

Bilirubin-Liposome Interaction

BINDING OF BILIRUBIN DIANION, PROTONIZATION, AND AGGREGATION OF BILIRUBIN ACID*

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Interaction of bilirubin with phospholipid liposomes was studied at varying pH. Liposomes were prepared from egg-phosphatidylcholine, dipalmitoyl-phosphatidylcholine, phosphatidylethanolamine, egg-phosphatidylcholine, and phosphatidylethanolamine with 5% (w/w) cholesterol, phosphatidylserine, sphingomyelin, and a lipid preparation from cat brain. Experiments were also conducted with erythrocyte ghosts. Interaction with bilirubin was studied by observing quenching of fluorescence from 1,6-diphenyl-1,3,5-hexatriene, incorporated in the lipid bilayers, by gradient centrifugation of the product, by measuring light scattering during the process, and by studying the solubility and infrared spectra of the final aggregates.

At pH values above 8.2, the findings are consistent with formation of a bilirubin dianion-phospholipid complex. At pH 6-8, protonization of bilirubin in the complex is indicated and is followed by self-aggregation of bilirubin acid with formation of large bilirubin particles containing a small amount of phospholipid.

Results were qualitatively similar with all lipids studied; marked quantitative differences in degree of fluorescence quenching and aggregation rates were observed with liposomes from different phospholipids.

Reflections on affinities and available concentrations indicate that this train of processes may be part of the toxic mechanism of bilirubin.

Bilirubin is toxic to the central nervous system, causing encephalopathy, especially in low-birth-weight, premature infants with hyperbilirubinemia and acidosis. The toxic mechanism has been the object of numerous investigations, mostly with the aim of finding enzymatic processes inhibited by bilirubin. It has been shown that many different membrane-bound enzymes are inhibited or accelerated (see review (1)). It has not been possible, however, to point out any specific enzyme as the target of bilirubin toxicity. Mustafa and King (2) in 1970 found that bilirubin is bound to lipid structures in mitochondria and other membranes. These authors, as well as Cowger (3) in 1971, advanced the hypothesis that the enzymatic changes are a result of bilirubin binding to multiple membrane structures. Since that time, interaction of bilirubin with structured lipids has been studied by several investigators. Nagaoka and Cowger (4), in 1978, reported that binding of bilirubin to phospholipid liposomes results in quenching of fluorescence of an added probe, 1,6-diphenyl-1,3,5-hexatriene located within the hydrocarbon region of the lipid bilayer.

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Association of bilirubin with a range of phospholipids was thus investigated. In the present study, the method of Nagaoka and Cowger (4) was combined with other techniques in order to identify the products of bilirubin-phospholipid interaction. The molecular mechanism of bilirubin encephalopathy in neonates is discussed.

MATERIALS AND METHODS¹

Bilirubin was obtained from Sigma Chemical Co., Inc. (St. Louis, Mo.) and was purified as described by McDonagh and Assisi (14). By thin layer chromatography, the purified bilirubin contains more than 98% bilirubin IX- α and less than 2% III- α and XIII- α isomers. Stock solutions of bilirubin were prepared by dissolving 4.00 mg purified bilirubin in 200 μ l 0.5 M NaOH, followed by slow addition of 5 ml deionized water. Finally, 75 μ l 1M HCl was added with stirring, giving a solution of a pH around 11 and a bilirubin concentration of 1.29 mM. This solution was kept in the dark and used within 1 hour. Experiments with bilirubin were conducted with exclusion of daylight, using bulbs covered with a double layer of orange cellophane, transmitting less than 3% at 460 nm.

The phospholipids used were: Phosphatidylcholine (lecithin from eggs, Article 5331 from E. Merck, Darmstadt, Germany); DL-phosphatidylcholine-dipalmitoyl, Grade 1, approx. 99%, crystalline, synthetic (from Sigma); sphingomyelin from bovine brain, Type 1 (from Sigma); 1- α -phosphatidylethanolamine from egg yolk, 25 mg/ml in chloroform-methanol (4:1) solution (from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England); and 1- α -phosphatidyl-L-serine from bovine brain (from Sigma). In the gradient centrifugation experiments we used [¹⁴C]-phosphatidylcholine (from New England Nuclear, Boston, Massachusetts) in toluene-ethanol (1:1) solution, 25 μ l (1 μ Ci) added to 300 mg egg-phosphatidylcholine. Phospholipids from cat brain were extracted as described by Nagaoka and Cowger (4). The brain was cut into small pieces and suspended in 100 ml, 150 mM NaCl solution. The mixture was agitated for a few minutes and then decanted. This procedure was repeated five times. The tissue was then treated with chloroform-methanol (2:1 by volume) in a glass-teflon homogenizer and subsequently centrifuged at 1200 g (average) for 10 min. The supernatant was filtered through a sintered glass filter, Schott & Genossen, (Jena, DDR) No. 4. The remaining tissue was treated once more and the combined filtrates were evaporated to dryness under reduced pressure in a rotation-evaporator in N₂-atmosphere. The yield was 80 mg dried lipid from 2 g fresh brain.

