The liver is responsible for the clearance and metabolism of unconjugated bilirubin, the hydrophobic end-product of heme catabolism. Although several putative bilirubin transporters have been described, it has been alternatively proposed that bilirubin enters the hepatocyte by passive diffusion through the plasma membrane. In order to elucidate the mechanism of bilirubin uptake, we measured the rate of bilirubin transmembrane diffusion (flip-flop) using stopped-flow fluorescence techniques. Unconjugated bilirubin rapidly diffuses through model phosphatidylcholine vesicles, with a first-order rate constant of 5.3 s⁻¹ (τ₁/₂ = 130 ms). The flip-flop rate is independent of membrane cholesterol content, phospholipid acyl saturation, and lipid packing, consistent with thermodynamic analyses demonstrating minimal steric constraint to bilirubin transmembrane diffusion. The coincident decrease in pH of the entrapped vesicle volume supports a mechanism whereby the bilirubin molecule crosses the lipid bilayer as the uncharged diacid. Transport of bilirubin by native rat hepatocyte membranes exhibits kinetics comparable with that in model vesicles, suggesting that unconjugated bilirubin crosses cellular membranes by passive diffusion through the hydrophobic lipid core. In contrast, there is no demonstrable flip-flop of bilirubin diglucuronide or bilirubin ditaurate in phospholipid vesicles, yet these compounds rapidly traverse isolated rat hepatocyte membranes, confirming the presence of a facilitated uptake system(s) for hydrophilic bilirubin conjugates.

Unconjugated bilirubin is the principal degradation product of heme metabolism. Although the physiologic isomer, bilirubin IXα, is a dicarboxylic acid, the molecule has minimal aqueous solubility at physiologic pH (1, 2) due to the formation of intramolecular hydrogen bonds (3). For this reason, bilirubin undergoes biotransformation in the liver to more polar conjugates prior to secretion in the bile. However, while unconjugated bilirubin is not hydrophilic, neither can it be characterized as lipophilic (1), since this compound is equally insoluble in apolar solvents (e.g. n-hexane, 1-pentanol). These unique physical-chemical properties have generated controversy regarding the mechanism of bilirubin uptake by the liver. Based on the spontaneous leakage from multilamellar liposomes (4), it has been proposed that bilirubin is able to diffuse through cellular membranes (5, 6). On the other hand, in vivo (7) and whole organ (8, 9) studies indicate that hepatic bilirubin uptake is saturable and occurs against a concentration gradient, findings that support a protein-mediated transport mechanism. To date, four putative bilirubin transporters have been identified in liver cells: BSP/bilirubin-binding protein, organic anion-transporting polypeptide, bilitranslocase, and organic anion-binding protein. Each of these proteins facilitates uptake of the hydrophilic organic anion, sulfobromophthalein (BSP),¹ a process that is competitively inhibited by bilirubin (10, 11).

However, despite the substantial body of evidence in support of protein-mediated bilirubin uptake, none of the aforementioned candidate proteins has been directly shown to transport unconjugated bilirubin. This is due, in part, to the use of BSP as a surrogate for bilirubin, based on the long standing assumption that these two compounds share a common transporter (12, 13). However, the cellular uptake of bilirubin and BSP can be dissociated (14, 15), suggesting distinct transport mechanisms. While it has been argued that the unique “permeability” of hepatocytes to bilirubin confirms the presence of a specific transporter (12, 16), other studies have demonstrated nonsaturable and non-energy-dependent bilirubin uptake (17), indicative of a diffusional process. Moreover, fibroblasts transfected with cDNA for the bilirubin-conjugating enzyme, UDP-glucuronosyltransferase, do not express any of the candidate transporters yet are effectively able to take up and conjugate bilirubin (18).

In an attempt to reconcile these disparate results and to further delineate the mechanism whereby bilirubin traverses cellular membranes, we devised a stopped-flow fluorescence system to facilitate detailed kinetic analysis of bilirubin flip-flop in model and isolated native membrane vesicles. Our data suggest that unconjugated bilirubin is capable of rapid, spontaneous diffusion through lipid bilayers, findings with important implications for bilirubin clearance by the liver.

EXPERIMENTAL PROCEDURES

Materials—Nigericin, valinomycin, and essentially fatty acid-free bovine and human serum albumin were purchased from Sigma. Bilirubin IXα (purity >99.5% by absorbance in chloroform, ε₄₃3 = 62,000 M⁻¹·cm⁻¹) and bilirubin conjugate (ditaurate2Na) were obtained from BASF Foundation Child Health Research Award (to S. D. Z.), and a Harvard Digestive Diseases Center Pilot/Feasibility Grant (to S. D. Z.), and a Charles H. Hood Foundation Postdoctoral Research Award (to W. G.).

