Interaction of Bilirubin with the Synaptosomal Plasma Membrane*

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The interaction of the neurotoxic pigment bilirubin with synaptosomal plasma membrane vesicles (SMPV) isolated from rat brain was investigated. The interaction seems to involve three steps: (a) a rapid formation of an electrostatic complex between bilirubin and polar lipid head groups; (b) a slow inclusion of the pigment into the hydrophobic core of the membrane; and (c) a SMPV-induced bilirubin aggregation, observed when membrane capacity for bilirubin is exceeded. The association constant of the initial complex increased markedly when pH was lowered below 7.4, particularly in SMPV isolated from newborn rats. A preferential binding of bilirubin to pure gangliosides and sphingomyelin was observed, thus suggesting a role for these lipids as first targets of the pigment in the synaptic membrane. The inclusion of bilirubin into the membranes was gradually enhanced when decreasing the pH or the age of the rats from which SMPV were isolated. In addition, membranes from 2-day-old rats have a higher capacity for bilirubin incorporation compared to those from adult rats. Experiments with reconstituted liposomes of varying protein and cholesterol contents suggest that the effect of age may be related to changes in synaptosomal membrane fluidity during development. Our results support the hypothesis that the interaction of bilirubin with the synaptic membrane plays an important role in the molecular mechanisms of bilirubin neurotoxicity.

Bilirubin is a bile pigment produced in the catabolism of hemoproteins. Bilirubin deposition in the brain during the neonatal period causes irreversible brain damage, a condition known as kernicterus or bilirubin encephalopathy (1, 2). Low birth weight, prematurity, perinatal asphyxia, acidosis, and the use of certain drugs have been reported to be the major predisposing factors for the occurrence of bilirubin encephalopathy, even in newborn infants with low levels of serum bilirubin (3–8). Numerous toxic effects of bilirubin have been observed, most of them on membrane-related processes (see Refs. 2, 9, and 10). Thus, the pigment has been shown to alter several mitochondrial functions (11, 12), to inhibit membrane-bound enzymes such as (Na+,K+)-ATPase or protein kinase C (13–15), and to affect ion membrane permeability (16) and synaptosomal membrane potential (17). In concordance with these effects, bilirubin has been reported to interact with mitochondrial membranes (12), erythrocyte membranes (18, 19), phospholipid bilayers (20–23), and gangliosides (24). However, there is a lack of information about the interaction of bilirubin with its expected target in pathophysiological circumstances, i.e., with specific neuronal structures. Such information might be relevant in elucidating the poorly understood mechanisms of bilirubin neurotoxicity, particularly in explaining the enhanced susceptibility of specific regions of the nervous tissue to bilirubin-induced damage as well as the increased risk of pigment accumulation in the brain of newborn infants (1, 2, 5, 7).

Therefore, the purpose of this study has been to characterize the interaction of bilirubin with the synaptosomal plasma membrane. This was accomplished by investigating the interaction of the pigment with different isolated membrane components and particularly with preparations of plasma membrane vesicles derived from synaptosomes isolated from the brain of rats at different stages of development. The plasma membrane preparations from various sources and specifically from synaptosomal fractions (25–27) have proved to be extremely useful for the study of binding and transport mechanisms. Our results provide strong evidence that three different stages are involved in the interaction of bilirubin with the synaptosomal plasma membrane: a complex between bilirubin and membrane lipid head groups, a hydrophobic inclusion of bilirubin into the membrane lipid bilayer, and a membrane-induced aggregation of bilirubin acid. Our data support and further extend the results obtained by Nagaoa and Cowger (20) and Eriksen et al. (23) in liposomes, and show that the internal structure of the membranes and the age of the rats from which they are isolated are critical factors. These results could also be of interest in explaining the increased risk of accumulation of bilirubin in certain areas of the brain during the early stages of development, in agreement with a key role of the interaction of bilirubin with the neuronal membranes in the pathogenesis of bilirubin encephalopathy.*

EXPERIMENTAL PROCEDURES

Materials

Crystalline bilirubin, fatty acid-free bovine serum albumin, sphingomyelin, gangliosides, ceramide, sphingosine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphorylcholine chloride, glycerophosphorylcholine, N-acetylneur...
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Raminic acid, trypsin, 1,6-diphenyl-1,3,5-hexatriene, phospholipase D, and phospholipase C were obtained from Sigma. All other chemicals were of the highest purity commercially available. Stock solutions of bilirubin were prepared by dissolving small amounts of solid bilirubin in 0.1 M KOH essentially as described (122). These solutions were diluted just before use with SPMV buffer, pH 7.4, and incubated in the same ionic medium, except for the change in pH, when indicated. During some experiments pH was "jumped" by a rapid addition of a small calibrated volume of concentrated KOH or HCL. Protein concentration was assayed by the fluorometric method of Resch et al. (30), using BSA as standard.