Erythrocyte ghosts were prepared as described by Dodge et al. (15). Preparation of liposomes has been described by Huang (16) and Bause (17) and was done as follows: The lipid was dissolved in chloroform and the solvent was evaporated in a current of N₂. The thin phospholipid film was suspended in Tris buffer 0.05 M, pH 7.4. The suspension was sonicated in an Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd., London SW1, England, 500 W-model, 20 kHz, Power Output 8). The tube was immersed in an ice-water mixture under nitrogen. The sonicated suspension was centrifuged at 149,000 g (average) for 1 hour in a Beckman L 5-65 ultracentrifuge, rotor SW-50, 1, from Beckman International (Geneva, Switzerland), to separate unilamellar vesicles from multilamellar vesicles and nonvesicular material. The supernatant was used without further treatment and stored in the refrigerator for a maximum of 2 weeks.

Fluorescent labelling of liposomes was carried out as described by Nagaoka and Cowger (4). The fluorescent label, 1,6-diphenyl-1,3,5-hexatriene, was obtained from Sigma. A stock solution, 1 mM, was prepared in tetrahydrofuran. This solution, 0.2 ml, was added to 2 ml liposome suspension, prepared as above, in 58 ml Tris buffer with vigorous stirring. The mixture was allowed to stand for 3 hours for incorporation of the fluorescent label. The suspension was used within 24 hours. The molar ratio of lipid to fluorescent label was about 500 to 1. The pH of the liposome suspension was adjusted with HCl or NaOH, as needed.

Fluorescence was measured, using an Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md. Slits 1, 3 and 4 = 2 mm, Slits 2 and 5 = 4 mm, Slit 7 = 5 mm, Sensitivity 100 percent, High Voltage = 700 V), excitation wavelength, 360 nm; emission wavelength 440 nm, in case of erythrocyte ghosts 430 nm. Correction of measured fluorescence intensities for filter effects was carried out after spectrophotometric determination of the absorbance. It was not technically possible to measure absorbance for each determination of fluorescence intensity when these were done at intervals of one or a few minutes, as in Fig. 1. In these experiments, the absorbance at 3 hours was used for filter effect correction at all times. The absorbance is nearly constant after 1 minute. Measurement of fluorescence intensities before 1 minute thus carries a certain error. Experiments have shown that this error changes the numerical value of fluorescence but not the qualitative shape of the curve.

Light scattering was measured with the same apparatus, incident wavelength = emission wavelength = 546 nm.

Gradient centrifugation of the different bilirubin-phosphatidylcholine mixtures was carried out in the ultracentrifuge. The sucrose gradients were made using a gradient former which assured a linear gradient from 10% (w/v) to 50% (w/v). The specimen, 0.5 ml, was placed on top of 12 ml gradient and centrifuged at 115,000 g (average) for 17 hours at 4°C. The centrifuge tubes were punctured

¹ Portions of this paper (including "Materials and Methods" and Fig. 5) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-1641, cite author(s), and include a check or money order for \$1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

and equal volumes collected, about 20 fractions. Bilirubin concentrations were monitored by spectrophotometry after dissolution of aggregates at pH 9.5–10.0. The amount of [14 C]phosphatidylcholine was determined in a Liquid Scintillation Spectrometer, Tri-Carb, model 2002 (Packard Instrument Co., Inc., Downers Grove, Ill.). Scintillation liquid: 5 g 2,5-diphenyloxazole + 0.3 g 1,4-di-2-(5-phenyloxazolyl)-benzene dissolved in 700 ml toluene and 300 ml abs. ethanol. Calculation of sedimentation constants was carried out as described by Funding and Steensgaard (18).