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1 The abbreviations used are: BSP, sulfobromophthalein; BDIT, bilirubin ditaurate; BDG, bilirubin diglucuronide; BSA, bovine serum albumin; cbBSA, Cascade Blue-labeled BSA; bLPM, basolateral (sinusoidal) liver plasma membranes; dansyl-PE, N-(5-dimethylamino-naphthalene-1-sulfonyl)dipalmitoyl-1,2-α-phosphatidylethanolamine; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid.
Porphyrin Products (Logan, UT). Grade 1 egg lecithin (phosphatidylcholine) was purchased from Lipid Products (Surrey, United Kingdom), and cholesterol was obtained from Nu-Chek Prep (Elysian, MN). Dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, and N-(5-diethylaminonaphthalene-1-sulfonfyl) dipalmitoyl-1-o-phosphatidylethanolamine (dansyl-PE) were purchased from Avanti Porcine (Birmingham, AL). The fluorescent probes 8-hydroxypropyrene-1,3,6-trisulfonic acid (pyranine) and Cascade Blue conjugated bovine serum albumin as well as anti-Cascade Blue antibodies were obtained from Molecular Probes, Inc. (Eugene, OR).

**Preparation and Loading of Unilamellar Phospholipid Vesicles**—Small unilamellar vesicles were prepared by probe sonication with a modification (19) of the technique of Barenholz et al. (20). A chloroform solution of phosphatidylcholine was evaporated to dryness under argon atmosphere, solubilized in ether, and re-evaporated to form a uniform film. The lipids then were desiccated overnight under vacuum to remove traces of ether, suspended in 0.1 mol% potassium phosphate solution at pH 7.4 (unless otherwise stated), and sonicated until clear. For the preparation of fluorescence-labeled vesicles, dansyl-PE was added to the lecithin-containing chloroform solution so as to comprise 1 mol% of total phospholipid. Large unilamellar vesicles were prepared using a modification (21) of the injection method of Kremer et al. (22). Constituent phospholipids were suspended in ethanol and slowly injected into a magnetically stirred aqueous solution of 0.1 mol% potassium phosphate (pH 7.4), with vesicle size regulated by the concentration of phospholipid in the injected ethanol. Cascade Blue-conjugated bovine serum albumin (cbBSA) was entrapped at the time of vesicle preparation by injecting the ethanolic phospholipid solution into phosphate buffer containing 50 μg cbBSA. The vesicle suspension (5 ml) was subjected to dialysis against 100 volumes of phosphate buffer three successive times to remove retained ethanol, and unentrapped cbBSA was separated from the vesicles by elution on a Sepharose 4B column (42 × 2.5 cm). Mean vesicle hydrodynamic diameter was determined by quasielastic light scattering (21).

**Isolation of Membrane Vesicles from Rat Liver**—Rat liver microsomal membranes were isolated from fasted male Sprague-Dawley rats, as described previously (21), and protein concentration was quantified by the Bio-Rad assay. Hepatocyte basolateral (bLPM) and canalicular plasma membranes were prepared by the method of Meier and Boyer (23), with appropriate enrichment documented by enzymatic assay (21), and by Western blotting with polyclonal antibodies to the α-subunit of the plasma membrane Na+/K+ ATPase and with monoclonal antibodies to the canalicular ecto-ATPase (generously provided by Dr. R. Green). Membranes were loaded with cbBSA (50 μg) by homogenization in the presence of the probe, followed by exhaustive washing.

**Analysis of Bilirubin Transmembrane Diffusion**—The flip-flop rate for unconjugated bilirubin was determined from fluorescence recordings of bilirubin equilibration between a suspension of dansyl-PE-labeled small unilamellar phosphatidylcholine vesicles and bovine serum albumin (Fig. 1A). The binding of bilirubin to dansyl-PE-labeled vesicles causes a reduction in fluorescence intensity due to resonance energy transfer between bilirubin and the dansyl moiety (19). Since unconjugated bilirubin is poorly soluble at neutral pH, incorporation into phospholipid vesicles was accomplished by dissolving bilirubin in alkaline (pH 12) potassium phosphate (19). The addition of a small aliquot (≤1%, v/v) of the bilirubin solution to a suspension of phospholipid vesicles buffered at pH 7.4 in 0.1 mol% potassium phosphate caused no detectable alteration in pH. Alternatively, when indicated, bilirubin was dissolved in dimethyl sulfoxide (Me2SO), and a small aliquot (≤1%, v/v) was added to the vesicle suspension (24). At this low concentration, Me2SO has no discernible affect on membrane physical properties (25, 26), and the use of either method to solubilize bilirubin yielded identical kinetic results. All steps were performed in diminished light to minimize bilirubin photodegradation.

An Aminco-Bowman II fluorescence spectrophotometer equipped with an SLM-Aminco MilliFlow stopped-flow reactor (mixing time: 3 ms) facilitated the rapid mixing of a suspension of dansyl-labeled vesicles containing bound bilirubin with an equal volume of a bovine serum albumin solution. Bilirubin equilibration is manifest by a time-dependent decrease in Cascade Blue fluorescence (excitation, 380 nm; emission, 430 nm), and flip-flop rates were calculated from the time course of the quenching of cbBSA. The rate of bilirubin transmembrane diffusion also was determined from the quenching of cbBSA entrapped within model or native membrane vesicles (Fig. 1B). Membranes were preincubated with anti-Cascade Blue antibodies in order to completely neutralize all residual unincorporated probe; therefore, the quenching of Cascade Blue fluorescence is predicated on bilirubin traversing the acceptor membrane and binding to the entrapped cbBSA. For these studies, bilirubin was incorporated into unlabeled small unilamellar phosphatidylcholine donor vesicles or, alternatively, solubilized in 0.1 mol% potassium phosphate (pH 7.4) containing Me2SO (20%, v/v). In order to minimize light-scattering effects,
Kinetic Analysis of Bilirubin Transmembrane Diffusion—We previously have shown that the equilibration of bilirubin between unilamellar phospholipid vesicles and BSA occurs via aqueous diffusion (27). Assuming that bilirubin flip-flop is significantly slower than membrane dissociation, the equilibration rate (R) can be described by the expression (27, 28),

