Influence of pH on Distribution of Bilirubin Between Albumin and Mitochondria.* (30534)

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Recent studies from the author's laboratory have shown that bilirubin is concentrated within the microenvironment of mitochondria when the latter is suspended in isotonic solutions containing 0.08 mM bilirubin. It has also been found that the association of bilirubin with mitochondria is prevented if the aqueous medium contained equimolar amounts of bilirubin and albumin at pH 7.4. Inclusion of competing organic anions which are bound by albumin increases the association of bilirubin with the mitochondria(1). The present observations report the effect of changes in hydrogen ion concentration on bilirubin distribution between albumin and mitochondria. It has been previously demonstrated that lower pH values will enhance the dissociation of bilirubin from albumin(2,3,4). However, it is not known whether the mitochondria will take up the bilirubin dissociated from albumin in aqueous media of lower pH.

Methods. Mitochondria were isolated by the differential centrifugation of 10% rat liver homogenates in 0.25M sucrose as previously reported(1). The isolated mitochondria were washed twice by resuspension in 0.25M sucrose to minimize contamination of lysosomes and reisolated by centrifugation at 10,000 *g* for 10 minutes. The stock suspension of mitochondria was prepared in 0.25M sucrose so that 1 ml of suspension contained the mitochondria from 1 g of liver.

The distribution studies were conducted as follows: 0.4 ml of the stock mitochondrial suspension was added to 0.5 ml of 0.1M phosphate buffers of pH ranges 6.5-7.4. To this suspension was added 0.1 to 0.6 μmoles of crystalline human‡ or bovine§ albumin from concentrated albumin solutions in 0.15M KCl. The suspensions were then made up to 4.8 ml with 0.15M KCl. Finally 0.4-0.5 μmoles of freshly prepared bilirubin in 0.2 ml of solution, was added. The bilirubin was dissolved in 0.05 N NaOH and brought to pH 7.8 with 0.1M phosphate buffer. The tubes were inverted and allowed to stand in an ice bucket for 15-30 minutes. The mitochondria were subsequently isolated by centrifugation of the tubes at 10,000 *g* for 10 minutes. The supernatants were decanted to separate tubes and the mitochondrial pellets were resuspended in 2 ml of a 5% albumin solution. The bilirubin content of the supernatants and resuspended pellets was determined by the modification of the van den Bergh test previously described(1). The bilirubin recovery was checked by comparison to a control tube which contained all the constituents except the mitochondria. The bilirubin employed was obtained from Hoffmann-LaRoche and exhibited a molar extinction coefficient of 5.96 $\times$ 10^4 in chloroform at 453 mμ. The pH of the supernatant solution was measured with a Beckman model GS pH meter and the osmolality with a Fiske Osmometer. The protein concentration of the concentrated albumin solutions was determined by a biuret technique previously used(1).

Results. In all, 15 separate rat liver homogenates were studied. Representative values combined from 4 liver homogenates are shown in Fig. 1 which illustrates the distribution of bilirubin between the mitochondrial phase and the supernatant protein phase of the suspension at the pH values 7.4, 7.0, and 6.5. Very little bilirubin is associated with the mitochondrial phase of the suspension ($<$20 μg) when the molar ratio of bilirubin to albumin is less than one, but beyond the 1:1 molar ratio linearly greater amounts of bilirubin are associated with the mitochondrial phase of the suspensions. Fig. 1 also demonstrates that lower pH values increase the rate