RESULTS

Bilirubin Dianion-Liposome Complex—When an alkaline solution of bilirubin was added to a buffered suspension of

TABLE I

Fluorescence from 1,6-diphenyl-1,3,5-hexatriene-labeled liposomes on addition of bilirubin at pH 7.4 and 8.3

Fluorescence intensity (F) was measured before addition of bilirubin (F_0), immediately after addition, and again 3 h later. Figures reported are F/F_0 . All values are corrected for filter effects, although this correction is less accurate for the immediate readings (see "Materials and Methods"). Concentration of bilirubin, 12.8 μ M. Temperature, 37 $^{\circ}$ C.

Phospholipid	pH 7.4		pH 8.3	
	Imme- diate	3 h	Imme- diate	3 h
	F/F_0			
Egg phosphatidylcholine, 130 μ M	0.31	0.77	0.41	0.41
Egg phosphatidylcholine, 150 μ M + cholesterol, 15 μ M	0.18	0.75	0.44	0.46
Phosphatidylethanolamine, 106 μ M	0.32	0.81	0.28	0.38
Phosphatidylethanolamine, 102 μ M + cholesterol, 10 μ M	0.25	0.75	0.33	0.31
Sphingomyelin, 32 μ M	0.02	0.38	0.05	0.05
Phosphatidylserine, 55 μ M	0.47	0.89	0.45	0.57

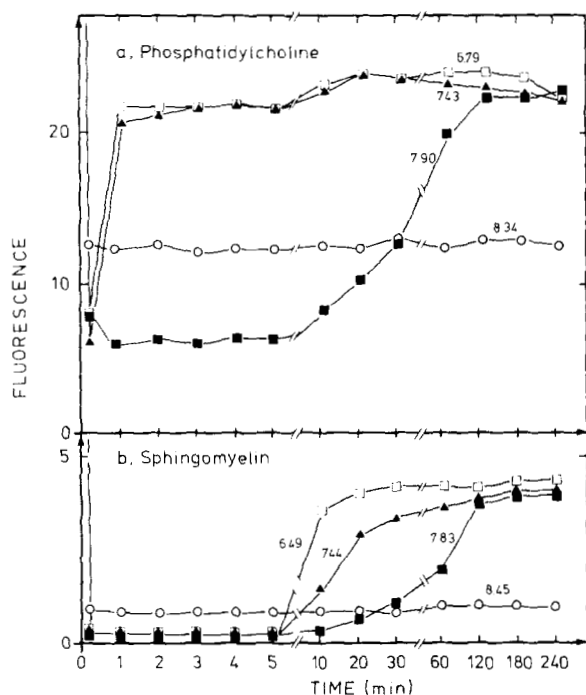


FIG. 1. Fluorescence from 1,6-diphenyl-1,3,5-hexatriene-labeled liposomes (ordinates, arbitrary units), as a function of time (abscissa) after addition of bilirubin. In *a*, liposomes were prepared from egg-phosphatidylcholine, in *b*, from sphingomyelin. Experiments were conducted at various pH values, as indicated in the figure. Before addition of bilirubin, phosphatidylcholine liposomes showed a fluorescence intensity of 29, while sphingomyelin liposomes gave 19 intensity units, in both cases independent of varying pH. Readings have been corrected for filter effects, but this correction is less accurate for readings at 1 min and before (see "Materials and Methods"). Concentrations were 130 μ M phosphatidylcholine, 32 μ M sphingomyelin, 13 μ M bilirubin, 0.05 M sodium phosphate. Temperature 37 $^{\circ}$ C.

liposomes, containing the fluorescent label diphenylhexatriene at pH values above 8.2, immediate quenching of the fluorescence was observed, indicating that bilirubin was bound to the phospholipid membrane. Significant quenching was seen with all phospholipids tested, strongest with sphingomyelin liposomes. Quenching increased with increasing bilirubin concentration. At pH values from about 8.2 and higher, the intensity of quenched fluorescence was independent of the pH and remained constant for several hours (see Table I, right half). It is concluded that stable complexes of bilirubin dianion and phospholipid are formed.

Similar results were obtained with liposomes prepared from cat brain lipid, although the final product was less stable. Also erythrocyte ghosts with fluorescent label showed quenching on addition of bilirubin, forming a stable complex.

