Effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes

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Effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes was studied by incubating the membranes at different pH and temperatures and determining the bound bilirubin. At all pH values, the amount of membrane-bound bilirubin increased with the increase in bilirubin-to-albumin molar ratios (B/As), being highest at lower pH values in all cases. Further, linear increase in bound bilirubin with the increase in bilirubin concentration in the incubate was observed at a constant B/A and at all pH values. However, the slope value increased with the decrease in pH suggesting more bilirubin binding to membranes at lower pH values. Increase in bilirubin binding at lower pH can be explained on the basis of increased free bilirubin concentration as well as more conversion of bilirubin dianion to monoanion. Temperature dependence of bilirubin binding to membranes was observed within the temperature range of 7°–60°C, showing minimum binding at 27°C and 37°C which increased on either side. Increase in bilirubin binding at temperatures lower than 20°C and higher than 40°C can be ascribed to the change in membrane topography as well as bilirubin-albumin interaction.

1. Introduction

Bilirubin is a yellow pigment produced by the catabolism of haemoglobin. Under physiological conditions, bilirubin remains bound to serum albumin which carries it to the liver for further metabolism. However, when bilirubin-to-albumin molar ratio (B/A) exceeds 1 : 1, free bilirubin binds to many types of cells including brain cells which is the cause of brain toxicity in premature neonates, a condition called kernicterus or bilirubin encephalopathy. In addition, jaundiced neonates with low plasma pH have been reported to be at greater risk of developing bilirubin encephalopathy (Kim *et al* 1980). Interaction of bilirubin with cells or cell membranes is well documented (Bratlid 1972; Sato *et al* 1987; Vazquez *et al* 1988; Hayer *et al* 1989; Leonard *et al* 1989; Tayyab and Ali 1995, 1997) and it is commonly accepted that the toxicity of bilirubin depends on its passage across the plasma membrane and its association with membrane lipids (Ali and Zakim 1993; Zucker *et al* 1994). However, the way in which bilirubin interacts with biological membranes is not fully understood. Erythrocytes being simple have been commonly used to study the interaction of bilirubin with cells or cell membranes as a model system (Hayer *et al* 1989). Further, erythrocyte-bound bilirubin has been suggested as a useful criterion for the risk of bilirubin encephalopathy in neonates (Bratlid 1972). It has been reported that the interaction of bilirubin with the membranes is greatly influenced by the physico-chemical properties of the interacting media such as pH and temperature (Sato and Kashiwamata 1983). Increased binding of bilirubin to biological membranes at physiological pH (i.e., 7· 0–7· 2) has been suggested to be due to increased precipitation of bilirubin on the surface of membranes (Cestaro *et al* 1983). However, Sato and Kashiwamata (1983) have reported that saturable bilirubin binding to erythrocyte membranes has a pH optimum at around pH 7· 1. They have suggested that the cellular susceptibility at lower pH may be determined not only by the physical state of bilirubin but also by the physico-chemical conditions of bilirubin binding substances on the membranes. Vazquez *et al* (1988) suggested that the increase in bilirubin binding to membranes at lower pH was mainly due to hydrophobic inclusion of bilirubin into membranes rather than aggregation of bilirubin on the surface of membranes. On the other hand, the influence of temperature on the binding of bilirubin is still not clear. A U-shaped thermal dependency of the total and saturable

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binding of bilirubin to erythrocyte membranes is reported with a minimum value near 37°C and a gradual increase below and above this temperature has been described (Sato and Kashiwamata 1983). Contrary to this, Leonard *et al* (1989) have reported no such thermal dependency of the binding of bilirubin to biological membranes over the temperature range of 10–40°C. All these studies of bilirubin binding to membranes were carried out either in albumin free medium (Cestaro *et al* 1983) or at a constant B/A i.e., 2·0 (Sato and Kashiwamata 1983; Sato *et al* 1987; Vazquez *et al* 1988). Therefore, these studies are limited in finding the answers of the role of albumin at various pH and temperatures on the interaction of bilirubin with membranes. Further, these studies are also limited in the data on the behaviour of bilirubin binding to membranes at various B/As (which are known to exist in physiological and jaundiced conditions) under different pH and temperatures. In this report, we have reinvestigated the effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes at different B/As.