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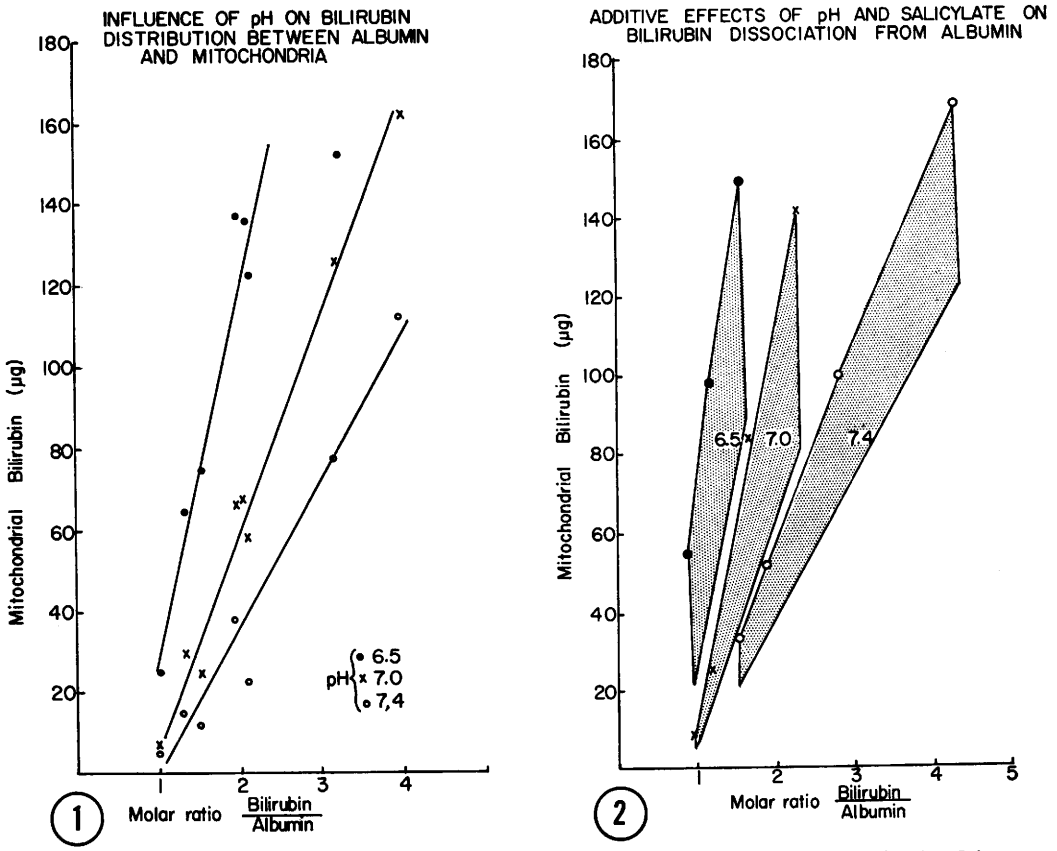

FIG. 1. Influence of pH on distribution of bilirubin between albumin and mitochondria: ordinate represents recovery of bilirubin in the mitochondrial phase of suspensions after equilibration. Abscissa indicates molar ratio of bilirubin to albumin added to suspension. Each series of tubes contained 260 µg (0.45 µMoles) of bilirubin in 5 ml. Albumin addition was adjusted to give the desired molar ratio of bilirubin to albumin. Regression lines were fitted by inspection to the points for the series of equilibrations at the 3 pH values.

FIG. 2. Additive effects of pH and salicylate on bilirubin distribution between albumin and mitochondria. Coordinates as in Fig. 1. Points along left-hand margin of stippled quadrilaterals represent bilirubin recovery in mitochondrial suspensions when 2.5 µMoles of salicylate were included in the equilibrations. Right-hand margin of quadrilaterals represents control values at the same pH. Each tube contained between 260-280 µg (0.5 µMoles) of bilirubin in 5 ml.

of bilirubin association with the mitochondria, but that the regression lines when extrapolated all intercept the abscissa at about the 1:1 molar ratio of bilirubin to albumin.

A similar series of equilibrations, simultaneously performed, also included the uniform addition of 2.5 µmoles of sodium salicylate to each tube. The results of these distribution studies are shown in Fig. 2. The enclosed stippled areas represent the additive effect of salicylate to the hydrogen ion concentration in increasing the association of the bilirubin with the mitochondria. Thus at pH 6.5 at the 1.5:1 molar ratio of bilirubin to albumin, salicylate increased by 65 µg the amount of bilirubin located within the mitochondrial phase (85 µg to 150 µg), whereas at pH 7.4 the salicylate only increased the amount by 15 µg (20 µg to 35 µg) for the same molar ratio. Extrapolation of the regression line describing the effect of salicylate to the abscissa indicate that at pH 6.5 salicylate would increase the bilirubin in the mitochondrial phase of the suspensions below the 1:1 molar ratio of bilirubin to albumin.

