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26. Accepted for publication July 26, 1974.

Pediat. Res. *8:* 963–967 (1974)

Albumin
bilirubin
pH

Effect of pH on the Interaction of Bilirubin with Albumin and Tissue Culture Cells

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Extract

Titrations of albumin with bilirubin were performed at pH 7.0 and pH 7.4. Unbound bilirubin concentrations were determined using a horseradish peroxidase assay. Titrations of infant serum indicated that 1 mol bilirubin was bound tightly/mol albumin at either pH with an association constant of approximately 6.2×10^7 M^{-1}. When binding occurred at secondary sites, data were more scattered, but no pH differences could be detected. The effect of bilirubin on cells was studied by determining the viability of plated L cells after a pulse of bilirubin. The lethal unbound bilirubin concentrations for 50% of cells at pH 7.4 was about 0.7 μM compared with 0.3 μM at pH 7.0. Cell uptake of unbound bilirubin was rapid at either pH. In the essential absence of albumin (20/1 molar ratio), about 66% of the added bilirubin appeared in the cell pellet in 10 min at pH 7.0; initial uptake at pH 7.4 was 44% ($P < 0.001$).

Our data do not support the hypothesis that acidemia decreases albumin binding of bilirubin. The increased sensitivity appeared to be related to a greater cell uptake of bilirubin at the lower pH.

Speculation

Although albumin binding of bilirubin is clearly an important factor in the pathogenesis of kernicterus, this study emphasizes the importance of cell-bilirubin interaction as a determinant of bilirubin distribution and toxicity. Intervention to decrease the serum bilirubin concentration in a jaundiced neonate must respond both to the status of albumin-bilirubin binding and to conditions which alter the cellular affinity for bilirubin.

The peroxidase method provides a sensitive new technique for analyzing protein bilirubin interaction.

Jaundiced infants with low plasma pH appear to have an increased risk of developing bilirubin encephalopathy (19, 20). This clinical observation is supported by experimental work in organ (39) and tissue (12) culture showing increased cell damage at low pH, and by animal experiments which have demonstrated an increased brain uptake of bilirubin in acidemic guinea pigs (16) and increased CSF bilirubin in acidemic rabbits (38). A decreased pH has also been shown to enhance the uptake of bilirubin by erythrocytes and mitochondria when incubated in media containing albumin (3, 32).

The increased toxicity of bilirubin at low pH has been attributed to a decrease in albumin binding of bilirubin (3, 11, 28, 32, 33). However, supporting experimental evidence for decreased binding within the physiologic pH range is conflicting, and the possibility exists that cells may be more sensitive to bilirubin at low pH (12, 16, 39, 45). As measurements of serum albumin binding and unbound bilirubin are becoming utilized increasingly in managing jaundiced infants, the effect of pH on albumin binding and bilirubin distribution is of practical as well as theoretical importance.

The following experiments examine whether the increased bilirubin toxicity in cell culture at low pH is due to a decrease in albumin binding or to an increased cell sensitivity to bilirubin.

METHODS

CELL TOXICITY

Strain L-929 cells (46) were grown under 5% CO_2 and air in Eagle's minimal essential medium containing Hanks' balanced salt solution and 5% fetal calf serum. The Hanks' solution was modified by substituting glutamine, 350 mg/liter, for glucose, and HEPES, 25 mEq/liter, for sodium bicarbonate, and adjusting to pH 7.4 with sodium hydroxide. Cells were

harvested by scraping and diluted to 100 cells/ml with minimal essential medium. One-milliliter aliquots of cell suspension were pipetted into 35-mm plastic Falcon dishes and allowed to attach overnight. On the following day, the plates were washed three times with 1 ml HEPES-buffered Hanks' solution, pH 7.0 or pH 7.4, containing varying concentrations of human albumin (*fraction V* (47)) and bilirubin (48) and incubated in the dark at 37° without stirring. Three tests plates and three control plates (exposed to albumin but not bilirubin) were incubated for each set of experimental conditions. After a 45-min incubation, the cells were washed twice with tissue culture medium and allowed to grow for 3 days. The cells were then fixed, stained with Giemsa, and colonies with two or more cells were counted. Viabilities were calculated by dividing the mean colony count of three test cultures by the mean colony count of control plates. For 68 sets (204 plates) with colony counts ranging from 7–81, the mean coefficient of variation in counting was 25.2%.

