fluorescence through resonance energy transfer. The movement of bilirubin from dansyl-labeled donor vesicles to unlabeled acceptor vesicles was monitored directly by the reemergence of dansyl fluorescence over time. Vesicle fusion and intervesicle transfer of the dansyl-PE probe were excluded by quasielastic light scattering and fluorescence resonance energy transfer studies. Stopped-flow analysis demonstrated that the transfer of bilirubin was described by a single-exponential function with a mean half-time of 2.0 ± 0.1 ms (±SD) at 37 °C. The rate of bilirubin transfer was independent of acceptor vesicle concentration and decreased with increasing buffer ionic strength, indicating that intermembrane transfer occurred via aqueous diffusion, rather than vesicle collisions. The free energy of activation ($\Delta G^{\ddagger}$) for the dissociation of bilirubin from donor vesicles was 14.2 kcal·mol^{-1}. These studies suggest that bilirubin is associated with phospholipid bilayers at the membrane–water interface. We postulate that the movement of unconjugated bilirubin between intracellular membranes occurs via spontaneous transfer through the aqueous phase.

The liver is responsible for the biotransformation of an array of hydrophobic substrates into more hydrophilic sugar, amino acid, or sulfate conjugates, which are subsequently eliminated in the bile or urine. Hence, the hepatocyte must transport a variety of poorly soluble endogenous compounds, drugs, and xenobiotics from the sinusoidal plasma membrane to the endoplasmic reticulum (ER),[1] where the enzymes (e.g., cytochromes P-450, UDP–glucuronosyltransferases) responsible for the metabolism of these substrates are located. Bilirubin-IXα, the hydrophobic end product of heme degradation, is commonly employed in studies of hepatic organic anion metabolism (Crawford et al., 1987; Tazuma et al., 1988; Ookhtens et al., 1988; Apstein & Robins, 1982) because it is an endogenous compound with well-characterized physicochemical and physiologic properties (Carey & Spivak, 1986). It has been hypothesized that, following uptake from plasma, unconjugated bilirubin binds to cytosolic binding proteins (e.g., glutathione S-transferase B) and is then transported via these soluble proteins to intracellular sites of metabolism (Berk & Stremmel, 1986; Sellinger & Boyer, 1990; Tipping & Ketterer, 1981). However, studies suggest that the intracellular movement of bilirubin and other small hydrophobic substrates may be mediated by direct intermembrane transfer and lateral diffusion within the plane of the membrane bilayer to the site of glucuronidation in the ER (Whitmer et al., 1984; Boyer et al., 1983; Rose et al., 1985).

Due to internal hydrogen bonding, bilirubin-IXα is an extremely hydrophobic molecule, with an aqueous solubility of less than 100 nM at pH 7.4 (Brodersen, 1979; Ostrow et al., 1990). Indeed, at neutral pH, unconjugated bilirubin partitions into phospholipid membranes, rather than the bulk aqueous phase (Tipping et al., 1979; Nagaoka & Cowger, 1978), suggesting that a substantial portion of intracellular bilirubin is membrane bound. It also has been shown that small hydrophobic molecules are capable of rapid movement within the lateral plane of native membranes (Edidin, 1974). Experimental evidence from our laboratory has demonstrated that glucuronidation of bilirubin-IXα occurs more efficiently when bilirubin is presented to hepatic microsomal UDP–glucuronosyltransferase associated with phospholipid vesicles rather than bound to cytosol or purified binding proteins, i.e., glutathione S-transferases (Whitmer et al., 1984). Thus, since the network of tubules, vesicles, and lamellae which constitutes

† This study was supported by National Institutes of Health Research Grants DK02047, DK07533, DK34854, DK36887, DK38398, and DK43955 and by a Postdoctoral Senior Research Fellowship Award from the American Gastroenterological Society (S.D.Z.). Dr. Zeidel is the recipient of Career Development and Merit Review Awards from the Department of Veterans's Affairs. Preliminary reports of this work have been published in abstract form (Zucker et al., 1990, 1991).

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[1] Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; dansyl-PE, *N*-(5-(dimethylamino)naphthalene-1-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; rhodamine-PE, *N*-(Lissamine rhodamine B sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; Texas Red-PE, *N*-(Texas Red sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; QLS, quasielastic light scattering; ER, endoplasmic reticulum.

0006-2960/92/0431-3184$03.00/0

the endoplasmic reticulum occupies approximately 15% of total cell volume (DePierre et al., 1988), it is likely that intracellular membranes play a significant role in the binding and transport of small hydrophobic compounds, such as unconjugated bilirubin, in the hepatocyte.

Fluorescence resonance energy transfer has been utilized to examine the interactions of various lipophilic molecular species with native and model membranes (Nichols & Pagano, 1982; Nagaoka & Cowger, 1978; Doody et al., 1980; Storch & Kleinfeld, 1986; Rose et al., 1985). In the present study, we have employed this technique to determine the rate of transfer of bilirubin-IXα between small unilamellar phosphatidylcholine vesicles in the absence of soluble proteins. The incorporation of dansyl-PE into donor vesicles enabled monitoring of bilirubin binding and movement, since the intrinsic absorbance properties of bilirubin result in the quenching of dansyl emission. The current investigations were designed to measure the kinetics and thermodynamics of the spontaneous intermembrane transfer of unconjugated bilirubin.

Experimental Procedures

Materials. Egg lecithin (grade 1) and phosphatidylserine (bovine spinal cord) used in the preparation of phospholipid vesicles were obtained from Lipid Products (Surrey, England). The fluorescent phospholipid probes *N*-(5-(dimethylamino)-naphthalene-1-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (dansyl-PE) and *N*-(Lissamine rhodamine B sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (rhodamine-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). *N*-(Texas Red sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (Texas Red-PE) was purchased from Molecular Probes (Eugene, OR). Bilirubin-IXα was obtained from Porphyrin Products (Logan, UT), and purity was documented at 98.5% by absorbance in chloroform solution (ϵ_{453} = 62 000 $M^{-1}\cdot cm^{-1}$). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Only the highest grade phospholipids and ligands available were employed in these studies, and all glassware was chloroform washed.