\[
R = \frac{k_{\text{BSA}}^{\text{Bil}}}{K_a^{\text{BSA}}} + \frac{[V]}{[\text{BSA}]} \left( 1 - \frac{k_{\text{BSA}}^{\text{Bil}}}{K_a^{\text{BSA}}} \right)
\]

(Eq. 2)

where \(k_{\text{BSA}}^{\text{Bil}}\) represents the flip-flop rate constant, \(k_{\text{BSA}}^{\text{Bil}}\) represents the dissociation rate constant from BSA, \(K_a\) represents the association constant, and [BSA] and [V] represent the concentrations of bovine serum albumin and vesicle phospholipid, respectively. Based on this equation, a plot of \(R\) versus \([V]/[\text{BSA}]\) intersects the y axis at \(k_{\text{BSA}}^{\text{Bil}}\) and asymptotically approaches \(k_{\text{BSA}}^{\text{Bil}}\) at high molar ratios of phospholipid to BSA. Values for the various kinetic parameters were determined by measuring the bilirubin equilibration rate over a range of phospholipid:albumin molar ratios and fitting the data to Equation 2.

Preparation of Bilirubin Diglucuronide—Bilirubin diglucuronide (BDG) was isolated from bile enriched by the intravenous administration of 20 mM unconjugated bilirubin in 3.2% bovine serum albumin to anesthetized male Sprague-Dawley rats (29). BDG was extracted with chloroform/ethanol and NaCl-saturated acidified glycine and isolated by thin layer chromatography. The purity of the BDG preparation (>95%) was measured by reverse-phase high pressure liquid chromatography (30), with the initial mobile phase consisting of a 35% solution of 1% acetic acid and 65% 25 mM sodium acetate in methanol/chloroform (95:5, v/v). After 30 s, the sodium acetate solution was increased in a linear fashion to 100% over 8 min, followed by a linear decrease to 80% over 5 min. The flow rate was maintained constant at 1 ml/min.

Effect of Bilirubin on Membrane Vesicle pH—In order to determine the effect of bilirubin on the internal pH of membrane vesicles, 0.5 mM pyranine, a hydrophilic pH-sensitive fluorescent probe, was dissolved in 25 mM HEPES/KOH (pH 7.4) and entrapped within small unilamellar phosphatidylcholine vesicles by sonication (31). Unentrapped pyranine was removed from the medium by elution on a Sephadex G-25 (coarse grade) column (40 × 1 cm). Pyranine-loaded vesicles were diluted to a concentration of 0.5 mM phospholipid, and 2 ml of total volume was placed in a temperature-controlled, stirred cuvette. The internal pH of the vesicles, as reflected by the fluorescence intensity of the entrapped pyranine, was monitored continuously at 25 °C using steady-state techniques. Excitation (405 nm) and emission (520 nm) wavelengths were selected so as to minimize bilirubin inner filter effects. At these wavelengths, pyranine fluorescence intensity correlates inversely with pH such that an increase in hydrogen ion concentration manifests as a corresponding increase in probe fluorescence.

Flip-flop of bilirubin from the outer to the inner bilayer hemileaflet was monitored by changes in pyranine fluorescence following the addition of a small (10-μl) aliquot of unconjugated bilirubin. To facilitate distinction between fluorescence changes induced by bilirubin absorbance versus those due to alterations in the pH of the entrapped volume, vesicles were pH-clamped by pretreatment with 3 μl (1 μg/mg phospholipid) of an ethanolic solution of the proton/potassium ionophore, nigericin (31). Control experiments were performed to document that the small volume of added ethanol (0.15%, v/v) did not alter the permeability of the vesicles to protons and that there was no effect of the bilirubin vehicle on pyranine fluorescence, either in the presence or absence of nigericin. Flip-flop of bilirubin from the inner to the outer hemileaflet of the membrane bilayer was examined by adding a 10-μl aliquot of bovine serum albumin dissolved in 25 mM HEPES/KOH (pH 7.4) to bilirubin-containing vesicles, producing a final BSA concentration of 5 μM.

RESULTS

In order to determine whether unconjugated bilirubin is able to spontaneously traverse a membrane bilayer, we studied the equilibration of bilirubin between 1 mol % dansyl-PE-labeled small unilamellar phosphatidylycholine vesicles and bovine serum albumin (Fig. 2). The resultant fluorescence curve is best described by a double exponential function, suggesting that the equilibration process consists of two kinetically distinct events.