A similar series of equilibrations was done with bovine albumin and showed a qualita-

tively similar distribution, but the affinity of bovine albumin for bilirubin was less than the human albumin.

The osmolalities of the supernatant fluids were 285 m Osm/kg of water. The bilirubin recoveries in the suspension tubes were all within 95% of the control tube.

Discussion. The concentrations of bilirubin and albumin used in the present model system were selected as representative of the range of concentrations in interstitial fluid when the serum concentrations of bilirubin and albumin are 25 mg/100 ml and 3.2 g/100 ml respectively. This is a relatively common occurrence in the neonatal period in jaundiced infants, and is of concern because of the neurotoxicity associated with hyperbilirubinemia.

The mitochondria were chosen because bilirubin rapidly associates with these intracellular organelles in liver after the infusion of physiologic amounts of bilirubin to intact animals(5), and *in vitro* studies with tissue cultures or homogenates indicate that bilirubin is toxic to mitochondria(6,7).

The present study demonstrates that below a 1:1 molar ratio of bilirubin to albumin the distribution of bilirubin is confined primarily to the albumin space. However, above this ratio particularly at pH values less than 7.4, the distribution of bilirubin includes the mitochondria. The combination of low pH and the presence of organic anions which bind to albumin further enhances the association of bilirubin with mitochondria so that less than half of the added bilirubin is present in the albumin space of the equilibration system when the bilirubin to albumin ratio exceeds 1.5:1. These *in vitro* studies would suggest that *in vivo* more bilirubin would enter intracellular fluids in the presence of extracellular acidosis or when albumin is partially saturated with other organic anions which compete for the loci on which bilirubin normally locates.

In contrast to many organic anions bilirubin association with albumin is very sensitive to small changes in hydrogen ion concentration in the physiologic pH range(2,8, 9). Organic anions, such as salicylate, which interfere with the albumin binding of bilirubin, are believed to bind to albumin at its cationic amine groups, particularly the epsilon amino group of lysine(8,10). These groups have a pKa of 10, hence by inference, the sensitivity of bilirubin at lower pH values suggests that changes in the molecular configuration of bilirubin in aqueous solution primarily account for the pH sensitivity of its protein binding to albumin.

Summary. The distribution of bilirubin between albumin and mitochondria suspended in isotonic aqueous media was investigated between the range of 6.5-7.4. It was found that more bilirubin is associated with the mitochondrial phase as the hydrogen ion concentration of the medium increased. Salicylate was additive to the influence of pH on bilirubin distribution, and the combined effects of salicylate and pH 6.5 displaced bilirubin from albumin even at molar ratios of bilirubin to albumin less than one.

1. Odell, G. B., J. Pediat., in press.
2. Martin, N. H., J. Amer. Chem. Soc., 1949, v71, 1230.
3. Najjar, V. A., Childs, B., J. Biol. Chem., 1953, v204, 359.
4. Klatskin, G., Bungards, L., J. Clin. Invest., 1956, v35, 537.
5. Brown, W. R., Grodsky, G. M., Carbone, J. V., Am. J. Physiol., 1964, v207, 1237.
6. Ernster, L. In Kernicterus, A. Sass-Kortsak, ed., Univ. of Toronto Press, Toronto, Canada 1961, p174.
7. Cowger, M. L., Labbe, R. F., Fed. Proc., 1964, v23, 223.
8. Klotz, I. M., in The Proteins, Vol. I B., H. Neurath, K. Bailey, eds., Academic Press, Inc., New York 1953, p727.
9. Odell, G. B., Cohen, S. N., Am. J. Dis. Child., 1960, v100, 525.
10. Davison, C., Smith, P. K., J. Pharm. Exp. Therap., 1961, v133, 161.

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