CELL UPTAKE OF BILIRUBIN

Cells were suspended overnight in minimal essential medium, washed three times with buffered Hanks' solution, pH 7.0 or 7.4, and diluted to 1×10^6 cells/ml. Five-milliliter aliquots were incubated in suspension at 37° for about 1 hr after which 5×10^{-9} mol human albumin and 0.1 μmol bilirubin were added (molar ratio of bilirubin to albumin, 20/1). The bilirubin stock solution was prepared by dissolving 5 mg bilirubin in 0.5 ml 0.1 N NaOH and diluting with 8.05 ml H_2O (1.0 mM). Efforts to perform the experiment in the total absence of albumin resulted in rapid colloid formation. At a molar ratio of 20/1, albumin served to disperse the bilirubin through nonspecific adsorption and thus inhibited rapid bilirubin aggregation and precipitation. Human serum albumin has three specific binding sites, but only one high affinity binding locus (21).

After incubation with bilirubin in subdued light for 10–60 min, the suspensions were centrifuged and the supernatants separated from the cell pellets. The cells were lysed and bilirubin extracted with 1.0 ml 1% sodium deoxycholate and 1.0 ml 1.0 N NaOH. Nonturbid bilirubin solutions were obtained by centrifuging at 32,000 × *g* for 5 min. Total bilirubin recovery was 82% ± 8% (SD) for cells and supernatant incubated at pH 7.0 (13 determinations) and 70% ± 8% at pH 7.4 (12 determinations).

Competititon between cells and albumin for bilirubin binding was studied by incubating suspended cells for 45 min in Hanks' solution containing 0.2 μmol bilirubin and sufficient albumin to give molar ratios (bilirubin to albumin) ranging from 0.8–4.0.

BILIRUBIN ASSAYS

Total bilirubin concentrations in all solutions were determined by directed spectrophotometry. The molar extinction coefficients for bilirubin were determined experimentally (see Table 1).

NONPROTEIN-BOUND BILIRUBIN CONCENTRATION

Unbound bilirubin concentrations were determined using a modification (23) of the horseradish peroxidase assay described by Jacobsen (21), and Jacobsen and Fedders (22). Titrations of albumin with bilirubin were performed at pH 7.0 and pH 7.4 using both purified human albumin (*fraction V*) and pooled infant sera diluted in HEPES-buffered Hanks' solution. The sera were obtained from cord blood at the delivery of three normal term infants with uncomplicated labor and delivery. The serum albumin concentration was determined by the bromocresol green method (17). All binding studies were performed at 37° in a thermostable cell compartment, using a Cary model 16 recording spectrophotometer.

RESULTS

ALBUMIN BINDING OF BILIRUBIN

Titrations of infant serum with bilirubin at pH 7.0 and pH 7.4 yielded nearly identical Scatchard plots (Fig. 1). One mole of bilirubin was bound tightly per mole of albumin at either pH with an association constant of approximately 6.2×10^7 M^{-1}. As the molar ratio of bilirubin to albumin approached 1, data in Figure 1 deviated from a straight line because of binding at secondary sites. The value n_2k_2 has been estimated previously as 1.2×10^6 M^{-1} where $n_2 = 2$ (21). The presence of weaker secondary sites introduces an insignificant error in the determination of k_1. The association constant of purified albumin-bilirubin complexes was considerably lower than that of native serum albumin. Binding constants of 2.8×10^7 M^{-1} at pH 7.0 and 3.2×10^7 M^{-1} at pH 7.4 were observed; the variation with pH was slight and within the error of the method.

The total and unbound bilirubin concentrations were also determined in test media containing various concentrations of bilirubin and albumin after incubation with plated L cells. The effects of molar ratio and pH on unbound bilirubin concentration are summarized in Figure 2. Although the concentration of unbound bilirubin rose more rapidly after saturation of the primary binding site on albumin, the increase in unbound bilirubin was far less than the amount added,

Table 1. *Molar extinction coefficients for bilirubin*

Solution	Maximum, nm	E^M_{1cm}
Chloroform	452	60,100
Buffered Hanks', pH 7.0		
With albumin	460	45,800
Without albumin	440	48,000
Buffered Hanks', pH 7.4		
With albumin	460	48,500
Without albumin	440	48,500
NaOH 0.5 N + NaDOC,[1] 0.5%	408	49,800

[1] Sodium deoxycholate.