We postulate that the fast phase reflects bilirubin dissociation from the external hemileaflet of the vesicle bilayer, while the
slow phase, which is readily resolved using a longer sampling interval (Fig. 2, inset), represents bilirubin flip-flop from the inner to the outer hemileaflet of the phospholipid bilayer. In support of this hypothesis, the rate constant for the fast component (k_{fast}) is identical to previously reported bilirubin off-rates from small unilamellar phosphatidylcholine vesicles (19). The finding that the ratio of the amplitudes of the fast versus slow phases of transfer (A_{fast}/A_{slow}) is identical to the outer:inner surface area of the vesicles (1.8:1) further supports the contention that the slower process corresponds to transmembrane diffusion from the internal to the external hemileaflet of the membrane bilayer.

A fluorescence system utilizing phosphatidylcholine vesicles loaded with cbBSA also was employed to verify that the slow component of bilirubin transfer represents bilirubin transmembrane flip-flop. Small unilamellar phosphatidylcholine donor vesicles were preincubated with bilirubin and then rapidly mixed with a suspension of cbBSA-loaded acceptor vesicles (Fig. 3, left panel). Alternatively, when bilirubin was solubilized in phosphate buffer containing 20% Me_{2}SO (in the absence of donor vesicles), a similar flip-flop signal was observed, since bilirubin must traverse the acceptor vesicle bilayer in order to quench cbBSA fluorescence. While the curve obtained in the presence of Me_{2}SO equilibrates at a slightly higher baseline due to the enhanced aqueous solubility of bilirubin, the finding that the equilibration rate constant is unaffected by the presence of donor vesicles indicates that diffusion of bilirubin across the acceptor membrane is rate-limiting. Control experiments demonstrated that neither vesicles nor Me_{2}SO have a direct effect on cbBSA fluorescence (Fig. 3, left panel).

The transfer of bilirubin from unlabelled phosphatidylcholine donor vesicles to free cbBSA (Fig. 3, right panel) exhibits kinetics comparable with cbBSA-loaded acceptor vesicles, although, in this case, the fluorescence signal reflects bilirubin flip-flop from the inner to the outer hemileaflet of the donor vesicles. This hypothesis is supported by the nearly instantaneous decline in fluorescence that occurs when bilirubin is solubilized in 20% Me_{2}SO, since there is no intervening membrane to impede bilirubin access to cbBSA, and the association rate of bilirubin for cbBSA is more rapid than can be resolved by the stopped-flow apparatus. Hence, the quenching of cbBSA by bilirubin is nearly complete before the first data point is recorded. These findings confirm that a flip-flop signal is observed only when bilirubin and cbBSA are separated by a membrane bilayer. Moreover, the similarity in the equilibration rate between unlabelled (Fig. 3, right panel) and dansyl-labeled (Fig. 2, inset) vesicles indicates that the presence of the dansyl-PE probe does not affect bilirubin flip-flop kinetics.

**Transmembrane Diffusion of Bilirubin Conjugates**—We examined whether the non-hydrogen-bonded, water-soluble bilirubin derivative, bilirubin ditaurate (BDT), is able to diffuse spontaneously through phospholipid bilayers. Since BDT is less efficient than unconjugated bilirubin at quenching cbBSA fluorescence (Fig. 4, inset), a 10-fold higher concentration was utilized to ensure that a flip-flop signal would be readily detectable. In contradistinction to unconjugated bilirubin, bilirubin ditaurate causes no significant attenuation in the fluorescence of entrapped cbBSA over a 150-s time course (Fig. 4), indicating that spontaneous diffusion of BDT across phosphatidylcholine bilayers is extremely slow. In control experiments, 20% Me_{2}SO had no effect on the rate of BDT flip-flop, verifying that Me_{2}SO does not alter membrane permeability to this bilirubin conjugate. Results identical to BDT were obtained with the principal physiologic bilirubin conjugate, BDG, while a bilirubin XIIa (kindly provided by Dr. D. Lightner), a bilirubin isomer that retains internal hydrogen bonding (26), exhibited a flip-flop signal identical to that of bilirubin IXa. These findings suggest that internal hydrogen bonds are permissive to bilirubin transmembrane diffusion.

**Kinetic Analysis of Bilirubin Flip-flop**—In order to determine the rate of bilirubin transmembrane diffusion, equilibration of bilirubin between dansyl-PE-labeled small unilamellar...
FIG. 4. Transmembrane diffusion of unconjugated bilirubin and bilirubin ditaurate. The flip-flop of unconjugated bilirubin (2 μM) and bilirubin ditaurate (20 μM) across unilamellar phosphatidylcholine vesicles (1 mM phospholipid) was monitored by the time-dependent quenching of entrapped cbBSA. A solution of unconjugated bilirubin (UCB) solubilized in 20% Me₂SO or BDT dissolved in 0.1 M potassium phosphate, was mixed with a suspension of cbBSA-loaded vesicles, using stopped-flow techniques. Each curve reflects the average of six stopped-flow injections performed at 25 °C. The inset demonstrates that unconjugated bilirubin (circles) and bilirubin ditaurate (squares) quench the fluorescence of free cbBSA (5 μM) in a linear manner up to a 1:1 molar ratio of bilirubin to albumin. Each point reflects the mean ± S.D. of three experiments and, following correction for bilirubin inner filter effects, is normalized to a scale of 0–1.