Preparation of Synaptosomal Plasma Membrane Vesicles (SPMVs)—SPMVs were isolated from rat brain as described (25, 26). This preparation is enriched in plasma membrane markers (27). SPMVs were finally preloaded with 120 mM KCl, 22 mM potassium phosphate buffer, pH 7.4, and incubated in the same ionic medium, except for the change in pH, when indicated. During some experiments pH was "jumped" by a rapid addition of a small calibrated volume of concentrated KOH or HCL. Protein concentration was assayed by the fluorometric method of Resch et al. (30), using BSA as standard.

Preparation of Lipids from SPMV and Preparation of Liposomes and Micelles—Lipids were isolated from SPMV by extracting with a chloroform/methanol (2:1) mixture as described by Folch et al. (31). Lipid/protein weight ratio was found to be 0.57 ± 0.04 for SPMV and 0.59 ± 0.11 for 2-day-old SPMV, expressed as the mean ± S.E. of three determinations. Liposomes of either SPMV lipids or commercial lipids were prepared daily by sonic oscillation in a MSE 100-watt ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London), as described (20). Electron microscopy and filtration through Sepharose CL-4B columns showed that liposomes were preswollen in 120 mM KCl, 22 mM potassium phosphate buffer, pH 7.4, and incubated in the same ionic medium, except for the change in pH, when indicated. During some experiments pH was "jumped" by a rapid addition of a small calibrated volume of concentrated KOH or HCL. Protein concentration was assayed by the fluorometric method of Resch et al. (30), using BSA as standard.

Preparation of Lipid-depleted SPMV—Lipid-depleted SPMV were prepared by lipid extraction with 10% water in acetone in the presence of amonia according to the method developed by Fleischer and Fleischer (53) for the extraction of approximately 95% of lipid from mitochondria.

Reconstitution of Liposomes with SPMV Proteins—1-ml minicol- umns were filled with Sephadex G-50 preswollen in 120 mM KCl, 22 mM potassium phosphate, pH 7.4 (buffer A). SPMV (2 mg of protein/ml) were incubated for 1 h at 4 °C with continuous stirring in buffer A plus 1% sodium cholate (buffer B) to solubilize membrane proteins and centrifuged at 100,000 g for 1 h. 100 μl of the supernatant were then added to 250 μl of a suspension of SPMV-extracted lipid (0.5 mg/ml) in buffer B, and the mixture was carefully applied onto the Sephadex minicolumn, recovering the proteoliposomes in the void volume eluate. Control liposomes were prepared in the same way, except that the 100 μl of supernatant were replaced by 100 μl of buffer B. Using this procedure less than 3% of the cholate initially added was recovered in the eluate, as measured by the method of Kagawa and Racke (34). An average of 45% of the initial SPMV proteins was found in the proteoliposome suspension.

Fluorescent Labeling of SPMV, Liposomes, and Micelles—A stock solution of 4 mM DPH was prepared in tetrahydrofuran. Small amounts of this solution were slowly added to 250 μl of SPMV or lipid suspension with vigorous stirring, and the mixture was than centrifuged at 27,000 g for 20 min, the pellet washed twice, and finally resuspended in the same medium, except for the change in pH, when indicated.

RESULTS

General Characteristics of the SPMV-Bilirubin Interaction—When bilirubin was added to a DPH-labeled SPMV suspension, fluorescence from the probe was quenched immediately (Fig. 1). The initial quenching extent appeared to be independent of the SPMV concentration in the range 10–200 μg of protein/ml, but depended strongly on the pH, being more pronounced at pH decreased. At pH values of 8.0 (Fig. 1B) and higher (not shown), the intensity of quenched fluorescence remained constant for the time considered. However, at lower pH a recovery of fluorescence was observed, and both its rate and extent augmented with increasing SPMV concentration. The profile of the recovery process gradually changed with the pH, being sigmoidal at pH 7.4 (Fig. 1B) and hyperbolic at pH 7.0 (Fig. 1A). Interestingly, when the interaction was monitored at pH 7.0 by absorption spectrophotometry, the maximum of the Soret band of the absorption spectrum of the pigment was shifted from 440 to 450–460 nm, and a shoulder around 490 nm appeared (Fig. 2, inset) simultane-
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**Fig. 1.** Time course of the bilirubin-induced quenching of fluorescence of DPH-labeled SPMV. The time course was started when bilirubin was added to the incubation mixture. Experimental conditions were as follows. A, SPMV concentrations: 200 (■), 100 (■), 50 (■), 25 (■), and 10 μg of protein/ml (●); pH 7.0. B, SPMV concentration: 30 μg of protein/ml, pH 8.0 (●); and SPMV concentrations: 200 (■) and 50 μg of protein/ml, pH 7.4. Bilirubin concentration was 10 μM in all the experiments.

**Fig. 2.** Differential absorption spectra of bilirubin upon interaction with SPMV. ———, 10 μM bilirubin in the presence of SPMV at 100 μg of protein/ml; — — — , 10 μM bilirubin in the presence of SPMV at 5 μg of protein/ml. pH was 7.0. Numbers in the figure indicate the value of λm in each case. *Inset,* absolute absorption spectra of 10 μM bilirubin alone (——) and in the presence of SPMV at 100 (——) and 5 (———) μg of protein/ml.