2. Materials and methods

Bilirubin, anhydrous caffeine, sulfanilic acid, sodium benzoate and sodium nitrite were purchased from SD Fine Chemicals, Boisar, India. Sodium potassium tartarate and sodium hydroxide were obtained from Qualigens Fine Chemicals, Mumbai, India. Bovine serum albumin, fraction V was procured from Sigma Chemical Company, St. Louis, MO, USA. Human serum albumin was isolated by the method of Tayyab and Qasim (1990). Other reagents used were of analytical grade. Human blood (in 1·32% sodium citrate and 1·47% dextrose) was obtained from the Blood Bank of JN Medical College, Aligarh Muslim University, Aligarh.

Protein concentration was determined by the method of Lowry *et al* (1951) using bovine serum albumin as the standard.

2.1 *Preparation of erythrocyte membrane suspension*

Human erythrocytes were collected by centrifugation of blood at 1000 *g* for 20 min, followed by three washes with 50 mM Tris/HCl buffer, pH 7·4 containing 100 mM NaCl. Erythrocytes were diluted with equal volume of 50 mM Tris/HCl buffer, pH 7·4 containing 100 mM NaCl to obtain 50% haematocrit value. The membranes were isolated at 4°C from these erythrocytes following the method of Palfrey and Waseem (1985). Finally, the membranes were washed with 50 mM Tris/HCl buffer of different pH i.e., 7·8, 7·6, 7·4, 7·2 and 7·0 and mixed with a volume of buffer equivalent to the starting volume of blood.

2.2 *Bilirubin binding experiments*

Bilirubin solution was prepared by dissolving few crystals of bilirubin in 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11·0. The concentration of bilirubin solution was determined by Fog's method (1958). The bilirubin solution was protected from light and used within 1 h. All the experiments were carried out under dim light.

Binding of bilirubin to erythrocyte membranes at different pH was studied by the following procedure. To 1·0 ml of albumin solution of known concentration in 50 mM Tris/HCl buffer having pH *X* (where *X* = 7·0, 7·2, 7·4, 7·6 or 7·8), different volumes (20–250 µl) of stock bilirubin solution were added to get different B/As and the volume was made up to 1·25 ml with 50 mM Tris/HCl buffer having same pH as that of albumin solution. Then, 250 µl of erythrocyte membrane suspension in the same buffer was added and the tubes were incubated for 30 min at 37°C after gentle shaking. The mixture was centrifuged at 10,000 *g* for 5 min at room temperature and the supernatant discarded. Membranes were washed 3–4 times with 50 mM Tris/HCl buffer, pH 7·4 until the last supernatant was devoid of yellow colour. After final washing, the membrane-bound bilirubin was extracted and determined in the same way as described earlier (Tayyab and Ali 1999) which is based on the determination of bilirubin as azobilirubin using Fog's method (1958) after SDS solubilization of membranes. The method is sensitive to measure a minimum amount of 3 nmol of bilirubin in a given sample of 1·0 ml. In another set of experiments, both the bilirubin and albumin concentrations were varied to obtain a constant B/A. Effect of temperature was studied by pre-incubating the membrane suspension in 50 mM Tris/HCl buffer, pH 7·4 and bilirubin/albumin mixture of different B/As, independently at different temperatures (i.e., 7°C, 20°C, 27°C, 37°C, 40°C, 50°C and 60°C) for 30 min followed by mixing of bilirubin/albumin solution with membrane suspension and further incubation for 30 min at their respective temperatures. Each experiment was carried out three times.

Statistical analysis of the data included calculations of dispersion. Difference of means were tested for significance using two-tailed '*t*' test. The '*t*' values were used to calculate the *P*-value.