Preparation of Small Unilamellar Vesicles and Incorporation of Fluorescent Probes. Small unilamellar vesicles were prepared by a modification (Whitmer et al., 1984) of the sonication procedure of Barenholz et al. (1977). Egg lecithin, solubilized in chloroform solution, was dried under argon and desiccated overnight under vacuum. For the preparation of fluorescent vesicles, appropriate molar concentrations of the probe molecule (e.g., dansyl-PE or rhodamine-PE) were thoroughly mixed with lecithin in chloroform solution prior to drying and desiccation. The dried phospholipids were suspended in a 0.1 M KCl/10 mM Tris-HCl buffer solution (pH 7.4) and sonicated on ice under an argon atmosphere, using the standard flat tip probe of a Branson (Danbury, CT) sonifier cell disrupter 200 (16 W for 30 min). Sequential centrifugation steps then were performed using a Beckman SW 40 Ti rotor to remove sonifier probe titanium particles (100000*g* for 30 min) and to sediment large vesicle contaminants (150000*g* for 180 min). The phospholipid concentration in the final vesicle preparation (3–6 mM) was quantified using the lipid phosphorus assay method of Bartlett (1959). All vesicles were stored in the dark, under argon at 4 °C, and were used within 10 days of preparation.

Incorporation of Bilirubin-IXα into Unilamellar Vesicles. Since unconjugated bilirubin is essentially insoluble in water at neutral pH (Brodersen, 1979), incorporation into unilamellar phospholipid vesicles was accomplished by a procedure involving solubilization of the bile pigment in alkali, followed by rapid neutralization to pH 7.4 (Whitmer et al., 1986). All steps were performed in the dark in order to minimize bilirubin photodegradation. The precise quantity of bilirubin-IXα to be added to the donor vesicles was determined by weight and confirmed by absorbance in chloroform, as described above. The chloroform subsequently was evaporated under argon and the bilirubin was desiccated in vacuo for a minimum of 24 h. The bilirubin then was solubilized in KCl/Tris-buffered solution (adjusted to pH 10 by the addition of potassium hydroxide), and small volumes were added to the vesicle preparation (buffered at pH 7.4), thereby bringing the final pH of the solution to 8.5. A volume of KCl/Tris buffer, acidified with hydrochloric acid and equal to the volume of bilirubin solution, was added immediately to the bilirubin–vesicle mixture in order to adjust the final pH to 7.4. Using this simple procedure, unconjugated bilirubin was introduced into membrane bilayers with molar ratios as high as 1:10 bilirubin:phospholipid, as determined by Sephadex G-25 column chromatography with [^{3}H]bilirubin-IXα (Whitmer et al., 1984). Similar molar ratios were obtained when bilirubin-incorporated vesicles were prepared by cosonication of [^{3}H]-bilirubin directly with the phospholipids (Whitmer et al., 1984).

Stopped-Flow Kinetic Analysis of Intermembrane Bilirubin Transfer. The spontaneous transfer of bilirubin between membrane populations was monitored by fluorescence resonance energy transfer. The experiments employed a model system in which the fluorescent probe, 1-(dimethylamino)-naphthalene-5-sulfonyl chloride (dansyl), covalently bound to the polar head group of phosphatidylethanolamine (PE), was incorporated into small unilamellar phospholipid vesicles composed of egg lecithin. The dansyl-PE probe was excited at 340 nm, and fluorescence was monitored using a 520-nm long-pass filter. At these excitation and emission wavelengths, the contribution of bilirubin fluorescence (Rosei, 1983; Cu et al., 1975) to the total fluorescence intensity of dansyl-PE was negligible (<0.1%). The addition of bilirubin-IXα to dansyl-labeled vesicles resulted in a reduction in probe fluorescence intensity due to resonance energy transfer between dansyl-PE and the bile pigment (Nagaoka & Cowger, 1978). When unlabeled acceptor vesicles were added to bilirubin-incorporated (quenched) donor vesicles, reemergence of fluorescence was observed as the bilirubin transferred from the fluorescent (dansyl-labeled) to the nonfluorescent (unlabeled) vesicle population. Acceptor vesicle concentrations were in marked (5–25-fold) excess of donor vesicles in order to ensure that desorption from the donor vesicles was the rate-limiting step for bilirubin exchange.

Since the spontaneous movement of bilirubin between vesicles was found to be rapid, a stopped-flow apparatus was employed to enable the kinetics of the transfer process to be determined. Experiments were performed using an Acorn (Cambridge, U.K.) Archimedes 410/1 computer driven Applied Photophysics (Leatherhead, U.K.) fluorometer equipped with an SPF-17 stopped flow device, which has a measured mixing time of 0.7 ms. Temperature was regulated by a Brinkman Instruments (Westbury, NY) Lauda K-2/R circulating water bath. Following the mixing of donor and acceptor vesicles, fluorescence intensity was monitored over time. The time course for the change in dansyl fluorescence was analyzed by fitting the observed intensities to the function (Storch & Kleinfeld, 1986):

$$F(t) = \sum_i A_i \exp(-k_i t) + C \quad (1)$$

where t is the time in seconds, k_i is the ith order rate constant,

A_i is the amplitude, and C is a constant term. In addition, data were fitted to a single-exponential equation which also included a linear steady-state term (mt):

$$F(t) = A \exp(-kt) + \mathrm{mt} + C \quad (2)$$

A standard nonlinear least-squares routine based on the Marquardt algorithm (Bevington, 1969) was used to fit the data to these functions. Curve fitting was determined to be complete when the normalized variance demonstrated less than 0.1% improvement. Fit quality was assessed by multiple regression analysis of variance using the F-statistic, which was calculated by dividing the regression mean square by the residual mean square of the curve fit function (Zar, 1984). An F-test also was applied to determine if the inclusion of an additional term significantly improved the curve fit (Zar, 1984).

Effect of Buffer Ionic Strength on Bilirubin Intermembrane Transfer. To determine the effect of buffer ionic strength on the rate of bilirubin intermembrane transfer, a series of buffers of graded ionic strength were prepared by serially increasing the concentration of KCl from 0.1 to 2.5 M, while the pH was maintained at 7.4 and the concentration of Tris-HCl remained constant. Appropriate volumes of unlabeled lecithin acceptor vesicles were added to each of the buffer solutions of varying ionic strength. These solutions were then incubated overnight (~18 h) to ensure that the equilibration of vesicle volume had occurred (Scarpa & De Gier, 1971). Bilirubin was incorporated into dansyl-labeled donor vesicles in 0.1 M KCl buffer (as outlined above) and mixed, using stopped-flow techniques, with the acceptor vesicle solutions of varying ionic strength. Changes in fluorescence intensity were monitored over time at 15 °C. Bilirubin transfer curves were normalized to a scale of 0–1.0 to facilitate comparison, since the dansyl quantum yield is sensitive to ionic strength.