Gradient centrifugation studies at pH 8.2 gave the following results (see later, Fig. 3, for details). A solution of bilirubin alone, 64 μ M, was incubated for 3 h at 37 $^{\circ}$ C, layered on top of a sucrose gradient and then centrifuged. Bilirubin remained largely in the specimen volume on top of the gradient, indicating that it was present as the dianion, or as aggregates too small for sedimentation, although the concentration was far in excess of the solubility of bilirubin which is about 0.2 μ M at pH 8.2 (5). Liposomes without bilirubin gave a narrow peak of phospholipid, just beneath the top of the gradient, with the sedimentation coefficient, 2–4 S, in agreement with the relatively low density of the lipid. Finally, the reaction mixture of liposomes and bilirubin, incubated for 3 h, gave an unchanged distribution of phospholipid and a bilirubin peak coincident with that of liposomes. The latter distribution was significantly different from that obtained with bilirubin alone, confirming that an interaction of bilirubin and phospholipid had taken place.

Bilirubin Acid-Liposome Complexes—At pH values lower than 8.2, a more complex pattern of fluorescence was observed when bilirubin was added to fluorescent-labeled liposomes. Immediate quenching was followed by a slow return of fluorescence, as seen in Table I and exemplified in Fig. 1.

The immediate quenching was in several cases more pronounced than at pH 8.3. This is presumably due to protonization of the bilirubin dianion-phospholipid complex. The

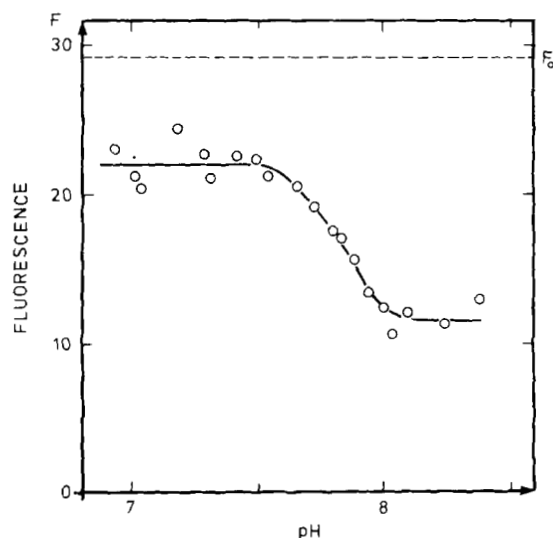
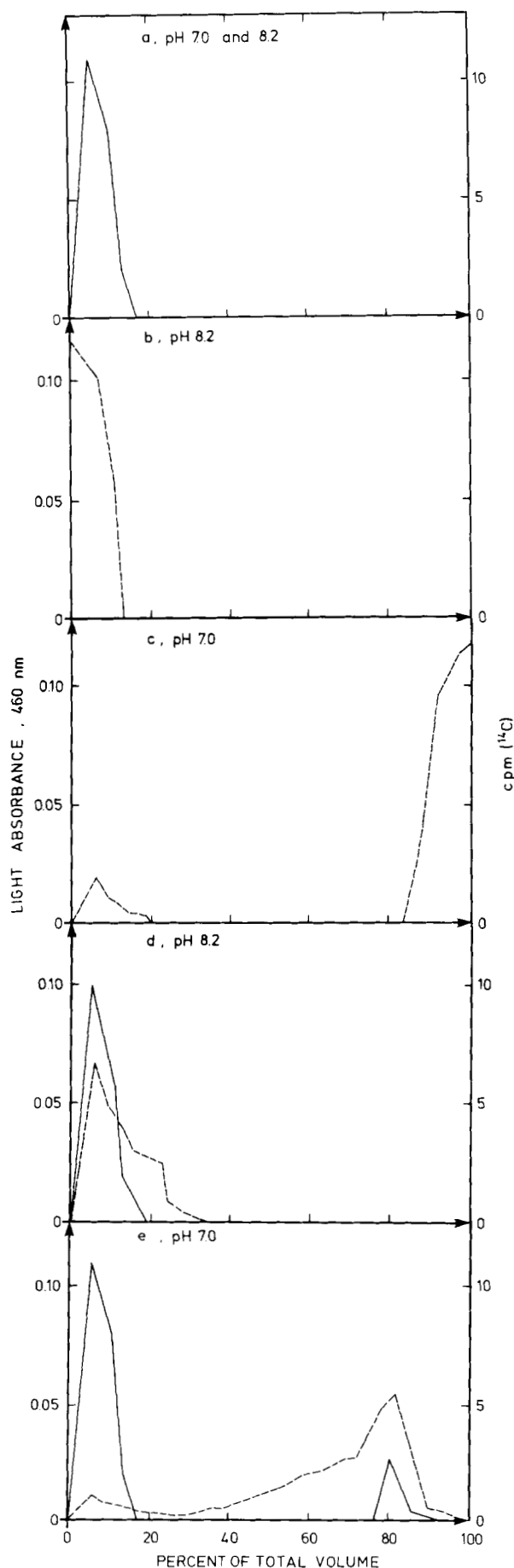


