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Calcium-induced bilirubin-dependent hemolysis of human erythrocytes

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Abstract

Human erythrocytes, preincubated with different concentrations of calcium chloride (0.17–1.67 mM) showed hemolysis after addition of bilirubin (72 μM). Hemolysis was observed only when cells were incubated first with calcium followed by bilirubin and not vice versa. This hemolysis was found to be dependent upon several factors such as concentration of bilirubin, time of incubation of erythrocytes with calcium and time of incubation of bilirubin with the calcium-loaded erythrocytes. Inclusion of EDTA in the incubation medium reduced the percentage hemolysis to a significant extent. Involvement of activated oxygen species in hemolytic process seems to be unlikely as inclusion of sodium azide and catalase did not prevent hemolysis. A comparison of other bivalent cations such as Ba^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+} with Ca^{2+} for their ability to hemolyse cells in presence of bilirubin shows that Ba^{2+} and Mg^{2+} are ineffective, whereas both Mn^{2+} and Cu^{2+} induce hemolysis both in the absence as well as in the presence of bilirubin. However, their mechanism of hemolysis is different from that of calcium-induced hemolysis. Formation of calcium-induced hydrophobic aggregates of phospholipid molecules in erythrocyte membrane may open the new binding sites for bilirubin on these membranes which may perturb the membrane conformation.

Keywords: Human erythrocyte; Bilirubin; Calcium; Hemolysis

1. Introduction

Calcium is known to play important role in many cellular functions. The ability of calcium to aggregate phospholipids of membranes, especially those containing high phosphatidylserine (PS) and phosphatidic acid (PA) is well documented [1–3]. These phospholipids respond characteristically to calcium and are aggregated by calcium through intramolecular chelation. This calcium-induced aggregation reduces the lateral diffusion of lipid molecules and retards the motion of alkyl chains which leads to the reduction in the transverse movement of molecules and ions across the aggregates [3]. Further, activities of proteins associated with lipid especially with PS is also affected. This type of effect has also been reported with Ba^{2+}, Sr^{2+} and Mg^{2+} but its magnitude is smaller than that of calcium [3]. The aggregation is reported to be dependent upon the percentage PS content of biomembranes. Further, calcium also changes the shape and size of erythrocytes which is species-dependent. For example, human erythrocytes are more prone to calcium and are transformed into echinocytes, whereas goat erythrocytes do not [4]. In addition, calcium also affects various uptake processes. Increased bilirubin binding has been reported by human erythrocyte ghosts in the presence of 1 mM $CaCl_2$ [5]. In the presence of bilirubin (mono or

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dianion), the calcium-induced phospholipid aggregation might be effected. In this report we present our data on the calcium-induced changes in human erythrocytes in the presence of bilirubin.

2. Materials and methods

Bilirubin, caffeine anhydrous, sulphanilic acid, sodium benzoate, sodium nitrite, sodium azide and cupric chloride were purchased from SD's Fine Chemicals, India. Calcium chloride, magnesium chloride, barium chloride and manganous chloride were obtained from Qualigens Fine Chemicals, India. Catalase was purchased from Sigma Chemical Company, USA. Other reagents were of analytical grade. Human blood was obtained from the Blood Bank of J.N. Medical College, Aligarh Muslim University, Aligarh.

Erythrocytes were isolated by centrifugation of collected blood at $1000 \times g$ for 20 min. The cells were then washed three times with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, after removing the buffy coat by centrifugation at $1000 \times g$ for 20 min. The final packed cell volume was diluted with the same buffer in a ratio of 1:1 to get 50% hematocrit value of human erythrocytes.

Bilirubin solution was prepared by dissolving few crystals of bilirubin in 38 mM sodium carbonate solution containing 0.15 M NaCl (pH 11.0) (adjusted with 0.1 N HCl). The concentration of bilirubin solution was determined by Fog's method [6]. The bilirubin solution was protected from light and used within 30 min. All the experiments were carried out under yellow light.

The solutions of calcium chloride, barium chloride, magnesium chloride, cupric chloride and manganous chloride were prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl.