Measurement of Vesicle Solution Viscosity. The kinematic viscosity of lecithin vesicle solutions was determined over a range of phospholipid concentrations (0–4000 μM) using a Cannon Instrument (State College, PA) viscometry apparatus equipped with a Haake (Saddle Brook, NJ) FK circulating water bath for temperature control. A calibrated size 50 Cannon-Manning semi-micro viscometer (A710) was employed for all viscosity determinations. The viscometer was loaded with an appropriate volume (0.57 mL) of vesicle solution and incubated at constant temperature for 10 min prior to viscosity determination. The kinematic viscosity (centistokes) of the sample was calculated by multiplying the efflux time (seconds) by the viscometer constant, after correction for temperature. The accuracy of the viscosity readings was confirmed by control experiments using distilled, deionized water. The observed results were found to be within 0.6% of published values for H_2O (Weast, 1985).

Quasielastic Light Scattering Analysis of Vesicle Size. Fusion of unilamellar vesicles was assessed by quasielastic light scattering (Cohen et al., 1990a,b). Mean vesicle hydrodynamic diameter and size variance were monitored using a quasielastic light scattering (QLS) apparatus consisting of a Spectra Physics (Mountain View, CA) Model 164 argon ion laser tuned to 5145 Å and a 64-channel Langley-Ford (Amherst, MA) Model 1096 correlator interfaced with a Digital (Maynard, MA) DEC Pro-350 computer (Cohen et al., 1989). Vesicle size and polydispersity in the presence of bilirubin (0–5.0 μM) were determined at constant temperature (25 and 37 °C), which was controlled by a thermostated sample holder based on a Peltier-effect module (Melcore Model CP2-31-06L; Trenton, NJ). A significant increase in vesicle size and/or polydispersity is indicative of membrane coalescence or fusion (Cohen et al., 1989, 1990a,b; Schurtenberger et al., 1985).

Fluorescence Quenching Measurement of Membrane Fusion and Phospholipid Probe Transfer. Fluorescence resonance energy transfer is a sensitive method for detecting vesicle fusion or intervesicle transfer of fluorescent phospholipid probes (Struck et al., 1981; Vanderwerf & Ullman, 1980). Therefore, fluorescence quenching techniques were employed to monitor the potential mixing of bilayer phospholipids. In these studies, headgroup-labeled fluorescent derivatives of phosphatidylethanolamine, dansyl-PE and rhodamine-PE, were utilized. The latter fluorophore, which has been shown to be exchangeable only over the course of hours to days (Struck et al., 1981; Pagano et al., 1981), was incorporated into a population of unilamellar acceptor vesicles at a concentration of 2.0 mol %. Intermembrane transfer of dansyl-PE or fusion of dansyl-labeled donor vesicles with rhodamine-labeled acceptor vesicles results in resonance energy transfer between the dansyl-PE and the rhodamine-PE, with consequent quenching of dansyl fluorescence. Following the mixing of dansyl-labeled and rhodamine-labeled vesicles (in the presence or absence of 0.5 μM bilirubin), fluorescence intensity was monitored over time using a Perkin-Elmer (Norwalk, CT) MPF-66 fluorescence spectrophotometer interfaced with a Perkin-Elmer 7300 computer. Constant temperature was maintained by a Haake FK circulating water bath. Excitation and emission wavelengths were 340 nm and 525 nm, respectively. These wavelengths were selected in order to reduce the contribution of rhodamine fluorescence (Struck et al., 1981; Pagano et al., 1981; Nichols, 1985) to negligible levels. Control experiments using phosphatidylserine (rather than phosphatidylcholine) vesicles labeled with dansyl-PE or Texas Red-PE, which has similar properties to rhodamine-PE, also were conducted. Phosphatidylserine vesicles, which fuse in the presence of 1.0 mM calcium chloride (Struck et al., 1981; Ababei & Hildenbrand, 1984; Papahadjopoulos et al., 1976), were utilized in order to verify the sensitivity of this assay system. Experiments were performed in the presence of unconjugated bilirubin (0–2.5 μM), and data were corrected for inner filter effects due to bilirubin and acceptor vesicle absorbance (Birdsall et al., 1983).

Determination of Thermodynamic Activation Parameters for Bilirubin Transfer. Thermodynamic activation parameters for the dissociation of bilirubin-IXα from donor vesicles were determined by measuring the rate of bilirubin intermembrane transfer at temperatures ranging from 5 to 37 °C. Both bilirubin-incorporated donor vesicles and unlabeled acceptor vesicles were maintained in the dark at 5 °C under an argon atmosphere. Aliquots of each vesicle solution were removed and incubated separately in the dark at the appropriate temperature for approximately 5 min prior to stopped-flow mixing.

The relationship between the off-rate constant for the dissociation of a molecule from the membrane bilayer (k_{off}) and temperature is described by the Arrhenius equation

$$\mathrm{d} \ln k_{off}/\mathrm{d}t = E_a/RT^2 \quad (3)$$

where R is the gas constant, T is absolute temperature, and E_a is the energy of activation. Upon integration, the Arrhenius equation yields

$$\ln k_{off} = -E_a/RT + C \quad (4)$$

where C is a constant. E_a provides a measure of the energy required to convert a membrane-bound molecule to its activated state and can be determined from a plot of $\ln k_{off}$ versus $1/T$, which yields a straight line with slope $-E_a/R$. By applying the transition-state theory developed by Eyring to the dissociation of a population of molecules, [A], from the

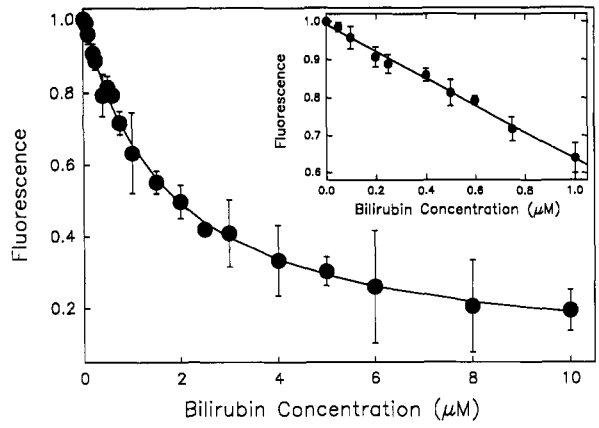

FIGURE 1: Quenching of dansyl-labeled vesicle fluorescence by bilirubin. Unconjugated bilirubin was incubated for 1 min at 25 °C with 200 μM dansyl-labeled phosphatidylcholine vesicles, and fluorescence intensity was measured using steady-state techniques. Each point represents the mean ± SD of three to five separate experiments. The addition of increasing concentrations of bilirubin to unilamellar vesicles containing 0.5 mol % dansyl-PE progressively quenched the fluorescence intensity of the dansyl-PE probe. The quenching of dansyl fluorescence was linear (r = 0.996) up to 1.0 μM bilirubin (inset); hence, bilirubin concentrations within this range were utilized for all subsequent intermembrane transfer experiments. The data are corrected for inner filter effects due to bilirubin absorbance.