FIG. 2. Plateau value of fluorescence intensity from diphenylhexatriene-labeled phosphatidylcholine liposomes and bilirubin, measured 3 h after admixture (ordinates, arbitrary units), as a function of pH (abscissa). Fluorescence intensity before addition of bilirubin was 29 (F_0). Concentrations and temperature as in Fig. 1a.



product of the prompt reaction is probably a complex of the phospholipid and bilirubin acid or bilirubin acid monoanion. The acid, which is less polar than the bilirubin dianion, seems to bury deeper into membranes of some of the lipids and thus more effectively quenches fluorescence from the lipophilic diphenyl-hexatriene.

The instant quenching was followed by a relatively slow return of fluorescence, observed in all experiments in the pH range 6–8. The rate of return of fluorescence increased with lowering of the pH, as seen in Fig. 1. This figure also illustrates that the rate varied from one phospholipid to another. A final, constant level of fluorescence intensity was in all cases reached within 3 h. The final level varied with the lipid used, but was in all cases below the fluorescence intensity of the labeled liposomes without bilirubin (Table I).

Similar results were obtained with lipid from cat brain and erythrocyte ghosts.

A marked increase of light scattering paralleled the slow increase of fluorescence, as further detailed below (see Fig. 4 *a* and *b*), indicating formation of large aggregates. The light absorption spectrum, recorded at the final plateau of fluorescence, was identical with that of a suspension of aggregated bilirubin acid, as previously reported (5).

Further insight into the mechanism of this process was obtained by measuring the level of fluorescence intensity, reached in 3 h, as a function of pH. Results obtained with phosphatidylcholine are depicted in Fig. 2 and are qualitatively similar to those obtained with other phospholipids. A sigmoid relationship was seen, as expected from a reversible process depending upon protonization of the bilirubin-phospholipid complex. The inflexion point is at pH 7.8, and the curve is steeper than expected from a process of independent protonization of discrete acceptors. Hill plots drawn from such curves, obtained with different phospholipids and varying bilirubin concentrations, were linear with slopes around 6. This indicates a cooperative nature of the process, as expected when several protons are taken up during formation of a large aggregate of an insoluble acid. However, too much emphasis should not be placed on the numerical value of the Hill coefficient as determined in this case, since reversibility is incomplete according to the experiments reported below (Fig. 4, *a* and *b*).

The results of gradient centrifugation of the final product are seen in Fig. 3, *a*, *c*, and *e*. Phosphatidylcholine liposomes and bilirubin were incubated together, for 3 h at 37 °C. In these experiments, pH was 7.0. After centrifugation, ¹⁴C-phospholipid was found in two peaks, one at 2–4 S, similar to the peak obtained at pH 8.2, and a smaller peak at 60 S. Bilirubin was found throughout the gradient, with a small peak at 2–4 S and a large one at 60 S. A control run with bilirubin without

FIG. 3. Gradient centrifugation of [¹⁴C]phosphatidylcholine liposomes, —; and bilirubin, - - -. Liposomes alone, shown in *a*, sedimented slowly, 2–4 S, equally at pH 7.0 and 8.2. Bilirubin alone at pH 8.2, *b*, remained at the top of the gradient and at pH 7.0, *c*, gave a small peak close to the top and a large amount of bilirubin at the bottom of the tube, >110 S. A mixture of liposomes, 68 μM lipid P, and bilirubin, 64 μM, at pH 8.2, shown in *d*, gave a peak containing both compounds at 2–4 S, with a tail of bilirubin extending into the gradient. At pH 7.0, a mixture of both components, as shown in *e*, gave two peaks, one at 2–4 S, containing phosphatidylcholine with a small amount of bilirubin and another at 60 S, consisting of bilirubin with some phosphatidylcholine. All solutions were incubated for 3 h at 37 °C and then layered on top of a sucrose gradient, 10–50%, w/v, and centrifuged at 4 °C for 17 h at 115,000 × *g* (average). After separation into about 20 fractions, phosphatidylcholine was monitored by the radioactivity (right ordinate) and bilirubin by light absorption at 460 nm after alkalization to dissolve bilirubin acid aggregates (left ordinate).