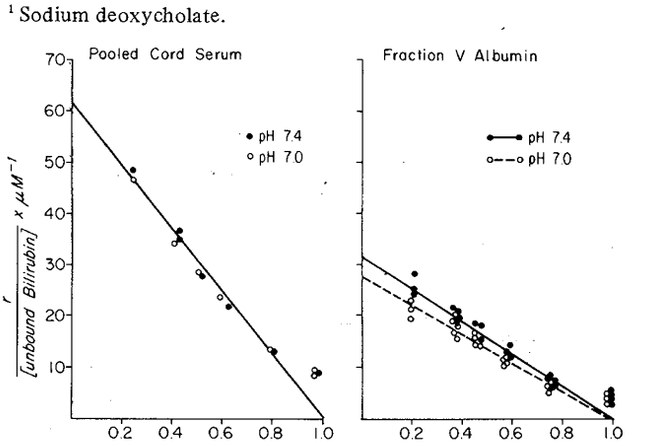

Fig. 1. Scatchard plots describing the high affinity binding of bilirubin by newborn serum albumin and Cohn *fraction V* at 37°. *r*: ratio of bound bilirubin to total albumin. Both albumin preparations could bind 1 mol bilirubin/mol albumin tightly. Newborn serum albumin had an association constant $k_1 = 6.2 \times 10^7$ M^{-1}; *fraction V* had binding constants, $k_1 = 3.2 \times 10^7$ M^{-1} at pH 7.4 and 2.8×10^7 M^{-1} at pH 7.0. As r approached 1.0, the data deviated from a straight line, reflecting weaker secondary binding.

confirming the presence of weaker secondary binding. The unbound concentration bilirubin was the same at both hydrogen ion concentrations when binding occurred at the primary site. When binding occurred at secondary sites, data were more scattered, but no pH difference could be detected.

CELL SENSITIVITY

The effect of bilirubin on cells was first studied by determining the viability of plated cells after a pulse of bilirubin (20-fold excess over albumin) at pH 7.0 or 7.4. The concentration of bilirubin did not change during the 45-min incubation; *i.e.*, the initial concentration of unbound bilirubin was essentially equal to the equilibrium concentration. At pH 7.0 no treated cells survived even with bilirubin concentrations as low as 2.2 μM, whereas the threshold for toxicity at pH 7.4 lay between 2.2 and 4.4 μM bilirubin. Solutions of bilirubin at pH 7.0 were quite unstable at bilirubin concentrations below 2 μM, and the threshold for bilirubin toxicity at pH 7.0 could not be established under these experimental conditions. Control cells at pH 7.0 and 7.4 grew equally well.

At lower molar ratios of bilirubin to albumin, bilirubin remained more stable, and predictable levels of unbound bilirubin could be achieved by altering the ratio. Plated cells were incubated with 180 μM bilirubin and appropriate albumin concentrations to give bilirubin to albumin ratios of approximately 0.8, 1.2, and 1.9 at each pH level studied (Fig. 3). Although some cells were killed at pH 7.0 at a molar ratio of 0.8 statistically significant differences in killing between pH 7.0 and pH 7.4 were found only at a molar ratio of 1.2. These differences are more clearly illustrated in Figure 4, which shows the relation between cell viability and unbound bilirubin levels in the test solutions. The lethal unbound bilirubin concentration for 50% of cells at pH 7.4 was about 0.7 μM compared with 0.3 μM at pH 7.0.

In these studies, cell death was defined as the inability to divide after exposure to bilirubin. Failure to divide could simply be due to inhibition of DNA synthesis (42) without actual killing. However, the distribution of single cells and clones of varying size (usually ranging from 2–16 cells) was similar in both treatment and control groups. Thus, it is probable that the failure to divide reflects a lethal insult.