3. Results and discussion

3.1 *Interaction of bilirubin with erythrocyte membranes at different pH*

At a given pH, increase in the B/A from 0·5 to 3·0 led to an increase in the membrane-bound bilirubin (figure 1). This increase was smaller up to a B/A of 1·5:1 but became more significant ($P < 0·05$) at high B/As at all the pH values used in this study. Nearly two-fold ($P < 0·05$) increase in membrane-bound bilirubin was noticed on increasing the B/A from 1·0 to 2·0. At all the five different pH values, i.e., 7·0, 7·2, 7·4, 7·6 and 7·8, the patterns of bilirubin binding to membranes were qualitatively similar. However, the amount of membrane-bound bilirubin at any B/A in the range of 0·5 to 3·0 was

different at different pH values, being negatively correlated with pH of the medium. In other words, lowering the pH from 7· 8 to 7· 0 resulted in increased binding of bilirubin to erythrocyte membranes at all the B/As tested even if B/A was below 1 : 1. In an earlier study, cellular binding of bilirubin has also been shown to vary with pH in the same fashion (Bratlid 1972).

At a given constant B/A (i.e., 2 : 1), the binding of bilirubin to erythrocyte membranes increased linearly with increase in bilirubin concentration at all the pH values used as shown in figure 2. Similar results were found with other B/As. Linear increase in bound bilirubin with the increase in bilirubin concentration in the incubate at a constant B/A and pH was also observed earlier with human erythrocytes (Hayer *et al* 1989; Tayyab and Ali 1997). Increase in membrane-bound bilirubin per unit increase in bilirubin concentration in the incubate was calculated from the slope values of the straight line plots obtained at different pH values and at different B/As. This slope value was found to be different for different pH values and different B/As, being highest at pH 7· 0 (figure 3). However, at B/As 2· 5 and 3· 0, the value of slope became constant between pH 7· 4 and 7· 0. In other words, the increase in membrane-bound bilirubin per unit increase in bilirubin concentration in the incubate was similar between pH values 7· 4 and 7· 0 at B/As 2· 5 and 3· 0. As the binding of bilirubin to erythrocytes and erythrocyte membranes follows a Michaelian saturation curve (Hayer *et al* 1989; Tayyab and Ali 1995), the slope value calculated from curves shown in figure 2 will depend on the range of free bilirubin concentration available in the incubate in such a way that with the increase in the range of free bilirubin concentration, the change in the slope value will be minimum. For example, at pH 7· 4, the difference in slope value obtained was maximum between B/As 2· 0 and 1· 5 followed by between 2· 5 and 2· 0 whereas the value was minimum between B/As 3· 0 and 2· 5 (see figure 3). Since the available range of free bilirubin concentration at B/A 1· 5 is minimum whereas it is maximum at B/A 3· 0, these results are in accordance with the above explanation. One possible factor for the higher binding observed at pH 7· 0 compared to pH 7· 4 or higher may be the large increase in free bilirubin concentration in the incubate at pH 7· 0 compared to pH 7· 4 due to decreased albumin binding as reduced binding of bilirubin to albumin has been shown earlier by different workers after lowering the pH from 7· 4 to 6· 5 (Odell *et al* 1969). Another factor may be the increased susceptibility of erythrocyte membranes towards bilirubin at lower pH. This is because decrease in pH may convert bilirubin dianion (B^{2-}) to monoanion (BH^-) which seems to be responsible for the increased binding of bilirubin to membranes at lower pH (Vazquez *et al* 1988). It may be noted that out of the three species of bilirubin i.e., bilirubin dianion (BH^{2-}), bilirubin monoanion (BH^-) and bilirubin acid (BH_2), erythrocyte membranes are capable of binding both BH_2 and BH^- (Vazquez *et al* 1988; Brites *et al* 1997). Keeping in view all the above points, consistency in the slope value observed in the pH range 7· 4–7· 0 at higher B/As (2· 5 and 3· 0) can be