liposomes gave one bilirubin peak close to the top of the gradient and a large amount of precipitated bilirubin acid at the bottom of the tube, at more than 110 S. In the presence of liposomes, the main peak of bilirubin thus consists of large bilirubin acid aggregates, attached to a smaller amount of phospholipid, while the main peak of phospholipid contained little bilirubin. The process initiated by lowering of the pH, therefore, is protonization of the bilirubin dianion-phospholipid complex followed by aggregate formation. Most of the bilirubin is transferred to a few liposomes where it is precipitated as large structures of bilirubin acid, retaining an amount of phospholipid from the original liposome.

This interpretation of the gradient centrifugation results may also explain the slow return of fluorescence which follows the immediate quenching. Bilirubin acid molecules, during the process of aggregation, are removed from intimate contact with the lipophilic interior of the membrane, causing fluorescence to return to a higher level.

Affinity of Formation of the Final Bilirubin Acid Aggregate-Phospholipid Complex—The final product might consist of a bilirubin acid-phospholipid complex with lower solubility than bilirubin acid itself. This would explain that the composed aggregates are formed in preference to a pure bilirubin acid precipitate. Alternatively, the solubility of the product might be similar to that of bilirubin acid. In the latter case, formation of the aggregates would occur with the same affinity as precipitation of the acid and would be explicable by faster aggregation around bilirubin molecules, already attached to a liposome. In order to distinguish these possibilities, the upper limit of pH for formation of the acid aggregates was determined with different phospholipids. Aggregate formation was observed by increase of light scattering within 3 h. The

limiting pH could be determined within ± 0.1 pH unit and was 8.0–8.2 for all phospholipids tested as well as for bilirubin alone.

The reversibility of this process was tested by letting the aggregates form at pH 7.0 and then observing dissolution after adjustment of pH to higher values. The results of both types of experiments with bilirubin-phosphatidylcholine liposomes, and with bilirubin alone, are seen in Fig. 4, *a* and *b*. The limit of dissolution was slightly higher, pH 8.4, than the limit for aggregation and was equal for bilirubin-phosphatidylcholine and for bilirubin alone. This indicates that reversibility was incomplete. Exact conclusions on the affinity can accordingly not be drawn, but it is obvious that the presence of phospholipid liposomes did not significantly influence the limit of pH, neither for formation nor for dissolution of the bilirubin acid aggregates.

The rate of aggregation, on the other hand, is considerably higher when phospholipid vesicles are present, as previously found (5).

In conclusion, the main process responsible for the increase of light scattering at pH values below 8.2 seems to be aggre-

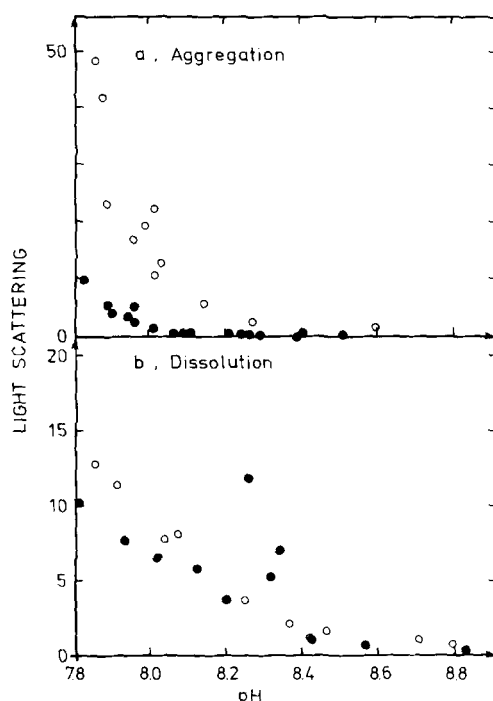


FIG. 4. Light scattering of phosphatidylcholine liposomes with added bilirubin (ordinates, arbitrary units), as a function of pH (abscissa). In *a*, the reactants were mixed and readings taken 3 h later. In *b*, the reaction mixture was left for aggregation during 3 h at pH 7.0, aliquots were adjusted to the pH values of the abscissa and were left for another 2 h for dissolution of the aggregates. ○ readings from liposomes with bilirubin, ● from bilirubin alone. Concentrations, 35 μ M phosphatidylcholine, 25 μ M bilirubin, 0.05 M sodium phosphate. Temperature 37 °C.