phosphatidylcholine vesicles and BSA was studied over a broad range of donor and acceptor concentrations, and the measured first-order rate constants were plotted against the phospholipid:BSA molar ratio (Fig. 5). It is notable that individual transfer curves (Fig. 5, inset) constitute approximately one-third of the total change in fluorescence signal from base line (0 μM BSA), consistent with the internal:external surface area of the vesicles. According to Equation 2, at low ratios of phospholipid to BSA, the equilibration rate (R) approaches $k_{on}$, the value of which is calculated to be $5.3 \pm 0.6 \text{ s}^{-1}$ ($t_{1/2} = 130 \pm 15$ ms; ±S.E.) based on the best fit of the data. Conversely, at high ratios of phospholipid to albumin, R asymptotically approaches $k_{off}$. The value of 0.16 s$^{-1}$ obtained from the curve fit is consistent with previously reported bilirubin off-rates from BSA (27). Comparable results were obtained when bilirubin equilibration between unlabelled (nonfluorescent) phosphatidylcholine vesicles and cbBSA was examined (data not shown).

While it previously has been shown that membrane lipid packing and cholesterol content are key determinants of bilirubin solvation (21), we found that neither vesicle hydrodynamic diameter (Table I), nor phospholipid:cholesterol ratios up to 50 mol % (data not shown) significantly altered the rate of bilirubin flip-flop. There also was no difference in the flip-flop rate for vesicles composed of dioleoylphosphatidylcholine or dipalmitoylphosphatidylcholine as compared with egg phosphatidylcholine, suggesting that acyl chain length and saturation have little impact on the flip-flop process. To investigate why membrane composition and lipid packing do not alter the rate of bilirubin flip-flop, we determined thermodynamic activation parameters by examining bilirubin equilibration between small unilamellar phosphatidylcholine vesicles and BSA over a temperature range of 10–40 °C (19). From an Arrhenius plot of the data (Fig. 6), the free energy of activation ($\Delta G^\ddagger$) at 25 °C was calculated to be 16.9 kcal/mol, the principal determinant of which is the activation enthalpy ($\Delta H^\ddagger$) of 12.6 kcal/mol, with only a minor entropic contribution ($\Delta S^\ddagger$ = -4.3 kcal/mol). These findings suggest a relatively modest energy barrier to bilirubin transmembrane diffusion, with minimal
The effect of membrane lipid packing on bilirubin flip-flop was determined by comparing the first-order rate constant for bilirubin equilibration between unilamellar phosphatidylcholine vesicles (small versus large) and cbBSA (10 μM). The mean hydrodynamic diameter ± S.D. of the vesicle preparations (1 mM phospholipid) was determined by quasielastic light scattering. Equilibration rate constants, which reflect the mean ± S.D. of four separate sets of 10 stopped-flow injections performed at 25 °C, were not statistically different between the two groups of vesicles (p = 0.72).

| Vesicle hydrodynamic diameter | First-order rate constant |
|-----------------------------|--------------------------|
| nm                          | s⁻¹                      |
| Small                       | 31 ± 1                   | 1.68 ± 0.20 |
| Large                       | 148 ± 22                 | 1.72 ± 0.05 |

The changes in fluorescence associated with equilibration of bilirubin (2 μM) between small unilamellar donor vesicles (1 mM phospholipid) and cbBSA-loaded phosphatidylcholine acceptor vesicles (upper panel) or 0.5 mg of protein/ml cbBSA-loaded rat liver microsomes (lower panel) were recorded at 25 °C. Each curve represents the average of six stopped-flow injections. At comparable membrane phospholipid concentrations, the rate of bilirubin flip-flop is similar between model and native vesicles, with rate constants of 0.071 ± 0.003 s⁻¹ and 0.077 ± 0.004 s⁻¹ (± S.E.), respectively.

The rate of bilirubin (2 μM) equilibration between membrane vesicles and free cbBSA was measured over a range of phospholipid (PL):albumin molar ratios. Small unilamellar phosphatidylcholine vesicles (●, short dashed line), microsomal membranes (■, solid line), basolateral liver plasma membranes (△, dotted line), or canalicular liver plasma membranes (▲, long dashed line), at concentrations ranging from 25 to 500 μM phospholipid, were utilized as the bilirubin donor. Each point reflects the mean ± S.D. of five sets of experiments performed at 25 °C, with lines generated from best fit parameters for Equation 2.
rates at low phospholipid:cbBSA molar ratios further suggests that $k_{ff}$ is similar for model and native membranes, consistent with the hypothesis that bilirubin transport across hepatocyte membranes occurs via spontaneous diffusion. We postulate that the divergence in equilibration rates at high ratios of phospholipid to cbBSA is due to variations in the binding affinity of the membrane preparations for bilirubin. While it also is conceivable that these differences reflect the effect of membrane lipid composition or vesicle size on bilirubin diffusion, this possibility seems unlikely in light of our demonstration that membrane hydrodynamic diameter, acyl chain saturation, and cholesterol content do not alter flip-flop kinetics (see above).