The concentration of Ca^{2+} was measured by O.C.P.C. method, using calcium measurement kit (Code No. 25952) supplied by Span Diagnostics Ltd., India.

The procedure of incubation was followed as described by Bratlid [7], except that instead of 0.5 ml, 1.0 ml of bilirubin solution was taken. The incubation was carried out in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl at 37°C and the final volume of the incubation mixture was 6.0 ml. The final hematocrit value of human erythrocytes was 8.3% and the final pH and ionic strength of the incubation medium was 8.1 and 0.17 respectively. Although the calculated solubility of bilirubin at pH 8.1 is less (about 91 nM) [8], the aggregation of bilirubin is checked with the hydrophobic inclusion of bilirubin into membranes which seems to be much higher at pH 8.0 [9]. In all experiments, the cells were incubated first with various salt solutions, i.e., calcium chloride, barium chloride, magnesium chloride, manganous chloride and cupric chloride of desired concentration for 30 min at 37°C and then a constant amount of bilirubin (72 μM) (otherwise stated) was added. The percentage hemolysis was calculated by the following formula:

$$\text{Percentage hemolysis} = \frac{\text{O.D.}_2}{\text{O.D.}_1} \times 100$$

where O.D._1 is the O.D. at 540 nm of hemolysate of human erythrocytes with distilled water in 6.0 ml (8.3% hematocrit value) after 1 h of incubation and O.D._2 is the O.D. at 540 nm of bivalent ion-induced hemolysate in presence or absence of bilirubin in 6.0 ml (8.3% hematocrit value) after 1 h of incubation.

3. Results and discussion

Human erythrocytes when incubated with different concentrations of calcium chloride ($CaCl_2$) (0.17–1.67 mM) for 30 min were found to be hemolysed in presence of bilirubin (72 μM) as shown in Fig. 1a. A marked increase in percentage hemolysis (58.3%) was found at 0.17 mM $CaCl_2$ concentration, beyond which it sloped off. The induction of such hemolysis by $CaCl_2$ in the presence of bilirubin seems to be a conditional requirement as no hemolysis was observed when the erythrocytes were incubated with either $CaCl_2$ or bilirubin alone, even at a higher concentration (i.e., 3.0 mM $CaCl_2$; 125.5 μM bilirubin) (data not shown). As can clearly be seen from Fig. lb, when $CaCl_2$ (0.17–1.67 mM) and bilirubin (72 μM) were simultaneously added to human erythrocytes, only 14% hemolysis was observed at 1.67 mM $CaCl_2$ concentration, compared to 62.5% when erythrocytes were incubated first with 1.67 mM $CaCl_2$

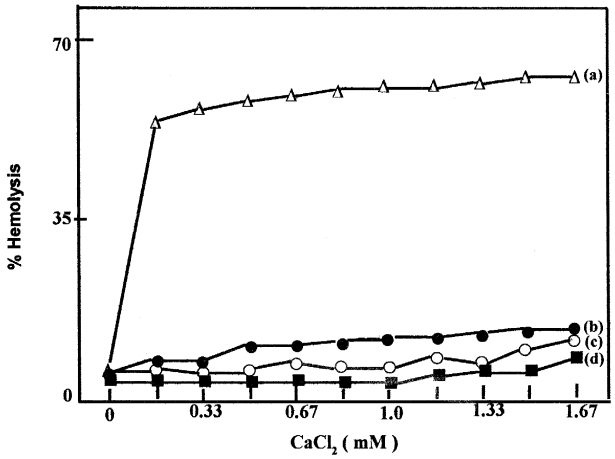

Fig. 1. Effect of calcium on human erythrocytes in the presence of bilirubin. (a) Erythrocytes first incubated with different concentrations of $CaCl_2$ (0.17–1.67 mM) for 30 min at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C. (b) Erythrocytes incubated with different concentrations of $CaCl_2$ (0. 17–1.67 mM) and bilirubin (72 μM) at the same time for 1 h at 37°C. (c) Erythrocytes first incubated with bilirubin (72 μM) for 30 min at 37°C followed by further incubation with different concentrations of $CaCl_2$ (0.17–1.67 mM) for 30 min at 37°C. (d) Erythrocytes first incubated with bilirubin (72 μM) for 30 min at 37°C then washed the unbound bilirubin followed by further incubation with different concentrations of $CaCl_2$ (0.17–1.67 mM) for 30 min at 37°C.