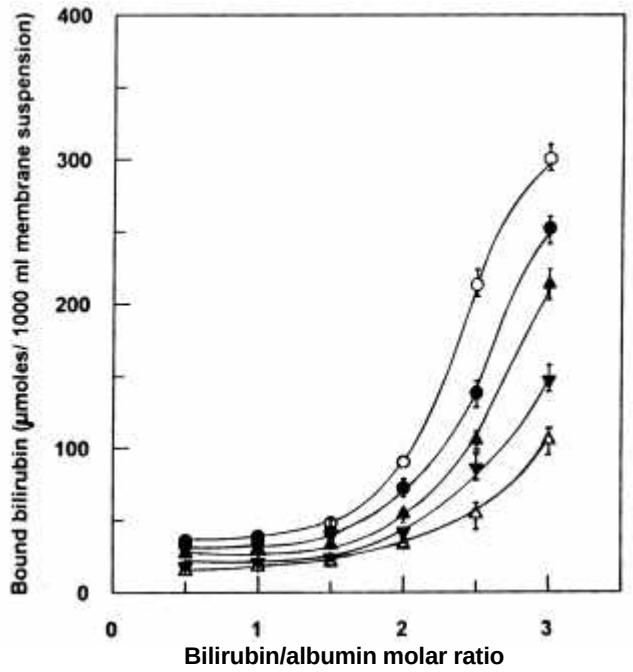

Figure 1. Binding of bilirubin to human erythrocyte membranes at different B/As and at different pH values [pH 7· 0 (○), pH 7· 2 (●), pH 7· 4 (▲), pH 7· 6 (▼) and pH 7· 8 (Δ)]. Each point is the mean ± SEM of three independent observations.

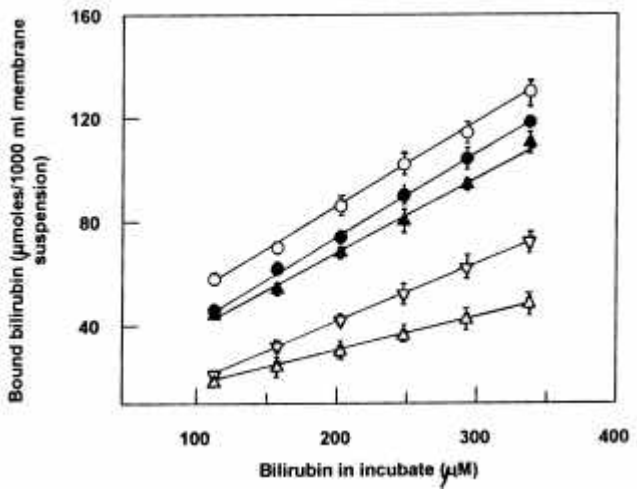

Figure 2. Binding of bilirubin to human erythrocyte membranes at a constant B/A (2 : 1) but increasing bilirubin concentrations and at different pH values [pH 7· 0 (○), pH 7· 2 (●), pH 7· 4 (▲), pH 7· 6 (▽) and pH 7· 8 (Δ)]. Each point is the mean ± SEM of three independent observations.

ascribed to a higher free bilirubin concentration in the incubate. Since membrane binding of bilirubin is significantly increased with decreasing pH even at lower B/A i.e., 1· 5, the results shown here are of clinical significance as B/A of as high as 1· 37 has been reported in jaundiced neonates (Cashore and Oh 1982) and also in the presence of various bilirubin displacing ligands including drugs, the B/A may reach a higher value which may be fatal under acidosis conditions.

3.2 *Interaction of bilirubin with erythrocyte membranes at different temperatures*

Incubation of erythrocyte membranes with bilirubin at different temperatures ranging from 7°C to 60°C showed that at any given B/A above 1 : 1, the amount of membrane-bound bilirubin was greatly influenced by the temperature of the incubation medium as shown in figure 4. Within the temperature range of 27°C to 37°C, the amount of membrane-bound bilirubin at any given B/A above 1 : 1 was found to be minimum. Increase in temperature on either side led to an increase in bilirubin binding to erythrocyte membranes at all B/As. Sato and Kashiwamata (1983) also reported minimum binding of bilirubin to erythrocyte membranes at 37°C using a B/A of 2· 0. A comparison of bilirubin binding patterns of human erythrocyte membranes at different temperatures and B/As shows that at each temperature, the binding was higher at higher B/As. This unusual behaviour of bilirubin binding to erythrocyte membranes at different temperatures may be either due to the effect of temperature on the binding of bilirubin to albumin or due to the change in the physical state of bilirubin binding sites of the erythrocyte membranes. The lipid bilayer of human erythrocyte membrane exists in gel state below 18· 5°C while above 40°C it mainly exists in liquid-crystalline state (Barenholz and Thompson 1980). Cestaro *et al* (1983) have reported that in the gel state both disordered phospholipid bilayers and protrusion of apolar regions of membrane proteins highly increased the hydrophobicity of outer sides of the membranes which greatly potentiates the binding of bilirubin to the membranes. On the other hand, above 40°C, the membranes are in liquid-crystalline state and the lipids are randomly arranged (Vigh *et al* 1998) and both the surface area per molecule and bilayer spacing are increased due to the hydration of phospholipids (Barenholz and Thompson 1980). Under such conditions, bilirubin can freely penetrate the internal apolar core of the membrane bilayer from both surfaces (Hayward *et al* 1986). However, within the temperature range of 20–40°C, the arrangement of lipid in the bilayer of the membrane is not random, i.e., most of the choline phospholipids are confined to the outer surface while negatively charged phospholipids to the inner surface. Under such condition, it is likely that the interaction of bilirubin with the outer surface is more favourable than that of the inner surface which may account for the low binding of bilirubin within the temperature range of 20–40°C. In addition to this, the role of temperature in decreasing the bilirubin-albumin interaction thereby increasing the free bilirubin concentration for binding to the membranes cannot be ruled out.