In order to establish whether water-soluble bilirubin conjugates are able to traverse hepatocyte membranes, we measured the rate of bilirubin diglucuronide equilibration between small unilamellar phosphatidylcholine vesicles (Fig. 9, upper left panel), rat liver microsomes (Fig. 9, upper right panel), basolateral plasma membranes (Fig. 9, lower left panel), and canalicular plasma membranes (Fig. 9, lower right panel). In contrast, bilirubin diglucuronide (20 μM) exhibits no spontaneous flip-flop in model vesicles but rapidly traverses native hepatocyte membranes. At these donor and acceptor concentrations, the first-order rate constant for BDG flip-flop in microsomal membranes of $0.29 \pm 0.02$ s$^{-1}$ (±S.E.) is 5 times faster than unconjugated bilirubin ($k = 0.058 \pm 0.002$ s$^{-1}$).

We subsequently examined the effect of a potassium gradient on the rate of bilirubin equilibration between rat bLPM and free cbBSA. Since these experiments monitor bilirubin flip-flop from the inner to the outer hemileaflet of the membrane bilayer, we applied an outwardly directed potassium gradient by

**Mechanism of Bilirubin Transmembrane Diffusion—**Increased uptake of unconjugated bilirubin by basolateral liver plasma membranes in the presence of an inwardly directed potassium gradient and the potassium ionophore, valinomycin, has been cited as evidence for a bilirubin-specific electrogenic transporter (24). We examined the effect of valinomycin on bilirubin transport by preincubating isolated rat bLPM with 10 μM valinomycin (or the ethanol vehicle) prior to recording bilirubin equilibration, using cbBSA as the bilirubin acceptor. Initial experiments were performed in the absence of a potassium gradient ($K_{in} = K_{out}$) by suspending the membranes and cbBSA in 0.1 M potassium phosphate, 0.15 M sucrose, 10 mM HEPES (pH 7.4). In the presence of valinomycin, bilirubin equilibration was best described by a single exponential function, while the equilibration curve was best fit by a double exponential equation in the absence of valinomycin (Fig. 10). In the latter experiments, the rate constant for the fast component of transfer (1.8 $\pm$ 0.8 s$^{-1}$) is essentially identical to the first-order rate constant measured in the presence of valinomycin ($k_{val} = 1.9 \pm 0.3$ s$^{-1}$), suggesting that the fast process represents bilirubin flip-flop. In the absence of potassium, (0.25 M sucrose, 10 mM HEPES), bilirubin equilibration was best described by a single exponential function, while the equilibration curve was best fit by a double exponential equation in the absence of valinomycin (Fig. 10). In the latter experiments, the rate constant for the fast component of transfer (1.8 $\pm$ 0.8 s$^{-1}$) is essentially identical to the first-order rate constant measured in the presence of valinomycin ($1.9 \pm 0.3$ s$^{-1}$), suggesting that the fast process represents bilirubin flip-flop. In the absence of potassium, (0.25 M sucrose, 10 mM HEPES), bilirubin equilibration was best described by a single exponential function irrespective of whether the vesicles are pretreated with valinomycin (data not shown), demonstrating that abrogation of the slow component requires the presence of both potassium and valinomycin.

We subsequently examined the effect of a potassium gradient on the rate of bilirubin equilibration between rat bLPM and free cbBSA. Since these experiments monitor bilirubin flip-flop from the inner to the outer hemileaflet of the membrane bilayer, we applied an outwardly directed potassium gradient by
Due to a...

These findings strongly suggest that the enhanced uptake of bilirubin flip-flop. The equilibration of bilirubin (2 μM) between isolated rat bLPM (0.125 mg/ml protein) and cbBSA (20 μM) was monitored after preincubating the membranes in the presence (+ val) or absence (− val) of valinomycin (10 μM). Each curve reflects the average of six stopped-flow injections performed at 25 °C and is normalized to a scale of 0–1. When no valinomycin is present, the fluorescence signal is best fit by a double exponential function (solid line), while a single exponential best describes the curve generated in the presence of valinomycin (dashed line). Similar results are obtained when the flip-flop of bilirubin across small unilamellar phosphatidylcholine vesicles (100 μM phospholipid) is examined under identical conditions (inset).

In order to test this hypothesis, we incorporated pyranine, a water-soluble pH-sensitive probe, within small unilamellar phosphatidylcholine vesicles and monitored pH through changes in pyranine fluorescence. The addition of bilirubin to pyanine-loaded vesicles induces a sharp decrease in pyranine fluorescence. The subsequent addition of BSA brings the fluorescence signal back to baseline, indicative of a decrease in the internal pH of the vesicles, an effect that is promptly reversed by nigericin. The subsequent addition of BSA brings the fluorescence signal back to base line. In contrast, when BSA is added to a suspension of pyranine-loaded vesicles prior to treatment with nigericin (Fig. 11, right panel), the fluorescence signal equilibrates at a level below baseline, consistent with an increase in vesicle pH. This bilirubin-induced pH gradient is dissipated by the addition of nigericin, which allows the vesicle pH to drop back to base line levels. These data indicate that flip-flop of bilirubin from the external to internal bilayer hemileaflet results in acidification, while movement of bilirubin out of the vesicle causes alkalinization of the entrapped volume, findings that are consistent with transbilayer diffusion of the bilirubin diacid.