for 30 min, followed by addition of bilirubin (72 μM). Moreover, incubation of erythrocytes with bilirubin (72 μM) for 30 min at 37°C followed by the addition of 1.67 mM $CaCl_2$ led to only 11% hemolysis (Fig. 1c). Similarly, incubation of erythrocytes with bilirubin (72 μM) for 30 min at 37°C, washing with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl to remove unbound bilirubin followed by the addition of $CaCl_2$ showed only 8% hemolysis (Fig. 1d). Taking the percentage hemolysis values (5%) in the control samples without $CaCl_2$, this hemolysis (Fig. 1b–d) seems insignificant. These results strongly suggest the pre-requirement of $CaCl_2$ to interact first with the erythrocytes followed by the interaction of bilirubin with the calcium-bound erythrocytes for the induction of hemolysis. Incubation of erythrocytes with calcium alone (0.33–2.0 mM) for 30 min led to a decrease in calcium level in the supernatant (see Fig. 2a). This suggests that calcium binds to erythrocyte membranes. The addition of bilirubin to this system released some calcium from these membranes as calcium level in the supernatant increased in the presence of bilirubin (Fig. 2b). However, the increase in the calcium level in the supernatant was significantly higher when cells were incubated first with bilirubin (72 μM) for 30 min followed by the addition of $CaCl_2$ (0.33–2.0 mM) for 30 min (Fig. 2c) compared to when the order was reversed (Fig. 2b). It seems that some of the calcium binding sites on erythrocytes are masked by bilirubin. The inability of bilirubin-bound erythrocytes to undergo hemolysis in the presence of Ca^{2+}, unlike the calcium-bound erythrocytes in the presence of bilirubin, further supports this theory. This contention is also supported by the fact that bilirubin has a high association constant for phosphatidylcholine (PC) [10] and its binding to PC prevents the phase separation of PS-PC bilayer membranes into a solid phase of PS aggregates bridged by Ca^{2+} chelation [1–3].

Calcium-induced hemolysis of erythrocytes in the presence of bilirubin was found to be dependent upon the concentration of bilirubin. This can be clearly seen from Fig. 3. Increase in bilirubin concentration

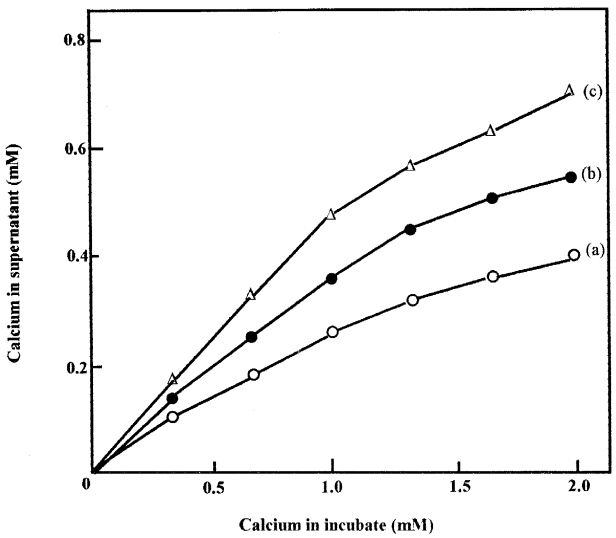

Fig. 2. Plot showing the calcium levels in the supernatant after incubation with human erythrocytes. (a) Erythrocytes incubated with different concentrations of $CaCl_2$ (0.33–2.0 mM) for 30 min at 37°C. (b) Erythrocytes first incubated with different concentrations of $CaCl_2$ (0.33–2.0 mM) for 30 min at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C. (c) Erythrocytes first incubated with bilirubin (72 μM) for 30 min at 37°C followed by further incubation with different concentrations of $CaCl_2$ (0.33–2.0 mM) for 30 min at 37°C.