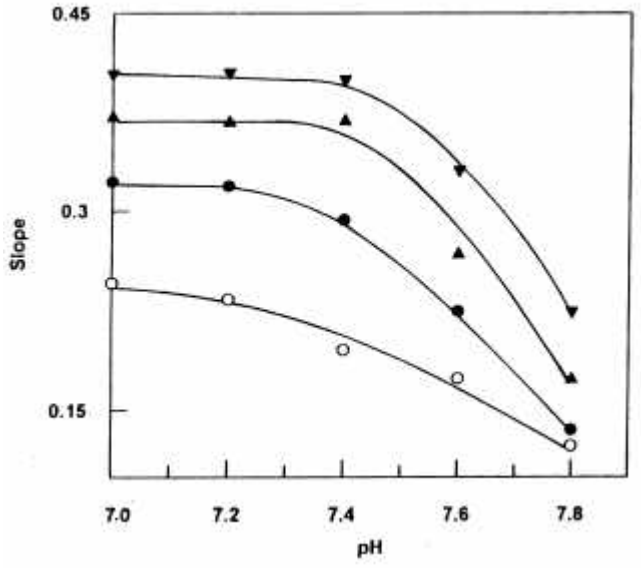

Figure 3. Plots of the slope values of the straight line plots obtained in figure 2 versus pH [B/A 1· 5 (○), B/A 2· 0 (●), B/A 2· 5(▲) and B/A 3· 0 (▼)].

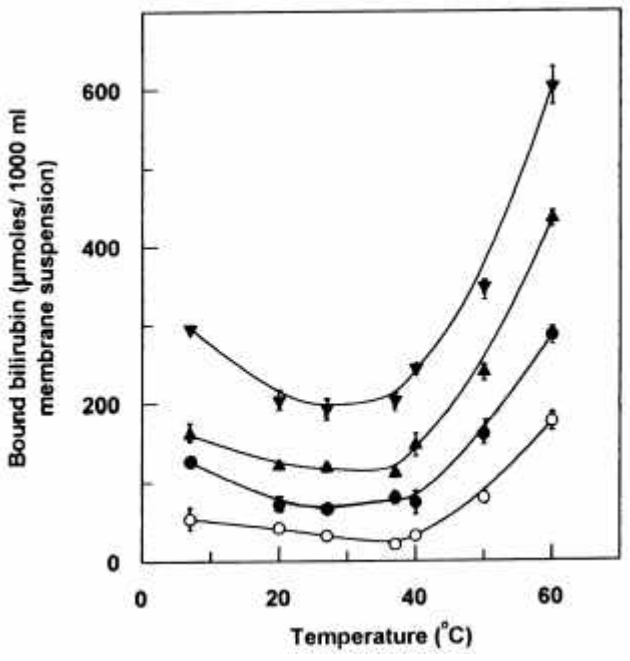

Figure 4. Binding of bilirubin to human erythrocyte membranes at a given B/A and pH but at different temperatures [B/A 1· 5 (○), B/A 2· 0 (●), B/A 2· 5 (▲) and B/A 3· 0 (▼)]. Each point is the mean ± SEM of three independent observations.

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