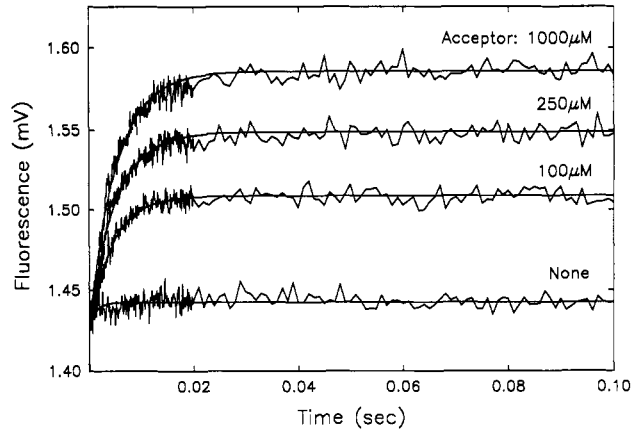

FIGURE 2: Effect of acceptor vesicle concentration on bilirubin intermembrane transfer. Data from a series of stopped-flow intermembrane bilirubin transfer experiments are presented. Fluorescence intensity (in millivolts) was measured over a time interval of 220 ms at 25 °C. Using a split time base, 200 readings were obtained over the initial 20 ms, and the subsequent 200 data points were recorded over the remaining 200 ms, during which time steady-state fluorescence intensity was reached. The time-dependent reemergence of fluorescence is due to the transfer of unconjugated bilirubin (0.5 μM) from 100 μM dansyl-labeled donor vesicles to increasing concentrations (0–1000 μM) of acceptor vesicles. Each curve represents the mean of five to eight repetitive stopped-flow injections and is fitted by a single-exponential function.

phospholipid bilayer, it can be shown that the off-rate is proportional to the concentration of molecules in the activated (transition) state [A‡]:

$$-\mathrm{d[A]}/\mathrm{d}t = k^{\ddagger}[\mathrm{A}^{\ddagger}] \quad (5)$$

where $k^{\ddagger}$ is the rate constant for the breakdown of the activated complex (Piszkiewicz, 1977). The ratio [A‡]/[A] defines the equilibrium constant for the formation of the activated complex ($K^{\ddagger}$). Since $k^{\ddagger}$ can be approximated by k_BT/h (Piszkiewicz, 1977), where k_B is Boltzmann's constant and h is Planck's constant, eq 5 may be rewritten as

$$-\mathrm{d[A]}/\mathrm{d}t = k^{\ddagger}K^{\ddagger}[\mathrm{A}] = (k_BT/h)K^{\ddagger}[\mathrm{A}] = k_{off}[\mathrm{A}] \quad (6)$$

The free energy for the formation of the activated complex ($\Delta G^{\ddagger}$) is represented by the standard thermodynamic equation

$$\Delta G^{\ddagger} = -RT \ln K^{\ddagger} \quad (7)$$

Substituting for K‡ using eq 6 gives

$$k_{off} = (k_BT/h) \exp(-\Delta G^{\ddagger}/RT) = (k_BT/h) \exp(-\Delta H^{\ddagger}/RT) \exp(-\Delta S^{\ddagger}/R) \quad (8)$$

since, at constant pressure $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, where $\Delta H^{\ddagger}$ is the enthalpy and $\Delta S^{\ddagger}$ is the entropy of activation. Assuming that $\Delta S^{\ddagger}$ does not vary significantly with temperature (Piszkiewicz, 1977), eq 8 can be differentiated to yield

$$\mathrm{d} \ln k_{off}/\mathrm{d}t = (\Delta H^{\ddagger} + RT)/RT^2 \quad (9)$$

Equation 9 is similar in form to the Arrhenius equation (eq 3). By combining these two equations, it can be shown that

$$E_a = \Delta H^{\ddagger} + RT \quad (10)$$

Thus, the activation energy, which is obtained experimentally by measuring k_{off} over a range of temperatures, may be utilized to determine the enthalpy, entropy, and free energy of activation for the dissociation of a molecule from the membrane bilayer.

Results

The addition of increasing concentrations of unconjugated bilirubin to unilamellar lecithin vesicles containing dansyl-PE (0.5 mol %) progressively quenched the fluorescence intensity of the dansyl-PE probe (Figure 1). The on-rate for bilirubin binding to dansyl-labeled vesicles was too rapid to be resolved with the available stopped-flow techniques. However, the equilibrium quenching of dansyl fluorescence by bilirubin-IXα was linear up to 1.0 μM bilirubin (Figure 1, inset) and, thus, bilirubin concentrations within this range were utilized for all subsequent intermembrane transfer experiments. The data were corrected for inner filter effects due to bilirubin absorbance, although corrections were trivial (<1%) for bilirubin concentrations below 1 μM. Since the fluorescent yield at any given concentration of dansyl-labeled phospholipid is temperature dependent (data not shown), all experiments were performed at constant temperature.

Kinetic Analysis of Intermembrane Bilirubin Transfer. The spontaneous transfer of unconjugated bilirubin (0.5 μM) from dansyl-labeled donor vesicles (100 μM lipid phosphorous) to increasing concentrations of unlabeled acceptor vesicles was monitored over time (Figure 2). The amplitude of the fluorescence change increased with increasing acceptor vesicle concentration. Since the quenching of dansyl fluorescence was linear at the bilirubin concentrations employed, these results demonstrate that the amount of bilirubin transferred from donor to acceptor vesicles increases with the quantity of acceptor vesicles. The fraction of bilirubin which transferred from dansyl-labeled donor to unlabeled acceptor vesicles over the time scale of these experiments was virtually identical to that predicted for the equilibrium distribution between two identical membrane populations. For example, 92 ± 4% (±SD) of the total bilirubin bound to 100 μM donor vesicles transferred to 1000 μM acceptor vesicles, as compared with a predicted value of 91% at equilibrium. These data indicate that the presence of 0.5 mol % dansyl-PE does not significantly alter the binding affinity of bilirubin-IXα for donor vesicles as compared with unlabeled acceptor vesicles.