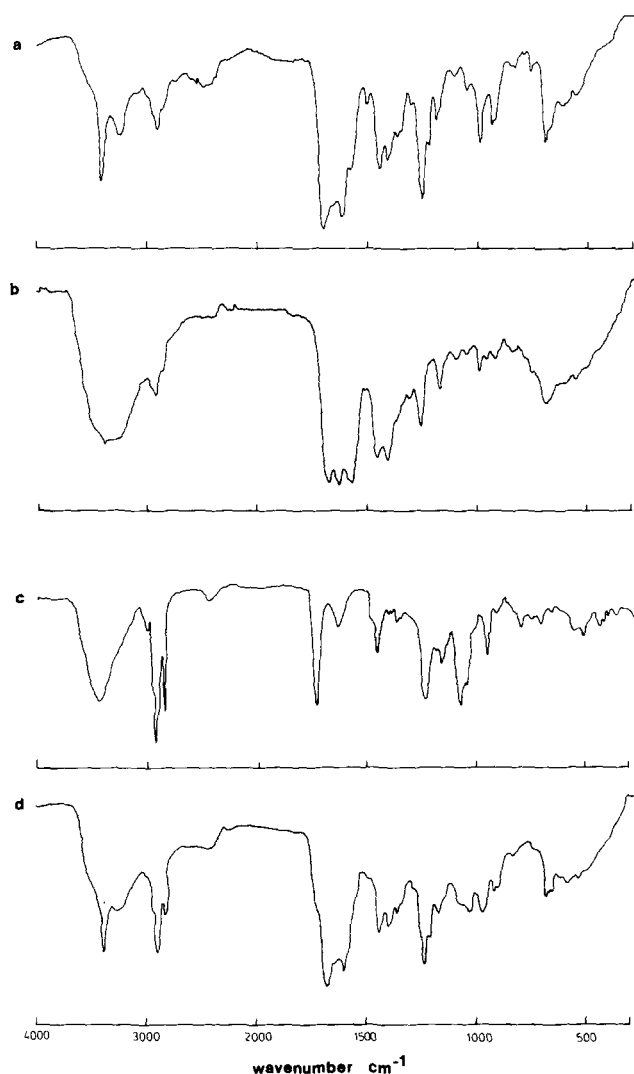


FIG. 5. Infrared light absorption spectra were recorded using a Beckman IR-20A Infrared Spectrophotometer from Beckman Instruments, Inc. (Fullerton, Ca.). *a*, bilirubin acid, *b*, bilirubin disodium salt, *c*, phosphatidylcholine, *d*, reaction product of phosphatidylcholine (64 μ M) with bilirubin (64 μ M), incubated for 3 hours at pH 7.4, temperature 37°C, sedimented by centrifugation, dried and incorporated in KBr tablets.

gation of bilirubin acid with bilirubin acid. Interaction of bilirubin with phospholipid takes place in the initial stages and results in enhanced aggregation. The final product in these experiments consists of large bilirubin acid aggregates containing a small amount of phospholipid, while the larger quantities of liposomes are left with only a small amount of bilirubin.

The solubility of the bilirubin acid aggregates, determined by dissolution within 2 h, is about 25 μM at pH 8.4 and does not vary measurably with the presence of phospholipids, in agreement with the above conclusion.

It is noteworthy that the solubility of the freshly precipitated bilirubin acid is considerably higher than that of bilirubin crystallized from chloroform-methanol. At pH 8.4, 37 °C, the solubility of crystalline bilirubin is 0.4 μM (5), approximately 60-fold less than the solubility of the freshly precipitated aggregates.

Infrared Light Absorption Spectra—The spectrum of bilirubin-phosphatidylcholine aggregates, formed at pH 7.4, was recorded (see Fig. 5). An identical spectrum could be obtained by combining spectra of phosphatidylcholine and bilirubin acid but not by combining the spectrum of bilirubin disodium salt with that of phosphatidylcholine. This finally confirms that bilirubin is present as the acid in the product of aggregation.