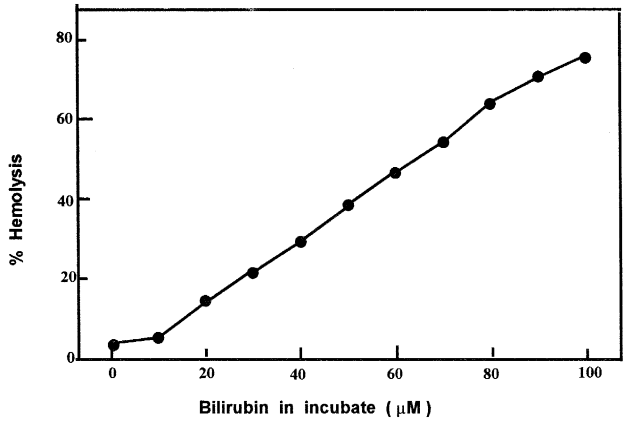

Fig. 3. Bilirubin dependence of calcium-induced hemolysis of human erythrocytes. Erythrocytes first incubated with $CaCl_2$ (1.0 mM) for 30 min at 37°C followed by further incubation with different concentrations of bilirubin (10–100 μM) for 30 min at 37°C.

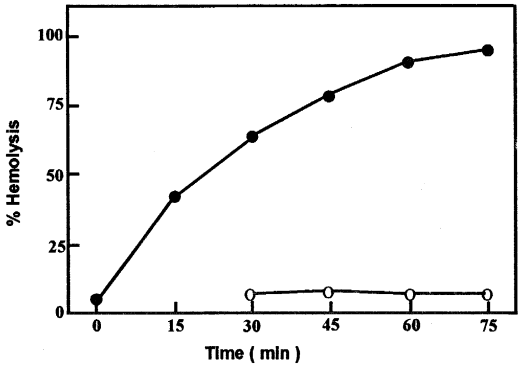

Fig. 5. Effect of time after the addition of bilirubin on the calcium-induced bilirubin-dependent hemolysis of human erythrocytes. Erythrocytes first incubated with $CaCl_2$ (2.0 mM) for 30 min at 37°C, followed by further incubation with bilirubin (72 μM) at 37°C for varying time periods (0–75 min). (0–0) shows the effect of incubation time of human erythrocytes with $CaCl_2$ (2.0 mM) on the hemolysis in the absence of bilirubin.

up to 100 μM after preincubation of erythrocytes with 1.0 mM $CaCl_2$ for 30 min at 37°C led to a linear increase in percentage hemolysis.

Hemolysis of erythrocytes induced by calcium in the presence of bilirubin was found to be dependent upon time of incubation with calcium. As can be seen from Fig. 4, the incubation of cells with calcium (1 mM) for different time periods followed by further incubation with bilirubin (72 μM) for 30 min yielded a different percentage hemolysis which became constant after 20 min. These results suggest that the interaction of calcium with human erythrocytes reached saturation within 20 min.

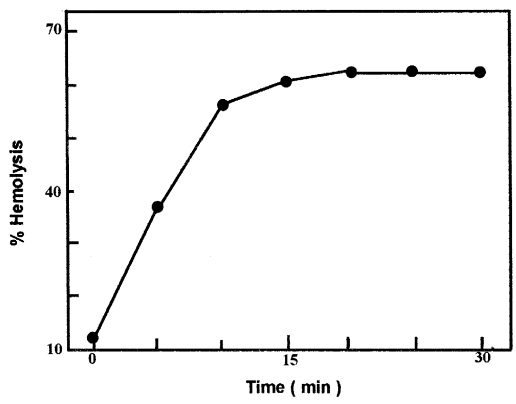

Fig. 4. Effect of incubation time on the calcium-induced bilirubin-dependent hemolysis of human erythrocytes. Erythrocytes first incubated with $CaCl_2$ (1.0 mM) for different time periods (0–30 min) at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C.