Although the amount of bilirubin that transferred from donor to acceptor vesicles was dependent on the number of acceptor vesicles, the rate of bilirubin transfer remained unchanged over a 25-fold range (100–2500 μM) of acceptor vesicle concentrations, at both 25 and 37 °C. No significant

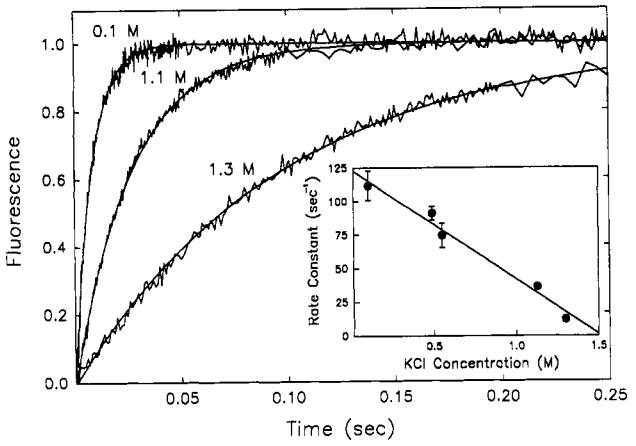

FIGURE 3: Effect of buffer ionic strength on the rate of bilirubin intermembrane transfer. Changes in fluorescence intensity over time were monitored, using stopped-flow techniques, to determine the effect of increasing KCl concentration (0.1–1.3 M) on the rate of unconjugated bilirubin transfer from dansyl-labeled donor vesicles (100 μM) to unlabeled acceptor vesicles (750 μM). The bilirubin concentration was 0.5 μM. Fluorescence intensity, which has been normalized to a scale of 0–1.0, was measured over an interval varying from 250 to 1200 ms depending on the time required to reach steady state. All experiments were performed at constant temperature (15 °C). Each curve represents the averge of five to ten separate stopped-flow injections and is fitted by a single-exponential function. A plot of the first-order transfer rate constant for bilirubin intermembrane transfer versus buffer KCl concentration (inset) demonstrates a linear decrease in transfer rate with increasing buffer ionic strength ($r = 0.990$). A 10-fold decrease in rate is observed as buffer ionic strength is increased over a 13-fold range. Each point represents the mean ± SD of three separate sets of transfer experiments.

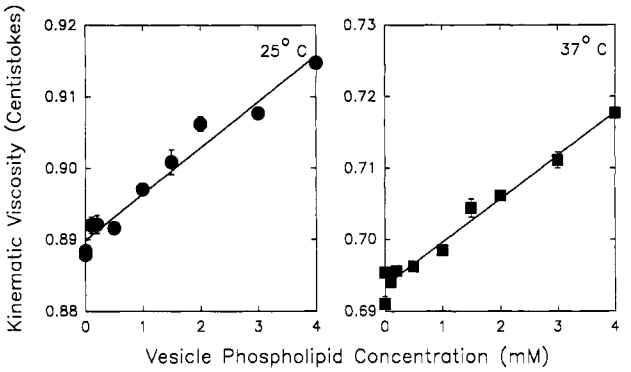

FIGURE 4: Effect of lecithin vesicle concentration and temperature on solution viscosity. The kinematic viscosity of aqueous buffer solution containing increasing concentrations of lecithin vesicles (0–4000 μM) was measured at both 25 °C (left panel, ●) and 37 °C (right panel, ■) using a calibrated Semi-Micro viscometer. Vesicle solutions were equilibrated at constant temperature for 10 min prior to viscosity determination. Each point represents the mean ± SD of three separate measurements. Kinematic viscosity increased linearly with vesicle concentration, with r values of 0.981 and 0.988 at 25 and 37 °C, respectively. Additionally, solution viscosity was inversely correlated with temperature.

bilirubin transfer was measurable in the absence of acceptor vesicles. Furthermore, similar transfer rates were observed when the movement of bilirubin from unlabeled donor to dansyl-labeled acceptor vesicles was studied (data not shown). These results demonstrate that the observed changes in dansyl fluorescence are due to the spontaneous transfer of bilirubin between donor and acceptor membranes. The independence of the transfer rate on acceptor vesicle concentration suggests that intervesicle bilirubin transfer occurs via aqueous diffusion rather than by vesicle collisions.

Multiple regression analysis of the curve fits revealed that intermembrane bilirubin transfer was adequately described by a single-exponential function ($p < 0.0005$). Significantly better fits ($p < 0.01$) were obtained using an equation containing a linear steady-state term in addition to a single-exponential term (eq 2). The improved fit is attributable to slight deviations of the equilibrium baseline from the horizontal, in either a positive or a negative direction. Such minor deviations were observed even when dansyl-labeled vesicles were mixed with unlabeled vesicles in the absence of bilirubin, and hence we conclude that this observation represents a mixing artifact, or possibly bleaching of probe fluorescence, rather than actual bilirubin transfer. Importantly, the contribution of the linear component to the transfer rate was consistently minor, such that the difference in the rate constants obtained using eq 1 versus eq 2 was ±7.5%. All rate constants presented in this report were determined using eq 2, since a double-exponential function offered no significant improvement in fit ($p > 0.05$). The mean first-order rate constant for bilirubin intermembrane transfer was 218 ± 8 s^{-1} (±SD) at 25 °C, which corresponds to a half-time of 3.2 ± 0.1 ms. At 37 °C, the mean rate constant was 355 ± 8 s^{-1}, with a $t_{1/2}$ of 2.0 ± 0.1 ms.

Effect of Buffer Ionic Strength on Intermembrane Bilirubin Transfer. To further test the hypothesis that bilirubin transfer between membranes occurs via aqueous diffusion, the effect of buffer ionic strength on the rate of bilirubin transfer from donor to acceptor vesicles was studied. As the KCl concentration of the buffer solution was increased from 0.1 to 1.3 M, a 10-fold decrease in transfer rate was observed (Figure 3). Increasing the buffer ionic strength resulted in a linear decrease in the rate of bilirubin movement between donor and acceptor vesicles (Figure 3, inset). Since high salt concentrations are known to decrease the aqueous solubility of hydrophobic compounds (Long & McDevit, 1952), these data provide additional support for the conclusion that intermembrane bilirubin transfer occurs by diffusion through the aqueous phase.

Effect of Solution Viscosity on Intermembrane Bilirubin Transfer. Vesicle solution viscosity exhibited a small positive linear correlation with phospholipid concentration, at both 25 and 37 °C (Figure 4). Since, as shown above, the bilirubin intermembrane transfer rate is independent of acceptor vesicle concentration, it follows that the rate also is independent of solution viscosity. Furthermore, since the diffusion coefficient for a molecule is inversely proportional to solution viscosity, these data suggest that diffusion through the aqueous phase is not the rate-limiting step for the transfer of bilirubin between donor and acceptor vesicles.