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Bilirubin-Synaptosomal Membrane Interaction

Fig. 3. λ<sub>max</sub> shifts in the interaction of SPMV with bilirubin at pH 7.0. Measurements were carried out after incubating for 30 s (A), 3 min (B), or 20 min (C). SPMV and bilirubin concentrations ranged from 0.5 to 100 µg of protein/ml and from 0.5 to 20 µM, respectively. Only concentration ratios are indicated.

Fig. 4. Effect of BSA on the interaction of SPMV with bilirubin. —, bilirubin alone in buffer; ---, bilirubin in the presence of BSA; ----, bilirubin incubated with SPMV for 20 min; -----, bilirubin and SPMV incubated for 20 min after an addition of BSA and a further incubation for the time indicated by the numbers. Bilirubin and BSA concentrations were 10 and 13 µM, respectively, in all the experiments. SPMV concentration was 180 (left graph) or 5 µg of protein/ml (right graph).

after bilirubin had been allowed to interact with SPMV for 1–20 min at pH 8.0 or right after the initial quenching at pH 7.0, the fluorescence immediately returned to the levels recorded in the case of the prior addition of BSA. However, if the addition was made once the fluorescence has been allowed to recover, only a slow reversion of fluorescence was detected (data not shown). Accordingly, when BSA was added to a mixture of bilirubin and “excess” SPMV (i.e. above the critical SPMV/bilirubin concentration ratio below which λ<sub>max</sub> starts to shift) previously allowed to interact for 20 min at pH 7.0, the spectrum of SPMV-bound bilirubin changed slowly toward that of BSA-bound bilirubin (Fig. 4, left panel). However, when the SPMV/bilirubin concentration ratio was lowered below the critical ratio, the ability of BSA to displace SPMV disappeared (Fig. 4, right panel). Taken together, these results indicate that the ability of BSA to displace bilirubin-bound SPMV to form the BSA-bilirubin complex depends on which interaction step has been reached. The first step (initial quenching of fluorescence) is rapidly reversed, and the fluorescence recovery or the spectral change corresponding to the second step is slowly reversed. Finally, no reversion is apparent when a high bilirubin/SPMV concentration ratio is used.

Different patterns of reversibility were also observed in pH jump experiments. A pH jump from 8.0 to 7.0 produced the same fluorescence recovery profile and dependence on SPMV concentration as when bilirubin was directly added at pH 7.0 (not shown). Within an incubation period of 20 min at pH 7.0, the process was completely reversible by pH jumps to 8.0. On the contrary, if a considerably longer period was considered (4 h), a heterogeneous profile indicating incomplete reversibility was found (results not shown). In addition, when SPMV and bilirubin (at a concentration ratio below the critical) were allowed to interact for 5–30 min at pH 7.0, the spectral characteristics of free bilirubin were recovered immediately after the pH “jumped” from 7.0 to 8.0 (data not shown), thus indicating complete reversibility. However, when the mixture was left to interact at pH 7.0 for longer periods of time (for instance, 2 h) under indentical conditions, the pH jump to 8.0 only reversed the interaction partially (not shown).

The Initial Bilirubin-Membrane Complex—The characteristics of the first stage of the interaction were analyzed by obtaining the quenching isotherms at various pHs and concentrations of SPMV immediately after adding bilirubin (Fig. 5). The modified quenching plots according to Lehrer and
Sogami (55, 56) yielded straight lines (not shown). As explained in the Miniprint Section, such linearity is consistent with a simple binding equilibrium model and a concentration of bound bilirubin very low compared to that of free pigment. Accordingly, a negligible dependence of quenching isotherms on SPMV concentration (in the range 7–100 µg of protein/ml) was observed (Fig. 5). As illustrated in this figure, the correspondence between the model and the experimental data is very good. Therefore, it is strongly suggested that a simple binding equilibrium is rapidly established between bilirubin and the membranes at the three pH values considered.

Table I (SPMV row) shows that both the association constant (K) and the maximum average quenching efficiency (η) markedly increase as pH is lowered from 8.0 to 7.0. Whereas the more important shift in K occurs when pH decreases below 7.4, η already reaches its maximum value when the pH is lowered from 8.0 to 7.4. A computer simulation study clarified this effect of pH comparing the theoretical proportions of protonated bilirubin species with the fraction of “additional” bilirubin molecules that bind to the membrane when pH is lowered below 8.0 and with the maximum quenching efficiency as a function of pH. The increase in the proportion of monoanionic bilirubin (BH−) was found to run parallel to the increase in the amount of bilirubin bound to the membranes. Thus, the presence of BH− seems to be responsible for the increased affinity at lower pHs. On the other hand, η was found to increase in a pH range where there is almost no change in the proportions of bilirubin ionic species. As suggested by Eriksen et al. (23) for the binding of bilirubin to phospholipid bilayers, the increase in the quenching efficiency could be explained by a fast protonization of bound bilirubin which, as a consequence of its lower polarity, would bury deeper into the bilayers, thus quenching more efficiently the fluorescence of the probe. If this explanation is to apply to our results, it will be necessary to demonstrate that bound bilirubin is protonated, despite absence of evidence for appreciable protonation of unbound bilirubin.