CELL UPTAKE OF BILIRUBIN

Cell uptake of unbound bilirubin (bilirubin to albumin, 20/1) was rapid at either pH but more bilirubin was bound at pH 7.0 than 7.4. About 66% of the added bilirubin appeared in the cell pellet in 10 min at pH 7.0 and little further change occurred over the next 50 min (Fig. 5). Initial uptake at pH 7.4 was 44%, although cells continued to extract bilirubin slowly throughout the incubation. The differences were statistically significant ($P < 0.001$). The capacity of cells to "bind" bilirubin was very large; at 10 min each cell contained $4-7 \times 10^{-14}$ mol or about $2-4 \times 10^{10}$ molecules/cell.

When cells were incubated for 45 min with a bilirubin to albumin molar ratio of 2/1, uptake was 58% complete by 5 min and 89% complete by 10 min. Thus, the equilibrium of bilirubin with cells and albumin was approached almost as quickly as with cells alone. In experiments where bilirubin uptake was studied as a function of molar ratio (45-min incubation), little bilirubin was found in cells until the bilirubin concentration exceeded that of albumin (Fig. 6). At any given molar ratio, more bilirubin was absorbed at pH 7.0 than pH 7.4.

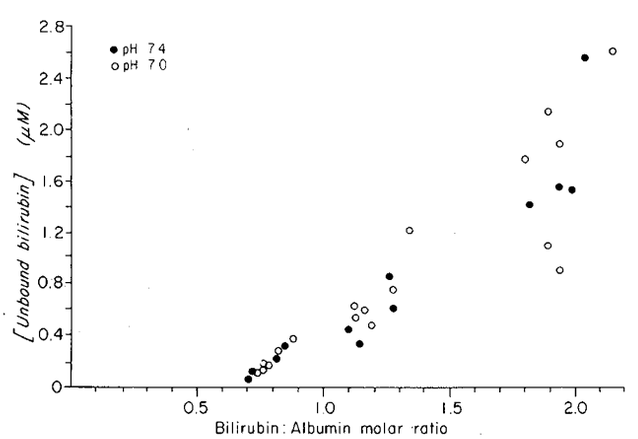

Fig. 2. Effects of pH and molar ratio on the nonalbumin-bound bilirubin concentration in test media. The bilirubin concentration was 180 μM and the albumin concentration varied. Media were analyzed after a 45-min incubation with plated L cells.

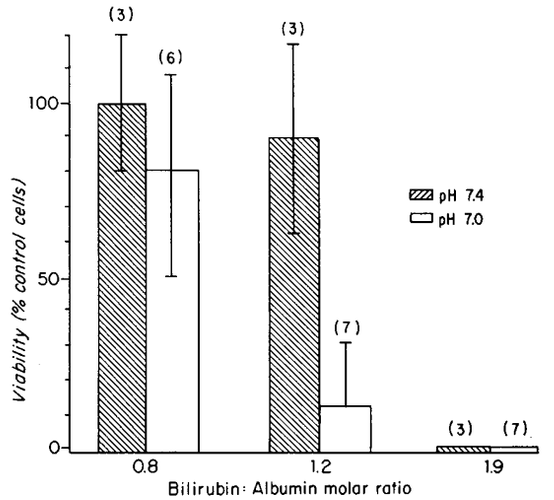

Fig. 3. Effects of bilirubin to albumin molar ratio and pH on L cell viability. Plated cells were incubated for 45 min in HEPES-buffered Hanks' solution, 37°. The bilirubin concentration was 180 μM (10.5 mg/100 ml) and the albumin concentrations varied. The number of observations are indicated in parenthesis. The SD are indicated. At a molar ratio of 1.2, more cells died at pH 7.0 than at pH 7.4 ($P < 0.001$).