Membrane Fusion Studies. In the above experiments, it has been assumed that dansyl-PE functions as a nonexchangeable fluorescent marker, thereby distinguishing donor from acceptor vesicles. This is likely a valid assumption, since the spontaneous transfer of head group labeled long-chain phospholipid molecules between unilamellar vesicles has been shown to be slow, on the order of hours to days (Nichols & Pagano, 1982; Struck et al., 1981; Pagano et al., 1981). Nevertheless, to exclude the possibility that membrane fusion contributed to the observed changes in fluorescence, quasielastic light scattering (QLS) was employed to monitor mean vesicle hydrodynamic diameter and size variance (Cohen et al., 1989, 1990a,b; Schurtenberger et al., 1985). No significant variation in mean vesicle diameter or polydispersity was observed when donor and acceptor vesicles were incubated at 25 °C in the presence of increasing concentrations of bilirubin-IXα (Table I). In addition, solutions of unlabeled lecithin vesicles, dansyl-labeled lecithin vesicles, and a mixture of the two populations were examined over a range of temperatures from 5 to 37 °C. No changes in vesicle size or polydispersity

Table I: Effect of Bilirubin Concentration on Vesicle Size as Determined by Quasielastic Light Scattering (QLS) Analysis[a]

final bilirubin concn (μM)	mean hydrodynamic diameter (Å ± SD)	variance (% ± SD)
none	320 ± 8	36 ± 3
1.5	329 ± 3	39 ± 2
2.5	321 ± 9	33 ● 4
5.0	326 ± 7	31 ± 2

[a] Fluorescent donor vesicles were preincubated with concentrations of bilirubin-IXα ranging from 0 to 10 μM. These quenched vesicles were then combined with an equal volume of unlabeled acceptor vesicles. After a 15-min incubation at 25 °C, the mean hydrodynamic diameter and size variance of the vesicle mixture were determined by QLS (see Experimental Procedures). Each value represents the mean ± SD of three experiments. Both donor and acceptor vesicle concentrations in the final mixture were 50 μM. There was no significant change in vesicle size or polydispersity, indicating that vesicle fusion did not occur over this time period.

were observed for any of the membrane populations during a 15-min incubation period, in both the presence and absence of 0.5 μM bilirubin (data not shown).

In addition to the QLS studies, fluorescence quenching analyses utilizing acceptor vesicles containing rhodamine-PE demonstrated no evidence of spontaneous intermembrane transfer of dansyl-PE over a period of 60 min (data not shown). The addition of 0.5 μM bilirubin-IXα had no effect, suggesting that at this concentration bilirubin does not significantly enhance the intermembrane transfer of dansyl-PE. In addition, stopped-flow studies revealed no evidence of rapid intervesicle exchange of the dansyl-labeled probe over a time course of 1–1000 ms, in both the presence and absence of 0.5 μM bilirubin. Control experiments demonstrated that the fluorescence of dansyl-PE-labeled (0.5 mol %) phosphatidylserine (PS) vesicles was quenched by Texas Red-PE-labeled (1.0 mol %) PS vesicles in the presence of 1.0 mM calcium chloride, a known fusogen for PS vesicles (Struck et al., 1981; Ababei & Hildenbrand, 1984; Papahadjopoulos et al., 1976). On the other hand, calcium had no effect on the zwitterionic phosphatidylcholine vesicles (data not shown). Taken together, these studies suggest that no significant intermembrane transfer of dansyl-PE, or fusion of membrane vesicles, occurs over a 1-h time course. Thus, the changes in dansyl fluorescence observed in the bilirubin transfer experiments arise solely from bilirubin quenching and intermembrane bilirubin transfer.

Thermodynamic Analysis of Bilirubin Dissociation from Phospholipid Vesicles. The transfer of bilirubin-IXα between unilamellar phosphatidylcholine vesicles was examined over a range of temperatures from 5 to 37 °C in order to determine the thermodynamic activation parameters for the dissociation of bilirubin from the phospholipid bilayer. The activation energy (E_a) for bilirubin dissociation from donor vesicles was calculated from the slope of an Arrhenius plot (eq 4) of the data (Figure 5) and was found to be 7.4 kcal·mol^{-1}. Equations 7–10 were used to determine the thermodynamic activation parameters for the desorption of bilirubin from the donor vesicle bilayer. At 25 °C, there was an approximately equal contribution of enthalpic ($\Delta H^{\ddagger}$ = 6.8 kcal·mol^{-1}) and entropic ($T\Delta S^{\ddagger}$ = −7.4 kcal·mol^{-1}) changes to the free energy of activation ($\Delta G^{\ddagger}$ = 14.2 kcal·mol^{-1}). The equilibrium constant for the activated complex ($K^{\ddagger}$) was calculated to be ~3.7 × 10^{-11}.

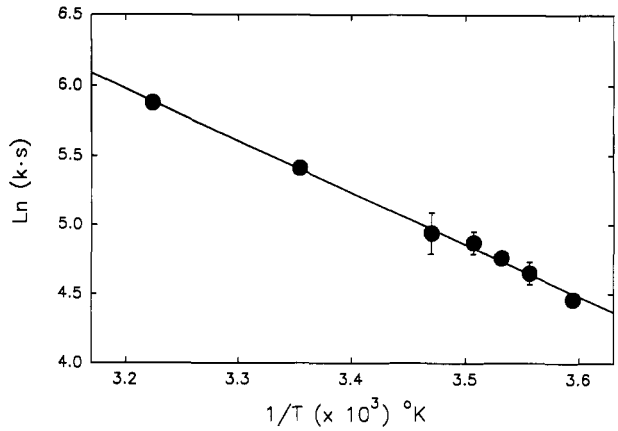

FIGURE 5: Bilirubin intermembrane transfer: Arrhenius plot. The natural log of the first-order rate constant (k) for bilirubin intermembrane transfer is plotted against inverse temperature (in Kelvin). Rate constants were determined over a range of temperatures from 5 to 37 °C. From the slope of the plot (r = 0.998), the activation energy (E_a) for the dissociation of bilirubin from donor vesicles was calculated to be 7.4 kcal·mol^{-1}.