In order to identify the nature of the membrane sites implicated in the binding process, the interaction of the pigment with different isolated membrane components was investigated. The general characteristics and binding parameters of the interaction of bilirubin with liposomes prepared from lipids extracted from SPMV were similar to those of the intact membranes at the three pH values considered (Table I). In contrast, when SPMV were treated with phospholipases C and D under the conditions described under “Experimental Procedures” (pH 7.4) the bilirubin association constants obtained for the treated membranes (K = 0.3 ± 0.03 µM−1, η = 0.75 for phospholipase C; K = 0.60 ± 0.05 µM−1, η = 0.8 for phospholipase D) were significantly different from those of untreated SPMV. These data would suggest that the polar protruding ends of the SPMV lipids are involved in the initial interaction.

The association constants of the bilirubin interaction with SPMV and SPMV lipids at pH 7.4 were similar to those reported with cow brain and pig skin lipids and phospholipids (20). However, the association constants for the interaction of bilirubin with both gangliosides and sphingomyelin at pH 7.4 (Table I) were found to be 1–2 orders of magnitude higher than those reported for phospholipids at pH 7.5 (20). The average quenching efficiencies were about 1 for both kinds of lipids and remained invariable with the pH, whereas the association constants increased more than 2-fold when pH decreased from 8.0 to 7.4. In contrast, the binding parameters for bilirubin interaction with their structural constituents ceramide (K = 0.5 ± 0.1 µM−1, η = 0.4) and sphingosine (K = 0.39 ± 0.01 µM−1, η = 0.6) at pH 7.4 were similar to those of SPMV and SPMV lipids at the same pH and those reported for phospholipids at pH 7.5 (20). Therefore, the different affinities displayed by bilirubin should be related to the polar head groups of sphingomyelin and the hydrophilic moieties of gangliosides. Further evidence in this regard was obtained by the fact that addition of 5 mM Ca2+ (which is known to bind to the sialic acid residues of gangliosides (37)) markedly augmented the bilirubin-gangliosides association constant at pH 8.0 (from 4.1 ± 0.1 µM−1 to 20 ± 5 µM−1, η = 1), presumably reflecting a favorable shielding of the negative charges of sialic acid residues.

In another set of experiments, the binding characteristics of bilirubin to 2-day-old SPMV were found to be qualitatively similar to that observed with SPMV. The average quenching efficiencies (Table I) and the proportion of binding sites (Table II) were not significantly different between the two preparations. However, lowering of the pH from 7.4 to 7.0 promoted a 4-fold increase in the association constant of 2-day-old SPMV as compared to 2-fold in the case of adults (Table I). This observation could indicate that interaction of bilirubin with synaptic membranes of newborn rats is more likely, in acidaemia, than in adults.

The Slow Interaction of Bilirubin with SPMV—Fig. 6A illustrates the time courses of the spectral change of bilirubin upon interaction with SPMV at different pH values, as monitored by differential absorbance measurements at 495 nm. Fig. 6, B–D, displays the effect of the age of the rats from which SPMV were isolated on the kinetic profiles of the spectral change. These data indicate that membranes from younger rats interact more “easily” with bilirubin. This is apparent from the increased rate and extent of spectral change at pHs 7.3–7.5 displayed by 2-, 10-, and 25-day-old SPMV, as well as from the interaction at higher pHs, for instance 7.65. When SPMV lipids were used, the interaction became even more favorable than with 2-day-old SPMV (see, for example, Fig. 7A, filled squares). These results strongly suggest that

Table I

<table>
<thead>
<tr>
<th>Association constants and average quenching efficiencies of the initial bilirubin interaction</th>
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<tr>
<td>K is expressed as the mean ± S.E. from at least three different membrane or lipid preparations. The usual range of η values is shown.</td>
</tr>
<tr>
<td>pH 8.0</td>
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</tr>
<tr>
<td>K</td>
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<tr>
<td>µM−1</td>
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<tr>
<td>SPMV</td>
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<tr>
<td>2-day-old SPMV</td>
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<tr>
<td>SPMV lipids</td>
</tr>
<tr>
<td>Gangliosides</td>
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<tr>
<td>Sphingomyelin</td>
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TABLE II
Calculated number of binding sites for the interaction of bilirubin with different membrane and lipid preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Number of binding sites</th>
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<tbody>
<tr>
<td>SPMV</td>
<td>9 ± 1 nmol/mg protein</td>
</tr>
<tr>
<td>2-day-old SPMV</td>
<td>11 ± 1 nmol/mg protein</td>
</tr>
<tr>
<td>SPMV lipid</td>
<td>5 ± 2 nmol/mg lipid</td>
</tr>
<tr>
<td>Gangliosides</td>
<td>20 ± 1 mmol/mol ganglioside</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>5 ± 1 mmol/mol sphingomyelin</td>
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Fig. 6. Time course of ΔA495 in the interaction of bilirubin with SPMV (A), 25-day-old SPMV (B), 10-day-old SPMV (C), and 2-day-old SPMV (D) as a function of pH. ΔA495 was monitored as detailed under “Experimental Procedures.” Bilirubin and SPMV concentrations were 10 μM and 100 μg of protein/ml, respectively, in all cases. The pHs of the incubation media are indicated.

the internal composition and/or structure of the SPMV plays an important role in the ability of bilirubin to interact with the membranes.