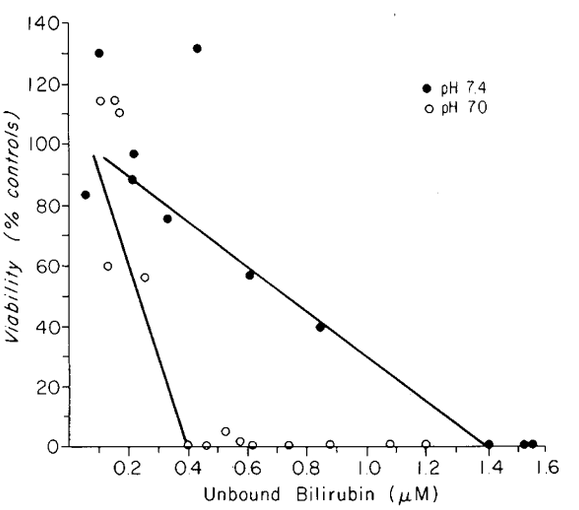

Fig. 4. Effect of nonalbumin-bound bilirubin concentration on L cell viability. Plated cells were incubated for 45 min in HEPES-buffered Hanks' solution, 37°. Test solutions contained 180 μM total bilirubin and various albumin concentrations.

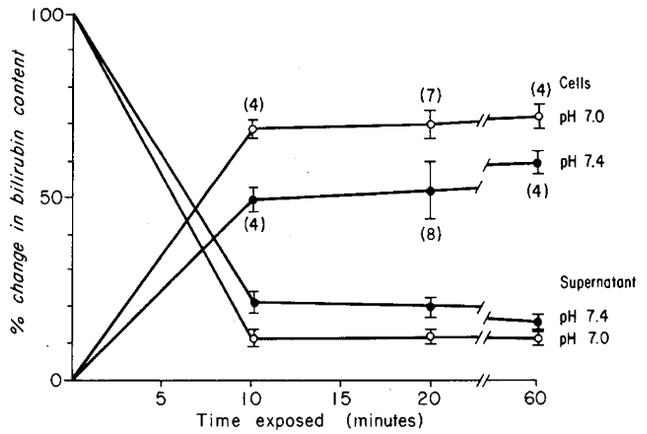

Fig. 5. Effect of pH on bilirubin uptake by L cells. Flasks initially contained 5 X 10^6 cells, 20 μM bilirubin, and 1 μM albumin in 5 ml HEPES-buffered Hanks' solution. The number of observations are indicated in parenthesis and the SD are indicated. Differences between pH 7.0 and pH 7.4 were all statistically significant ($P < 0.001$) except for the 10-min supernatant concentration where $P < 0.01$.

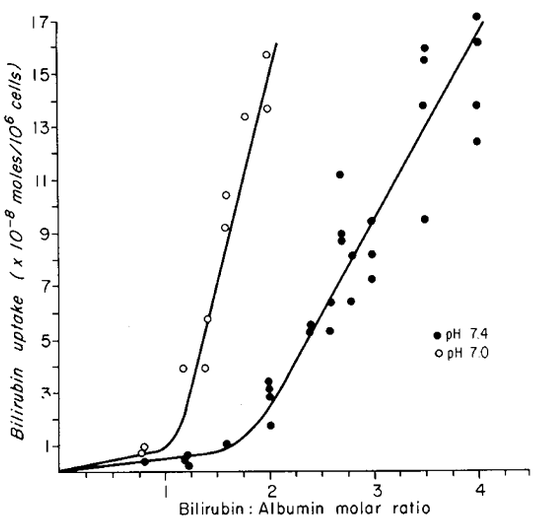

Fig. 6. Effect of pH and molar ratio on L cell uptake of bilirubin. Cells were incubated for 45 min with various bilirubin to albumin molar ratios. The initial bilirubin concentration was 40 μM.