Discussion

Little is known regarding the mechanism(s) of intracellular transport of unconjugated bilirubin and other hydrophobic substrates to their sites of biotransformation in the endoplasmic reticulum of the hepatocyte. Both in vivo (Gollan et al., 1981) and isolated perfused rat liver studies (Crawford et al., 1987; Gollan et al., 1981; Wolkoff et al., 1978) indicate that the entire process of bilirubin uptake, intracellular transit, glucuronidation, and excretion into bile occurs within minutes. Since unconjugated bilirubin has very low aqueous solubility at the intracellular pH of 7.06 in the hepatocyte (Renner et al., 1989), the rapid rate of bilirubin glucuronidation requires the existence of an efficient mechanism for transport from the sinusoidal plasma membrane through the cytosol to intracellular sites of metabolism. It has been generally assumed that, following uptake from plasma, bilirubin-IXα binds to cytosolic binding proteins and then is carried via these soluble proteins to the ER (Berk & Stremmel, 1986; Sellinger & Boyer, 1990; Tipping & Ketterer, 1981), where the conjugating enzyme, UDP-glucuronosyltransferase is localized (Hauser et al., 1984). Ligandin (glutathione S-transferase B), which accounts for up to 5% of soluble liver protein in rats and humans (Boyer, 1989), is presumed to be a major intracellular binding protein for both bilirubin and bile acids. It has been postulated that the glutathione S-transferases enhance the metabolism of hydrophobic molecules by facilitating their diffusion through the aqueous phase (Sellinger & Boyer, 1990; Tipping & Ketterer, 1981). However, there is evidence indicating that glutathione S-transferases do not have direct access to membrane-bound ligands (Boyer, 1989; Boyer et al., 1983). Moreover, these cytosolic proteins do not appear to increase the rate of bilirubin influx into the hepatocyte (Boyer, 1989; Wolkoff et al., 1979). Finally, we have previously demonstrated that the glucuronidation of bilirubin by hepatic microsomes occurs more efficiently when bilirubin is bound to phospholipid vesicles rather than to cytosol or purified binding proteins (Whitmer et al., 1984). Thus, we postulated that intracellular membranes may play an essential role in the hepatocellular transport of unconjugated bilirubin and, potentially, other small hydrophobic molecular species (Whitmer et al., 1984, 1987).

In order to further characterize the process of direct intermembrane transfer of hydrophobic ligands, we have studied the kinetics and thermodynamics of the spontaneous movement of bilirubin-IXα between small unilamellar vesicles composed of phosphatidylcholine, the predominant phospholipid in rat liver microsomes (Whitmer et al., 1986). Although the in vivo intracellular concentrations of bilirubin and its conjugates are

unknown, indirect evidence based on the calculated K_m and V_{max} values for UDP–glucuronosyltransferase (Crawford et al., 1990) suggests that, under physiologic conditions, unconjugated bilirubin levels are below 10 μM in the hepatocyte. Bilirubin concentrations of 0.5 μM, which are likely to be within the "physiologic" range, were utilized for these intermembrane transfer experiments. The results of these studies indicate that the movement of bilirubin between unilamellar vesicles is very rapid, with a mean half-time of 2.0 ms at 37 °C, and can be described by a single-exponential function. The rate of spontaneous intermembrane transfer of bilirubin was found to be independent of acceptor vesicle concentration, suggesting that transfer occurs via aqueous diffusion. If membrane collisions were required for bilirubin transfer, the rate would be proportional to the product of the concentrations of donor and acceptor vesicles, since more donor–acceptor collisions occur at higher vesicle concentrations (Roseman & Thompson, 1980; Doody et al., 1980).

The term "salting-out" describes a decline in the solubility of a nonelectrolyte with increasing salt concentration (Long & McDevit, 1952; Massey et al., 1982) and is the most likely explanation for the observed decrease in the rate of bilirubin intermembrane transfer in the presence of high KCl concentrations. Enhanced membrane lipid order induced by increased buffer ionic strength (Storch & Kleinfeld, 1986) also may account, in part, for the slower rate of bilirubin transfer. These data corroborate the previous findings indicating that spontaneous intermembrane transfer of bilirubin occurs via aqueous diffusion and are consistent with the results from studies of other hydrophobic molecules such as cholesterol (McLean & Phillips, 1984; Fugler et al., 1985), phospholipids (McLean & Phillips, 1984; Roseman & Thompson, 1980), and long-chain fatty acids (Storch & Kleinfeld, 1986). In these experiments, donor and acceptor vesicles were identical except for the presence of small quantities (0.5 mol %) of dansyl-PE, and hence on-rates were presumed to be equivalent. The validity of this assumption is supported by the demonstration that the equilibrium distribution of bilirubin between donor and acceptor vesicles was unaffected by the presence of the dansyl probe. Furthermore, the absence of any effect of viscosity on the intermembrane transfer rate indicates that the aqueous diffusion of bilirubin was not rate-limiting. Finally, since donor vesicle-bound bilirubin transfers in a first-order process with respect to acceptor vesicle concentration, and since bilirubin transfer between membranes occurs by aqueous diffusion, the rate-determining step is likely to be dissociation of the bilirubin molecule from the donor vesicle (k_{off}).

Despite an aqueous solubility comparable to that of cholesterol (Renshaw et al., 1983), unconjugated bilirubin transfers between phospholipid vesicles at a rate which is approximately 6 orders of magnitude faster (Table II) than that of cholesterol (McLean & Phillips, 1981, 1982, 1984; Lund-Katz et al., 1988; Fugler et al., 1985). Furthermore, fatty acids and retinol, which have greater aqueous solubility than bilirubin-IXα (Small, 1986), exhibit intermembrane transfer rates that are up to 10 000-fold slower than bilirubin (Storch & Kleinfeld, 1986; Doody et al., 1980; Pownall et al., 1983; Noy & Xu, 1990; Ho et al., 1989). Thus, other ligand physicochemical properties, in addition to aqueous solubility, appear to have an important influence on the rate of spontaneous intermembrane transfer. For example, at pH 7.4, although bilirubin is predominantly in the uncharged diacid form, approximately 20% is present as the monoanion (Ostrow et al., 1990). It is possible that the monoanion species is an important determinant of the intermembrane transfer rate. The binding of unconjugated bilirubin to the phospholipid bilayer also might result in an alteration in the pK_a of the carboxylic acid moieties, thereby facilitating the dissociation of bilirubin from the membrane. Furthermore, in addition to its low aqueous solubility, unconjugated bilirubin also is poorly soluble in apolar solvents, such as carbon hydrides, carbon tetrachloride, acetone, and triglycerides (Brodersen, 1979; Leonard et al., 1989). Hence, bilirubin-IXα is neither hydrophilic nor lipophilic, and, for this reason, it is plausible that this bile pigment may associate with the bilayer primarily at the membrane–water interface rather than intercalate between the phospholipid acyl chains.