As shown in Fig. 7A, the reconstitution of liposomes from SPMV lipids with proteins extracted from SPMV clearly slows the interaction and decreases the final extent of the spectral change. Moreover, the presence of cholesterol in the liposomes markedly diminishes the ability of bilirubin to interact with the lipids (Fig. 7B). Since proteins as well as cholesterol are known to increase microviscosity in membranes (38), it is strongly suggested that the slow interaction of bilirubin with the synaptic membrane is modulated by the membrane fluidity. The synaptic membrane is known to become less fluid with age, probably as a consequence of an increase in its cholesterol content and of a decrease in the unsaturation index of its fatty acid constituents (39). Such variations in microviscosity could thus explain the changing pattern of bilirubin interaction with SPMV of different ages.

In order to further characterize the slow interaction, two different experimental approaches were employed. First, the characteristics of the final state of fluorescence recovery at pH 7.0 were studied (Fig. 8). As shown, the fluorescence signal depended strongly on the SPMV concentration, as opposed to the initial interaction. We have interpreted these data in terms of a model which assumes the interaction of bilirubin with an additional population of binding sites (see Miniprint Section). Using the values of K1, n1, and n2 from Tables I and II and a computer simulation program, we found that the functional dependence of T/T0 (fluorescence quenching) on B7 (total bilirubin concentration) and P (SPMV concentration) estimated by the model adequately fitted the experimental data (see Fig. 8, A and B), using the sole set of parameters K2 = 0.9 μM⁻¹, n2 = 1, and n2 = 1.4 μmol/mg protein. Thus, although the binding parameters are similar, the number of binding sites predicted for the second population is more than 100 times higher than that for the first one. The plot of the initial rate of fluorescence recovery (ω0) versus the parameter α also yielded a straight line (Fig. 8A, inset), as predicted by the model. From this plot a value of k1 = 0.06 μm⁻¹ min⁻¹ was calculated and, since K2 = 0.9 μM⁻¹, k2 was estimated to be 0.07 min⁻¹.

The two-population model also explains why Nagaoka and Cowger (20), in their study of the interaction of bilirubin with brain and skin lipids, observed almost no lipid dependence of the quenching in the final state of fluorescence recovery, in contrast to our results. According to the model, the final quenching of fluorescence has first a steep dependence on the total number of binding sites present (Fig. 8A, lefthand side) and tends to reach a plateau as they are increased (Fig. 8A, righthand side). The amount of lipid present in our SPMV suspensions did not exceed 0.06 mg/ml (SPMV concentrations up to 0.1 mg of protein/ml). However, Nagaoka and Cowger (20) usually employed lipid concentrations up to 1.24 mg/ml which presumably correspond to the region in which the dependence of the quenching on the number of binding sites is negligible.

The second approach was to study the differential spectrophotometric titrations at 495 nm once the spectral change was completely developed at pH 7.0 (Fig. 9). Assuming a
Bilirubin-Synaptosomal Membrane Interaction

![Graph](image)

**Fig. 8.** Comparison of the results predicted by the model of two populations of binding sites with the experimental data. A, dependence of \(I/I_0\) in the final state of fluorescence recovery on SPMV concentration. Inset, dependence of \(I_0\) on the parameter \(\alpha\) (see text and Miniprint for details). B, bilirubin quenching isotherms in the final state of fluorescence recovery. Experimental conditions were as follows: A, bilirubin concentration, 10 \(\mu\)M; B, SPMV concentrations: 100 ( ), 25 ( ), 10 ( ), and 5 \(\mu\)g of protein/ml ( ). All the curves were generated by computer according to Equations 7 and 9 (Miniprint Section) using the parameters from Table I and the following values: \(K_1 = 0.95 \mu\)M\(^{-1}\), \(n_1 = 1\), and \(n_2 = 1.4 \mu\)mol/mg protein.

![Graph](image)

**Fig. 9.** Differential spectrophotometric titrations of SPMV with bilirubin at pH 7.0. \(\Delta \text{Cap}\) was measured after incubating bilirubin with SPMV for 20 min. SPMV concentrations were: 2 ( ), 7 ( ), 10 ( ), 15 ( ), 20 ( ), and 100 \(\mu\)g of protein/ml ( ). \(\Delta \text{Cap}\) was calculated as the limiting slope that results when SPMV concentration increases infinitely. Curves were generated by computer (see Miniprint) according to Equation 4 (solid lines) or Equation 5 (dashed lines) using the best fit parameters obtained in each case.

The model assumes that the association constant \(K\) and the number of binding sites \(n\) were calculated as explained in the Miniprint Section. Computer-generated curves showed a good correspondence between the predictions of the model and the experimental data (Fig. 7, solid lines). Interestingly, the number of binding sites calculated (0.7 ± 0.1 \(\mu\)mol of bilirubin/mg of protein) is of the same order as that predicted by the fluorescence recovery model (1.4 \(\mu\)mol of bilirubin/mg of protein). Moreover, the number of binding sites clearly agrees with the critical ratio (0.5-1 \(\mu\)mol of bilirubin/mg of protein) above which \(\lambda_m\) starts to shift.