**Discussion**

Our results indicate that unconjugated bilirubin is able to diffuse spontaneously through phospholipid bilayers. The first-order rate constant for bilirubin flip-flop (5.3 s⁻¹), although 40 times slower than solvation from the membrane surface (19), exceeds the bilirubin dissociation rate from human and bovine serum albumin by over 5- and 50-fold, respectively (27). These findings are consistent with the concept of dissociation-limited uptake, originally proposed by Weisiger (35), in which the rate-limiting step in bilirubin clearance is solvation from se-
rum albumin. The value obtained for the flip-flop rate constant for unconjugated bilirubin was determined from the fit of the equilibration data to Equation 2. While one cannot exclude the existence of alternative mathematical models that may potentially yield different kinetic parameters, the model utilized in the derivation of Equation 1 (28) is straightforward, and the parameters \( k_{off}^{BSA} \), \( K_{a}^{BSA}/K_{a}^{V} \) obtained from the fit of the data correspond closely with published values (27, 36).

Our results are discordant with the findings of Noy et al. (37) that the rate of bilirubin transmembrane diffusion exceeds bilirubin solvation from unilamellar dioleoylphosphatidylcholine vesicles \( k > 70 \, s^{-1} \). The log-order discrepancy between the rate constant for bilirubin flip-flop determined by these authors and the present study is unlikely to reflect differences in the phospholipid content of the vesicles, since we demonstrate that the rate of bilirubin diffusion across dioleoylphosphatidylcholine vesicles is the same as in vesicles composed of phosphatidylcholine. However, Noy et al. derive the rate of bilirubin flip-flop from a single exponential fit of bilirubin equilibration curves, while we found that bilirubin transfer is best described by a double exponential function. This raises the possibility that these authors may not have examined a long enough time course to identify a flip-flop signal or that their fluorescence system was unable to resolve the two components of equilibration. Indeed, the value for the bilirubin dissociation rate determined by these authors is intermediate between the off-rate and flip-flop rate constants obtained in our studies. Moreover, we have shown that it is imperative to examine equilibration rates over a range of phospholipid:albumin ratios in order to determine the flip-flop rate constant (Equation 2).

Thermodynamic analyses indicate a moderate energy barrier to bilirubin flip-flop, composed primarily of an enthalpic component. Spectroscopic studies have shown that the negatively charged propionate groups of bilirubin form ion pairs with the quaternary ammonium of sphingomyelin (38); hence, we postulate that the activation enthalpy reflects the energy required to disrupt this carboxylate/quaternary ammonium ion interaction (since phosphatidylcholine and sphingomyelin possess an identical head group). In contrast to long-chain fatty acids (32), unconjugated bilirubin traverses hepatocyte plasma membranes at a rate similar to that for phosphatidylcholine vesicles. This finding is consistent with the small entropic contribution to the free energy of activation, suggesting few steric or orientation constraints to bilirubin transmembrane diffusion. The lack of an effect of cholesterol, lipid packing, phospholipid acyl chain length, or saturation on the bilirubin flip-flop rate supports this hypothesis. Based on these data, the plasma membrane does not appear to pose a significant barrier to the diffusion of bilirubin into cells.

The proposition that bilirubin is able to freely enter all cells of the body raises questions as to the mechanism underlying the specificity of bilirubin clearance by the liver. While our

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**FIG. 11. Bilirubin effects on vesicle pH.** A representative series of experiments examining the effect of bilirubin (5 \( \mu M \)) on the fluorescence of pyranine-loaded small unilamellar phosphatidylcholine vesicles (0.5 mM phospholipid) is displayed. In the left panel, vesicles were pretreated with nigericin (NIG, at a concentration of 1 \( \mu g/\text{mg} \) phospholipid. Injection of unconjugated bilirubin (UCB) induces a sharp decrease in fluorescence intensity (A), while the subsequent addition of 5 \( \mu M \) BSA causes an increase in pyranine fluorescence (D). When bilirubin is added to pyranine-loaded vesicles in the absence of nigericin (middle panel), a lesser decline in fluorescence intensity (B) is observed, indicating acidification of the entrapped vesicle volume, as supported by the abrupt decrease in fluorescence (A) induced by nigericin. Injection of BSA is again associated with a rapid rise in fluorescence to the level (D) previously observed. In the right panel, following the addition of bilirubin, the fluorescence signal again settles at a level (B) consistent with acidification of the internal vesicle volume. Additionally, when BSA is added prior to nigericin, the fluorescence signal equilibrates below base line (C), indicative of alkalinization of the entrapped volume. Gradual dissipation of the proton gradient is observed before the addition of nigericin, which immediately collapses the pH potential and returns the fluorescence intensity to base line (D).
studies do not exclude the presence of specific sinusoidal membrane transporter(s) that may be particularly important at low plasma bilirubin concentrations, our findings are entirely consistent with a diffusional uptake mechanism for unconjugated bilirubin. Despite the large body of kinetic evidence supporting the existence of protein-mediated bilirubin transport and the identification of several putative bilirubin transport proteins in liver cells (12, 39, 40), none of the candidate transporters has been shown to facilitate bilirubin uptake. The only study to directly examine bilirubin transport (by organic anion-transporting polypeptide) reveals no enhancement in bilirubin uptake by transiently transfected HeLa cells over nontransfected controls (15). Evidence that bilirubin is transported by bili-translocase is limited to the induction of hyperbilirubinemia by injection of polyclonal anti-bili-translocase antibodies into rats (41), while support for a role for BSP/bilirubin-binding protein is derived from partial inhibition of bilirubin uptake by pretreatment of HepG2 cells (42) and cultured hepatocytes (10) with a monospecific antibody. These latter findings provide the most convincing proof of the existence of protein-mediated bilirubin transport, although antibody-inhibitable uptake accounts for less than one-half of total uptake. We speculate that the noninhibitable component of bilirubin uptake represents spontaneous transmembrane diffusion and that high affinity binding to glutathione S-transferase (the principal cytosolic bilirubin binding protein in the hepatocyte) and rapid bilirubin conjugation by UDP-glucuronosyltransferase provide sufficient driving force for efficient bilirubin clearance by the liver (43).