Furthermore, the percentage hemolysis was also found to be dependent upon the time of incubation of bilirubin with the calcium-loaded erythrocytes, as shown in Fig. 5. The percentage hemolysis increased continuously with the increase in the time of incubation of bilirubin with calcium-loaded erythrocytes, reaching a 95% value after 75 min. On the other hand, incubation of cells with 2 mM $CaCl_2$ alone for 75 min did not show any significant lysis (see Fig. 5).

It was found that inclusion of EDTA in the incubation mixture along with calcium did reduce the percentage hemolysis after the addition of bilirubin, but total inhibition was not observed (see Fig. 6). The percentage hemolysis decreased continuously (from a value of 65% in control) with the increasing concentration of EDTA reaching to a value of 35% at an EDTA concentration of 2.0 mM, (double the concentration of calcium used). This shows that all the calcium ions are not removed by 2 mM EDTA. Further, washing of erythrocytes pretreated with Ca^{2+} and bilirubin, with 50 mM Tris-HCl buffer (pH 7.4), containing 100 mM NaCl and different concentrations of EDTA, showed that subsequent hemolysis was prevented by removal of cell-bound calcium. As can be seen from Fig. 7, 2.0 mM EDTA led to a significant decrease in hemolysis. These results probably suggest that the basic reaction which results in

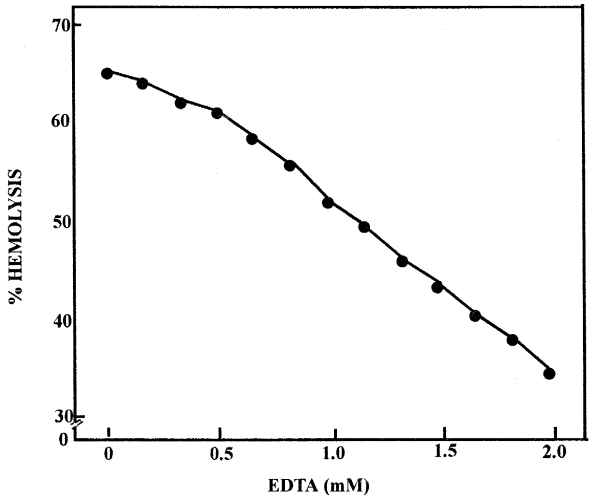

Fig. 6. Effect of EDTA on the calcium-induced bilirubin-dependent hemolysis of human erythrocytes. Erythrocytes incubated with $CaCl_2$ (1.0 mM) and different concentrations of EDTA (0–2.0 mM) at the same time for 30 min at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C.

the hemolysis probably involves the calcium bound to the erythrocyte membrane as this calcium can easily be washed out compared to the intracellular calcium.

To determine the role of activated oxygen species in bilirubin-dependent calcium-induced hemolysis, sodium azide and catalase were used in the incubation medium. It was found that neither catalase (13–120 mg/l) nor sodium azide (0.17–1.83 mM) inhibited hemolysis. Further, inclusion of hydrogen peroxide (0.16–1.6 mM) with human erythrocytes alone also failed to produce any significant hemolysis (see Fig. 8). Therefore, singlet oxygen, H_2O_2 and OH^- are not involved in hemolysis. Moreover, the question of generation of any free radical by the interaction of calcium with bilirubin did not arise as the incubation of calcium with bilirubin for 30 min at 37°C followed by addition of human erythrocytes did not produce any hemolysis. This is because the calcium bilirubinate formed in this reaction did not bind to the cells.

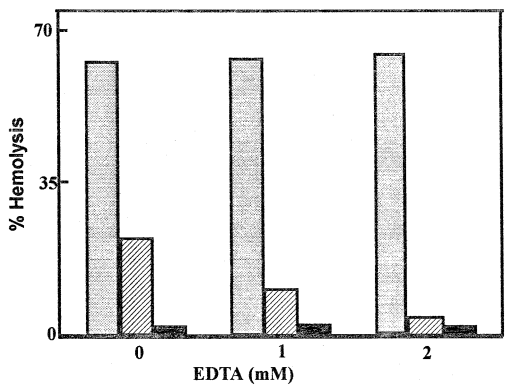

Fig. 7. Effect of removal of surface-bound calcium on the subsequent hemolysis of human erythrocytes pretreated with calcium and bilirubin. Erythrocytes first incubated with $CaCl_2$ (1.0 mM) for 30 min at 37°C followed by further incubation with bilirubin (72 μM), washing with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and different EDTA concentrations (0–2.0 mM). Shaded columns, control; hatched columns, first wash; filled columns, second wash.