The interaction of bilirubin with 2-day-old SPMV and with SPMV lipids was also studied by performing the differential spectrophotometric titrations at 495 nm. The number of binding sites obtained for 2-day-old SPMV (1.8 ± 0.3 \(\mu\)mol of bilirubin/mg of protein) was more than 2 times higher than that of SPMV, despite their similar lipid/protein weight ratios (see “Experimental Procedures”). These findings further emphasize the important role that the internal composition and/or structural arrangement of the membranes plays in the mechanism of bilirubin-SPMV interaction.

The association constants obtained (1-15 \(\mu\)M\(^{-1}\) for SPMV, 1-7 \(\mu\)M\(^{-1}\) for 2-day-old SPMV, and 0.2-4 \(\mu\)M\(^{-1}\) for SPMV lipids) were relatively high, approximately only 1 order of magnitude lower than the association constant of bilirubin to BSA (40, 41), and 10\(^5\) times higher than that of bilirubin-phosphatidylcholine interaction at pH 8.2 (22). However, given the apparent hydrophobic nature of this step of the interaction, the process would also be adequately described by a model which assumes a nonspecific partitioning of bilirubin molecules between aqueous and membrane phases. In that case, the limited capacity of SPMV for bilirubin would arise from the unfavorable electrostatic interactions among the ionized propionic acid groups of the bound bilirubin, as proposed by Rose et al. (42) for the interaction of heme with phospholipid bilayers.

We then alternately investigated if the partitioning model proposed by Rose (42) (see Miniprint Section) was able to fit the data shown in Fig. 9. As shown in this figure (dashed lines) the fitting of the experimental data to this model is also very good. The calculated parameter \(A\) fluctuated between 1.5 and 8 \(\text{m}^-1\). \(C_m\) (the surface capacitance) could, therefore, be estimated as lying between 900 and 4300 \(\mu\text{F/cm}^2\), assuming \(Z = -2\) for bound bilirubin. However, we could only conclude that the value of \(K\) (the partition constant) is at least \(10^6\). The model was also applied to the spectrophotometric titrations corresponding to 2-day-old SPMV and SPMV lipids. The surface capacitances obtained where estimated as lying between 2500 and 6400 \(\mu\text{F/cm}^2\) (\(A = 1-3 \text{ m}^-1\) for 2-day-old SPMV and between 600 and 3700 \(\mu\text{F/cm}^2\) for SPMV lipids (\(A = 2-13 \text{ m}^-1\)). As with SPMV, the values of \(K\) were at least \(10^5\) in either case.

The minimum values obtained for \(K\) resemble those reported by Rose et al. (42). In contrast, the calculated values of \(C_m\) are 1-2 orders of magnitude higher than those obtained by Andersen et al. (43) and by Rose et al. (42) for the binding of tetraphenylborate and heme, respectively, to phospholipid bilayers. This may likely be a consequence of a partial protonization of bilirubin bound to the bilayer, i.e., an average value of \(Z\) for bound bilirubin above -2, which would make the surface capacitance overestimated. This result is consistent with the fact that no interaction is observed at pH 8.0, at which the concentration of protonated species of bilirubin is negligible.

The Aggregation of Bilirubin—Fig. 10A shows that the amount of light scattered by a mixture of bilirubin and SPMV after an incubation for 20 min at pH 7.0 greatly increased when the capacity of the membranes for bilirubin (Table I) was exceeded. When SPMV were replaced by a suspension of phosphatidylserine liposomes (Fig. 10B), the increase in scattered light observed when the bilirubin/lipid concentration ratio exceeded a critical value was even clearer. On the other hand, at pH 8.0, no scattered light was detected in the presence of SPMV or liposomes (not shown).
The reversibility of the aggregation process of bilirubin in the presence of a low concentration of SPMV after pH jumps from 7.0 to 8.0 or BSA additions at pH 7.0 was also studied. BSA was found unable to influence the process. On the other hand, the pH jumps completely reversed the scattering signal only when short periods of incubation of bilirubin with the membranes (5-30 min) were considered. The reversion was incomplete for longer periods of incubation (2 h) (data not shown).

Taken together, these results clearly demonstrate that the \( \lambda_m \) shifts, the occurrence of sigmoidal kinetics, and the loss of the ability of BSA to displace SPMV-bound bilirubin are a consequence of the SPMV-induced aggregation of the pigment. Indeed, the \( \lambda_m \) shifts are, in spectrophotometric terms, analogous to the disappearance of the isosbestic point corresponding to the dimerization equilibrium of several porphyrins as a consequence of aggregation processes (44, 45). Cooperative changes in the bilirubin absorption spectrum in the presence of lipids have also been considered to be an aggregation phenomenon induced by bound bilirubin (20, 23).