DISCUSSION

Our data do not support the hypothesis that acidemia decreases albumin binding of bilirubin. Several electrophoretic studies have demonstrated dissociation of the bilirubin-albumin complex at extremely low pH (10, 25, 40, 45), but most reports indicated complete migration of bilirubin with albumin above pH 6.0 (25, 40, 41), and no study has observed dissociation within the physiologic pH range. Odell *et al.* (34) observed a blue shift in the absorption spectrum of bilirubin-albumin complexes with decreasing pH similar to that observed in the presence of competing anions and interpreted this to represent dissociation of the complex. However, more recent studies with optical rotary dispersion (2) (using bovine serum albumin), circular dichroism (1), and absorption spectra (44) indicate that the hydrogen ion-induced spectral shift is due to conformational changes in the complex rather than dissociation. Martin (29), who is frequently cited as demonstrating a decrease in binding at low pH with dialysis (11, 28, 32, 33), actually concluded that serum albumin forms a stable complex with bilirubin over a pH range extending well beyond physiologic limits. A single dialysis performed at pH 7.4 showed 1.9 mol bilirubin bound/mol albumin compared with 2.5 mol at pH 7.6 and 3.3 mol at pH 7.9. However, the total bilirubin concentration in solution allowed for maximum molar ratios of 2.0, 2.9, and 4.5, respectively, precluding any conclusion regarding pH effects on binding. Using Sephadex G-25 gel filtration, Chunga and Lardinois (11) demonstrated a decrease in binding at low physiologic pH when the bilirubin concentration exceeded that of albumin. Unfortunately, bilirubin has been shown to bind to Sephadex (11, 30), especially at lower pH, and thus, equilibrium conditions did not exist.

In contrast to the negligible influence of pH on bilirubin-albumin interaction, the concentration of bilirubin required to kill L cells at pH 7.0 was less than half the lethal concentration at 7.4. The increased sensitivity appeared to be related to a greater cell uptake of bilirubin at the lower pH and may be caused by changes in the physical state of bilirubin. The two propionic acid side chains on bilirubin have an unusually high pK (35), reported to be 7.95 at 37° and physiologic ionic strength (26). Two protons are accepted, and the titration curve has a single sharp inflection (26), which indicates nonindependence of the propionate groups. As a result, the concentration of ionized bilirubin is very low at pH 7.4 and even less so at pH 7.0. The high apparent pK could be explained if the bilirubin acid is removed from the equilibrium either by an internal conformational change or by aggregation and precipitation. Although there is disagreement as to the exact solubility of bilirubin at pH 7.4 (7, 8, 35), it appears that bilirubin solutions exceeding 0.1 μM exist in a supersaturated state (6, 7) and become increasingly unstable as the pH decreases to 7.0 (8, 44). The increased affinity between cells and bilirubin with decreasing pH might simply be explained by the increased hydrophobicity of bilirubin and consequent interaction with lipids and lipoproteins in the cell membranes (31). Cowger (12) and Silberberg *et al.* (39), using L cells and neural explants, respectively, found that bilirubin was essentially nontoxic at pH 7.8. These observations would suggest that the water-soluble conjugate base of bilirubin has a low affinity for cellular membranes.

Although increased hydrophobicity of bilirubin correlates with increased cell toxicity and uptake, colloid formation has been shown to inhibit the uptake of bilirubin by most tissues (5). At pH 7.4, cells appeared to be more sensitive to unbound bilirubin when the bilirubin was delivered in the presence of relatively high concentrations of albumin (Fig. 4) than in the essential absence of albumin (20/1 ratio). The difference may be due to undetected bilirubin aggregation at the 20/1 molar ratio. Alternatively, it is possible that interaction between albumin and cell membranes could enhance the transfer of bilirubin between protein and cells.

In addition to the influence of pH, there is evidence that uptake and toxicity of bilirubin vary with cell type (15, 18, 24, 42) and can be influenced by drugs and environmental factors which alter cellular metabolism (14, 36, 37) or possibly cellular affinity for bilirubin (37). The known toxic effects of bilirubin, however, involve basic cellular functions and would appear to transcend cell lines (4, 9, 12, 13, 27, 37, 38, 42). Thus, although the specific threshold of toxicity may be different, there is no evidence to suggest that principles involved in bilirubin distribution and toxicity in susceptible brain nuclei are in any way unique.

SUMMARY

The effects of pH on bilirubin binding to albumin and to cells in tissue culture have been studied. The binding of bilirubin to albumin is independent of pH within the physiologic pH range. The cell toxicity of bilirubin is enhanced at low pH both in the presence and essential absence of albumin and appears to be related to an increased cellular affinity for bilirubin.

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49. This research was supported by grants HD-04665 and HD-00095 from the United States Public Health Service. The authors gratefully acknowledge the skilled technical assistance of Ms. Marian Hunner.

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53. Accepted for publication July 18, 1974.