Much of the kinetic data supporting the existence of specific bilirubin transporters are derived from studies that utilize hydrophilic organic anions, such as BSP and BDG, as surrogate markers for unconjugated bilirubin (10, 11, 44). While unconjugated bilirubin competitively inhibits BSP transport (10, 11), hepatocellular uptake of bilirubin can be dissociated from BSP (14, 15), supporting the presence of distinct transport mechanisms. We have further demonstrated a marked difference in the uptake kinetics for unconjugated bilirubin and bilirubin diglucuronide in hepatic microsomes (Fig. 9), suggesting that these two compounds also are unlikely to share identical transport mechanisms, at least within this membrane compartment. These findings raise questions as to whether uptake kinetics for hydrophilic organic anions can be appropriately extrapolated to unconjugated bilirubin.

Protein-mediated transport has been inferred from the increased uptake of unconjugated bilirubin by isolated rat basolateral liver plasma membranes in the presence of valinomycin and an inwardly directed potassium gradient (24). While this finding has been construed as evidence for electrogenic uptake, it is equally consistent with a model of bilirubin transport across biologic membranes, originally proposed by Wennberg (5, 45), in which unconjugated bilirubin traverses the hydrophobic membrane core as the uncharged diacid. We have shown that bilirubin induces acidification of the entrapped volume of phospholipid vesicles, supporting a mechanism whereby bilirubin anions acquire a proton(s), diffuse through the lipid bilayer, and subsequently release hydrogen ions into the vesicle interior (31). Since the dissipation of a transmembrane proton gradient is limited by the slow counterflow of potassium ions (46), we propose that increased uptake of bilirubin by bLPM in the presence of potassium and valinomycin results from rapid dissipation of a bilirubin-generated pH gradient, as opposed to electrogenic transport of charged bilirubin species.

The UDP-glucuronosyltransferases are a family of enzymes that convert hydrophobic endo- and xenobiotics (e.g. bilirubin, drugs) into water-soluble glucuronides. Topographical studies of UDP-glucuronosyltransferase indicate that the active site is oriented toward the lumen (47, 48) of the endoplasmic reticulum, and it has long been postulated that specific transport systems exist to facilitate the movement of polar glucuronides back across the microsomal membrane into the cytosol. Although uptake of bilirubin diglucuronide by hepatocyte basolateral and canalicular plasma membranes has been well characterized (49), our demonstration of rapid BDG transport across microsomal membranes provides some of the first direct evidence for the presence of a glucuronide transporter in the endoplasmic reticulum (50). Based on these findings, we propose that unconjugated bilirubin gains access to active site of UDP-glucuronosyltransferase via spontaneous flip-flop across the microsomal membrane, while bilirubin glucuronide export is carrier-mediated.

In contrast with newborns who are at significant risk for the development of kernicterus (bilirubin encephalopathy), adults are resistant to the neurotoxic effects of bilirubin. The basis for this observation has been presumed to reside in the lipid composition of the adult blood-brain barrier, which renders it impermeant to bilirubin. Our findings regarding the relative ease with which bilirubin traverses membranes composed of a variety of lipid species shed doubt on this hypothesis. UDP-glucuronosyltransferase activity has been identified in brain microvessels (51) and choroid plexus (52), and we speculate that glucuronidation of bilirubin to form membrane-impermeant bilirubin conjugates is an essential mechanism to prevent bilirubin entry into the adult brain. In the newborn, hepatic UDP-glucuronosyltransferase activity is extremely low, approaching adult levels only after several days of life (53). Assuming that expression of UDP-glucuronosyltransferase in brain capillary endothelial cells parallels that in the hepatocyte, there exists a period during early postnatal development when the blood-brain endothelium is unable to effectively conjugate bilirubin, permitting access of unconjugated bilirubin to the central nervous system.

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