The aggregation presumably competes with the hydrophobic inclusion of bilirubin into the membranes. At pH 7.0, however, hydrophobic inclusion seems to be kinetically more favorable, and the aggregation of bilirubin is only clearly observed when the SPMV become saturated. The inability of pH jumps from 7.0 to 8.0 to reverse completely the process of bilirubin aggregation when long periods of incubation time are considered may most likely arise from an "aging" of bilirubin aggregates which would progressively form microcrystals of bilirubin acid on the SPMV surface. Such bilirubin acid microcrystals have been detected by several authors in albumin-bilirubin solutions (46), in cerebellar cell cultures (47), and even in the brains of icteric neonates at autopsy (48).

**DISCUSSION**

The aim of this paper was to contribute to a better understanding of the molecular mechanisms of bilirubin neurotoxicity. We have approached this problem by investigating in detail the interaction of the pigment with specific neuronal structures, using a preparation of synaptosomal plasma membrane vesicles isolated from rat brain (SPMV), as well as several isolated components of such membranes. Our purpose was to characterize the interaction and to identify the molecular species which are involved in the process. On the other hand, taking into account that the nervous tissue of the newborn shows a markedly enhanced risk of pigment accumulation and that asphyxia and acidosis are major predisposing factors for the occurrence of bilirubin encephalopathy (see Refs. 2 and 5 and references therein), we have focused our attention on the effects that the stage of development of the rats from which SPMV were isolated and the pH of the incubation media have on the interaction of bilirubin with the synaptic membrane.

The data presented in this report can be summarized in our proposed model for the molecular mechanisms of the interaction of bilirubin with the synaptosomal plasma membrane. This includes three different phenomena: (i) an initial complex between anionic forms of bilirubin and the polar lipid head groups on the membrane surface, especially those from gangliosides and sphingomyelin (Fig. 11A); (ii) an inclusion of bilirubin into the hydrophobic core of the membrane (Fig. 11B); and (iii) a membrane-induced aggregation of bilirubin acid (Fig. 11C).

The static fluorescence quenching method used in this paper has been employed by several authors for the study of bilirubin interaction with various kinds of phospholipid bilayers (20, 23). The fluorescence quenching data show that bilirubin interacts almost instantaneously (<15 s) with the SPMV. The experimental results can be explained by assuming a single binding equilibrium model. Several lines of evidence indicate that the interaction consists of the formation of a surface complex (with at least partial electrostatic nature) between anionic bilirubin and the polar head groups of the SPMV lipids. An ionic interaction between bilirubin and...
phospholipids has been proposed by several authors (20, 23, 49).

In its first interaction with the neuronal membrane, bilirubin probably binds to most kinds of the membrane lipids, since their affinities for the pigment are similar (Ref. 20 and this paper). However, our data show that sphingomyelin and gangliosides bind bilirubin with a high affinity, their association constants being 5-25 times higher than those of other lipids including sphingolipid precursors such as ceramide and sphingosine (20, 22). It is worth noting that sphingomyelin and gangliosides are asymmetrically localized on the outer surface of the neuronal membranes, particularly at the presynaptic nerve endings. Gangliosides, for instance, constitute about 10 mol % of the total plasma membrane lipid and 20 mol % of the lipid on the outer surface of neural synaptic membranes, thus occupying a significant fraction of the plasma membrane lipidic surface (50). In severe cases of neonatal jaundice, the concentration of albumin-bound bilirubin may be 150 μM, giving a calculated concentration of free bilirubin dianion of 0.25 μM (23). Our results suggest that at submicromolar concentrations bilirubin would bind preferentially to the gangliosides and sphingomyelin in the SPMV, thus indicating a role for both these lipids as first targets for the binding of the pigment to the synaptic membranes.

Another important fact is that the binding parameters of the SPMV-bilirubin initial interaction are markedly dependent on the pH of the incubation medium, in agreement with previous results with other preparations (18, 23, 51, 52). The presence of the bilirubin monoanion seems to be responsible for the increased affinity of the membranes for bilirubin at lower pHs. Interestingly, such an increase in affinity is much more marked in SPMV isolated from the brain of newborn rats, thus suggesting that the interaction of bilirubin with the nervous tissue in the newborn animals is more critically affected by changes in the pH below physiological values. It should be noted that a blood pH of 7.0 and lower can be attained as a consequence of episodes of asphyxia and that changes in the blood pH of 1-day-old rats during 20 min of anoxia and the following recovery correlate well with a transient increase of bilirubin levels in the brain of the newborn rats (53, 54).

At pH lower than 8.0, the other steps of interaction will take place. The fluorescence recovery data and the appearance of spectral changes are consistent with binding of the pigment to a second population of sites. The equilibrium between bilirubin and the latter sites is reached more slowly (>15 min) than in the case of the initial interaction (<15 s), and the number of binding sites is approximately 100 times higher. The ability of BSA to quickly reverse this interaction disappears when the pigment binds to the second population of sites, though the association constants for both populations are similar. This fact might reflect the binding of bilirubin to sites "masked" for BSA. In this regard, it is worth noting that, in order to prevent or reverse bilirubin effects "in vitro," BSA must be added before or shortly after bilirubin (11, 17).