Table II: Comparison of Aqueous Solubility, Free Energy of Activation, and Half-Time for the Spontaneous Intermembrane Transfer of Hydrophobic Molecular Species

molecular species	aqueous solubility[a] (moL·L^{-1})	free energy of activation ($\Delta G^{\ddagger}$)[b] (kcal·mol^{-1})	transfer half-time ($t_{1/2}$)[c] (s)
phospholipid	5×10^{-10}	22.9	1×10^{5}
cholesterol	1×10^{-8}	21.5	7×10^{3}
fatty acid (C_{18})[d]	2×10^{-6}	20.8[f]	3×10^{1}
retinol[e]	1×10^{-5}	17.3	1×10^{0}
fatty acid (C_9)[d]	1×10^{-3}	16.4	1×10^{-1}
fatty acid (C_6)[d]	8×10^{-2}	14.7	7×10^{-3}
bilirubin-IXα	9×10^{-8}	14.2	3×10^{-3}

[a] Values for aqueous solubility were obtained from the following sources: phospholipids, cholesterol, retinol, and fatty acids (Small, 1986; Renshaw et al., 1983) and bilirubin (Ostrow et al., 1990). [b] Literature values for $\Delta G^{\ddagger}$ were obtained from the following: phospholipids and cholesterol (McLean & Phillips, 1984), retinol (Noy & Xu, 1990), and fatty acids (Pownall et al., 1983; Doody et al., 1980). [c] The $t_{1/2}$ for intermembrane transfer was derived from the following: phospholipids (McLean & Phillips, 1984; Roseman & Thompson, 1980), cholesterol (McLean & Phillips, 1984; Fugler et al., 1985), C_{18} fatty acids (Storch & Kleinfeld, 1986), retinol (Noy & Xu, 1990; Ho et al., 1989), and C_6 and C_9 fatty acids (Pownall et al., 1983; Doody et al., 1980). [d] The reported fatty acid solubility data are for native fatty acids, while the $\Delta G^{\ddagger}$ and $t_{1/2}$ for intermembrane transfer is based on studies of fluorescent fatty acid derivatives. [e] Solubility data for retinol is extrapolated from that for an equivalent length fatty alcohol (Small, 1986). [f] The value of $\Delta G^{\ddagger}$ was determined using small unilamellar egg lecithin vesicles at 24 °C (A. M. Kleinfeld and J. Storch, personal communication).

The nature of the interaction of unconjugated bilirubin with phospholipid bilayers is complex and poorly understood (Leonard et al., 1989; Eriksen et al., 1981; Brodersen, 1979; Mustafa & King, 1970; Vazquez et al., 1988; Nagaoka & Cowger, 1978). Although infrared spectroscopy suggests that bilirubin-IXα associates with the acyl-chain region of liposomes (Zakim & Wong, 1990), data from air–water interface studies of mixed monomolecular films (Notter et al., 1982), nuclear magnetic resonance and calorimetric analyses (Cestaro et al., 1983), and absorbance spectra performed in the presence of bile salt micelles (Carey & Koretsky, 1979) and reverse micelles (Carey & Spivak, 1986; Ostrow et al., 1988) demonstrate that unconjugated bilirubin has significant surface activity. The observed first-order transfer kinetics with respect to acceptor vesicle concentration, as well as the rapid equilibration of bilirubin between donor and acceptor vesicles, indicate that membrane-bound bilirubin exists in a single, homogeneous population. The rapid transfer rate supports the concept that the major proportion of unconjugated bilirubin is located at the vesicle–water interface.

The thermodynamic analysis provides information on the strength of bilirubin–membrane interactions and thereby aids in the localization of bilirubin in the bilayer. The free energy of activation ($\Delta G^{\ddagger}$) for the dissociation of bilirubin from phospholipid vesicles was determined to be approximately 14.2

kcal·mol^{-1}, which is significantly less than measured values (Table II) for cholesterol (McLean & Phillips, 1981, 1982, 1984), phospholipids (McLean & Phillips, 1984; Nichols, 1985; Mutsch et al., 1986), medium- and long-chain fatty acids (Doody et al., 1980; Pownall et al., 1983; A. M. Kleinfeld and J. Storch, personal communication), and retinol (Noy & Xu, 1990; Ho et al., 1989). The low $\Delta G^{\ddagger}$ explains the high transfer rate constant for bilirubin, since studies of phospholipids (Nichols, 1985) and fatty acids (Pownall et al., 1983; Doody et al., 1980) have shown that the rate of formation of the activated complex determines the rate of intermembrane transfer. In addition, the relatively low free energy of activation for bilirubin dissociation from the membrane bilayer is inconsistent with deep hydrophobic interactions. Extrapolating the $\Delta G^{\ddagger}$ for bilirubin to a series of saturated fatty acids of increasing chain length (Pownall et al., 1983) suggests that the depth of bilirubin penetration into the bilayer is equivalent only to that of a 5-carbon fatty acid. Thus, it appears likely that a major portion of the bilirubin molecule is not localized within the hydrophobic domain of the membrane bilayer but is rather associated at the membrane surface.

The dissociation of bilirubin-IXα from phospholipid vesicles is characterized by an increase in enthalpy and an approximately equivalent decrease in entropy. In this regard, the thermodynamic behavior of unconjugated bilirubin is similar to that of fatty acids and retinol (Pownall et al., 1983; Noy & Xu, 1990), as opposed to that of cholesterol or phospholipids, which demonstrate little or no entropic change on desorption from a membrane bilayer (Nichols, 1985; McLean & Phillips, 1984). It is possible that bilirubin–membrane interactions may involve hydrogen bonding, as has been suggested for retinol (Noy & Xu, 1990), since the calculated $\Delta H^{\ddagger}$ (6.8 kcal·mol at 25 °C) is approximately equivalent to the energy of one hydrogen bond. The entropic contribution to the free energy of activation for bilirubin transfer most likely arises from the ordering effect of the molecule on the aqueous phase.

The interaction of bilirubin with cellular membranes may have important physiologic implications. The postulated, and as yet undefined, role of bilirubin-membrane binding in the pathogenesis of kernicterus (bilirubin-induced encephalopathy) in newborns (Eriksen et al., 1981; Mustafa & King, 1970; Cashore, 1988) emphasizes the importance of characterizing the precise nature of this interaction. The significant surface active properties of unconjugated bilirubin may, in part, explain its damaging effect on membrane structures (Mustafa & King, 1970). Further analyses of the kinetics of intracellular organic anion transport will provide insight into the mechanisms of bile formation and the dynamics of hepatic processing and elimination of a variety of xenobiotics and drugs. On the basis of the results of our studies, we conclude that unconjugated bilirubin transfers rapidly between membrane populations in the absence of soluble binding proteins. Furthermore, it is likely that bilirubin associates with membranes at the membrane–water interface, perhaps involving a single hydrogen bond. We postulate that the intracellular transport of bilirubin from the liver cell plasma membrane to its site of glucuronidation in the endoplasmic reticulum may involve spontaneous intermembrane transfer through the aqueous phase. It also is possible that the movement of a variety of other hydrophobic substrates in the hepatocyte is mediated in this manner.

Acknowledgments

We thank Drs. Joanne Donovan, David Cohen, and Fred Konakoff from the laboratory of Dr. Martin Carey for performance of the quasielastic light scattering analysis and for helpful discussions. We gratefully thank Julie Narciso and Ellen Wrchota for expert technical support and Dr. Eric Grossman for assistance with stopped-flow experiments. Special thanks are given to Dr. Bernard Ransil under whose guidance the data analysis was conducted.

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