DISCUSSION

Nagaoka and Cowger (4) have studied the interaction of bilirubin with vesicular dispersions of phospholipids in aqueous buffer, pH 7.5. Complexes were formed with all lipids tested. An especially high binding affinity was found with sphingomyelin, and these authors suggest that binding of bilirubin to sphingomyelin in membranes of neural cells may be one possibility for bilirubin toxicity in the brain. Weil and Menkes (6), similarly, suspect that gangliosides are involved in the mechanism of bilirubin toxicity.

Our results qualitatively confirm the findings of Nagaoka and Cowger and extend the investigations to interaction at varying pH, including ultracentrifugation, light scattering, and infrared spectroscopic studies. Thereby it has become possible to describe the products of interaction in terms of ionic status and aggregation. The present findings indicate that interaction of bilirubin with phospholipid vesicles takes place through several steps:

- I. Bilirubin dianion + phospholipid \rightleftharpoons bilirubin dianion-phospholipid complex,
- II. Bilirubin dianion-phospholipid complex + H^+ \rightleftharpoons bilirubin acid-phospholipid complex,
- III. Aggregation of bilirubin acid, attached to phospholipid.

At pH values higher than 8.2, only Step I is observed. In more acid solutions the subsequent steps take place. Steps I and II proceed promptly to equilibrium while the rate of Step III, aggregation of bilirubin acid, is low at pH values close to 8 and increases in more acid media. At physiologic and acidotic pH values, the total process is completed in less than 1 min with phosphatidylcholine and most other phospholipids tested. The reaction with sphingomyelin is slower. All phospholipids tested react similarly in qualitative respects. Results obtained with sphingomyelin are compatible with an especially high affinity of this lipid for binding of bilirubin dianion, in agreement with the results of Nagaoka and Cowger (4). Quantitative determinations of binding constants were not attempted in the present study since it proved difficult to establish true equilibria.

Tipping, *et al.* (7), studying the interaction of bilirubin with aqueous dispersions of egg phosphatidylcholine at pH 8.2, 25 °C, found that $\bar{\nu}_{\text{max}}$, i.e. the maximal number of bilirubin molecules bound per molecule of lipid phosphorus, is 0.1. At pH 7.4, 25 °C, the limiting value of $\bar{\nu}/c$, where c is the free bilirubin concentration, is about 1.2×10^4 liters mol^{-1} , as $\bar{\nu} \rightarrow 0$.

The quantitative findings of Tipping *et al.* (7) are consistent with our results, if the former are understood to describe interaction of bilirubin dianion with phosphatidylcholine.

In a severe case of neonatal jaundice, the concentration of bilirubin-albumin may be 150 μM with as little as 12 μM unoccupied albumin.^{2,3} The binding constant of bilirubin dianion to human albumin at 37 °C and physiologic ionic strength is 5.5×10^7 liters mol^{-1} (5) giving a calculated concentration of free bilirubin dianion about 0.25 μM . Using the figure of Tipping *et al.* for $\lim \bar{\nu}/c$ (although determined at 25 °C), we find $\bar{\nu} \approx 0.003$ at this bilirubin concentration, meaning that an order of 3 phosphatidylcholine molecules out of 1000 would bind a bilirubin dianion at equilibrium. Sphingomyelin in the brain may bind even more, so that a considerable amount of bilirubin would bind to tissues in this fashion, corresponding to Step I in the above mechanism. Steps II and III would then proceed, if pH is sufficiently low, resulting in gross aggregation of bilirubin acid in exposed membranes. Since clinical experience (8), as well as work on animals (9) and on tissue cultures (10, 11), have shown that toxicity depends upon a low pH, it seems reasonable to presume that this aggregation of bilirubin acid is responsible for the toxic effects of bilirubin. This is in agreement with the previous suggestion by Lee and Cowger (12), see also review (13).

The present investigations thus seem to confirm that precipitation of bilirubin acid in organelles of the central nervous system is thermodynamically possible and suggest a mechanism whereby such precipitation may take place. Several steps in the transport of bilirubin from plasma into the cells remain to be elucidated, since it is not known whether this occurs through free bilirubin, through direct contact of the plasma bilirubin-albumin complex with a membrane, or through an unknown carrier.

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