The slow interaction may be interpreted as an inclusion of bilirubin into the hydrophobic core of the membranes. This process appears to be saturable in the sense that synaptosomal plasma membranes seem to be able to accommodate a limited number of bilirubin molecules. This fact is evident no matter which interaction model (i.e. single binding equilibrium or partition) is assumed, since both predict that at pH 7.0 most bilirubin molecules would be included in the bilayer until the latter becomes saturated.

Finally, several observations indicate that the remaining bilirubin molecules tend to form aggregates on the surface of the membranes, gradually forming microcrystals. These observations include the reversibility patterns of the interaction after pH jumps from 7.0 to 8.0, the inability of BSA to reverse the spectral change below the critical SPMV/bilirubin concentration ratio, the λ<sub>a</sub> shifts, the occurrence of sigmoidal kinetic profiles, and the light-scattering data.

The occurrence of several steps in the interaction of bilirubin with liposomes (20, 23) or erythrocyte ghosts (19) has been proposed by other authors. In general, the findings of Nagao and Cowger (20) and Eriksten et al. (23) in liposomes are in agreement with our results. It is worth noting, however, that in our SPMV preparations aggregation of bilirubin is not the only phenomenon that takes place at pH below 8.0 after the formation of the initial surface complex. A distinct second phase of the interaction can be characterized, the inclusion of bilirubin into the membrane bilayer, probably reflecting the partitioning of the pigment as described by others for heme (42). In this regard, our results clearly point out the importance of parameters such as the membrane (or lipid)/bilirubin concentration ratio and the time of the interaction in the interpretation of the data. The experimental conditions (low liposomes/bilirubin concentration ratios and long times of incubation) used by Eriksten et al. (23) will favor the occurrence of aggregation phenomena.

There are several relevant aspects of the interaction of bilirubin with the synaptosomal membrane that could help to understand better the mechanisms of bilirubin accumulation and toxicity. First, the high content in gangliosides and sphingomyelin would dispose the synaptic and dendritic regions of the brain to interact preferentially with bilirubin, thus rendering the nervous tissue more susceptible to the toxic effects induced by the binding of the pigment. Second, very small pH changes (0.2-0.4 pH units) can bring about very marked changes in the affinity, extent, and kinetic characteristics of the different steps of the interaction. Therefore, the acidosis due to episodes of asphyxia in the perinatal period may be a critical risk factor for accumulation of bilirubin in the brain (2, 54). Factors such as regional differences in cerebral blood pH or in membrane lipid composition (i.e. proportion and/or presence of subclasses of sphingomyelin and gangliosides) might be relevant in explaining the preferential accumulation of bilirubin in different areas of the brain that is observed in bilirubin encephalopathy and after anoxic episodes (2, 5, 54).

Third, in connection with the effect of acidosis, the stage of development of the rats from which the synaptosomal plasma membranes are isolated also has a marked influence on the interaction. Given the effect of "ordering" agents inhibiting the interaction, our data suggest that this fact may be related not only to developmental changes in the chemical composition of the bilayers, but also to changes in membrane fluidity (39). Fourth, the existence of several phases in the interaction of bilirubin with the synaptosomal membrane would suggest that bilirubin neurotoxicity may result from multiple effects on synaptic functions.

Further studies of the above mentioned aspects will be needed in order to fully explain the preferential accumulation of the pigment in different areas of the brain of the newborn, as well as the varying degrees of neurological damage (ranging from severe cerebral palsy and mental retardation to minor learning disabilities) associated with both perinatal asphyxia and bilirubin encephalopathy.

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REFERENCES

Experimental Procedure

Analysis of fluorescence quenching data.—The formulation of Banack and Cooper (10) for the quenching of fluorescence from DPH-labeled liposomes by bilirubin was employed. According to these authors, the fluorescence 

$F_{b} = F_{b}^{0} e^{-k_{q} t}$

where $F_{b}$ is the fluorescence intensity at time $t$, $F_{b}^{0}$ is the initial fluorescence intensity, and $k_{q}$ is the quenching rate constant.

For our experiments, we employed the two models: 1. The partition model described by eq. 1, in which $B$ is the bound bilirubin concentration, $F$ is the free bilirubin concentration, and $K$ is the association constant.

$B = K F$.

2. The kinetic model described by eq. 2, in which $B$ is the bound bilirubin concentration, $F$ is the free bilirubin concentration, and $k_{1}$ and $k_{2}$ are the rate constants of the forward and reverse reactions, respectively.

$B = k_{1} F - k_{2} B$.

Analysis of spectrophotometric data.—Two models were employed: 1. The partition model described by eq. 1, in which $B$ is the bound bilirubin concentration, $F$ is the free bilirubin concentration, and $K$ is the association constant. 2. The kinetic model described by eq. 2, in which $B$ is the bound bilirubin concentration, $F$ is the free bilirubin concentration, and $k_{1}$ and $k_{2}$ are the rate constants of the forward and reverse reactions, respectively. These models are expressed in the following equations:

$B = K F$.

$B = k_{1} F - k_{2} B$.

where $B$ is the bound bilirubin concentration, $F$ is the free bilirubin concentration, and $K$ is the association constant.