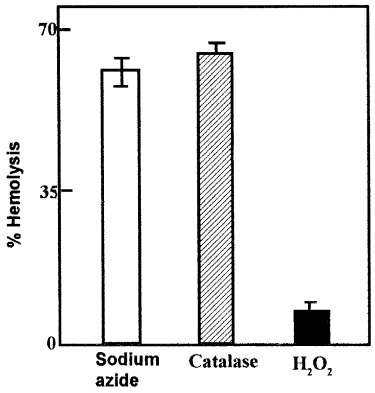

Fig. 8. Effect of sodium azide and catalase on calcium-induced bilirubin-dependent hemolysis of human erythrocytes. Erythrocytes first incubated with $CaCl_2$ (1.0 mM) and sodium azide (0.17–1.83 mM) or catalase (13–120 mg/l) for 30 min at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C. Effect of H_2O_2 (0.16–1.6 mM) on human erythrocytes is also shown.

The effect of other bivalent cations for their possible role of inducing hemolysis of human erythrocytes in the presence of bilirubin was also investigated. Ba^{2+} and Mg^{2+} are closely related bivalent cations to Ca^{2+} due to their ability to induce membrane aggregation through intramolecular chelation of phospholipid molecules [3]. However, under similar conditions of incubation procedure, both Ba^{2+} and Mg^{2+} (0.17–2.0 mM) were found to be unable to induce hemolysis of human erythrocytes as compared to Ca^{2+} (0.17–2.0 mM) in the presence of bilirubin (72 μM) (see Fig. 9d,e and Fig. 10). Even at higher concentrations (3.3–16.7 mM), both Ba^{2+} and Mg^{2+} failed to induce any hemolysis (data omitted for

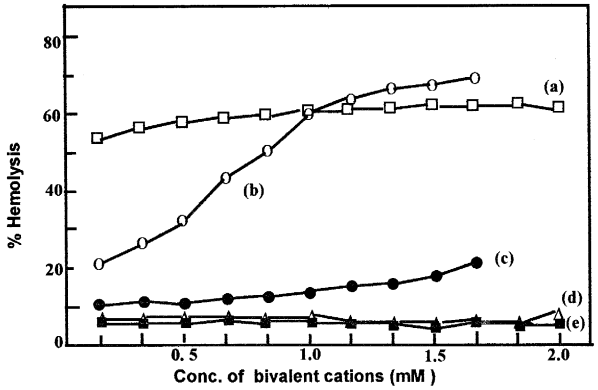

Fig. 9. Effect of various bivalent cations on human erythrocytes in the presence of bilirubin. Erythrocytes first incubated with various salts of bivalent cations (0–2.0 mM) for 30 min at 37°C followed by further incubation with bilirubin (72 μM) for 30 min at 37°C. The various salts used are: $CaCl_2$ (a); $CuCl_2$ (b); $MnCl_2$ (c); $BaCl_2$ (d) and $MgCl_2$ (e).

brevity). Although the effect of both Ba^{2+} and Mg^{2+} on biomembrane is reported smaller compared to Ca^{2+}, the failure to induce hemolysis even at a higher concentration of these cations, in the presence of bilirubin, suggests that either Ba^{2+} or Mg^{2+} alone, or Ba^{2+} or Mg^{2+} complexed with bilirubin (if formed) is unable to alter the permeability of human erythrocytes. On the other hand, Mn^{2+} and Cu^{2+} induced hemolysis of human erythrocytes both in the presence and absence of bilirubin, as shown in Fig. 9b,c and Fig. 10. In the absence of bilirubin, Mn^{2+} at a concentration of 1.67 mM produced 20% hemolysis (Fig. 10), whereas in the presence of bilirubin, the

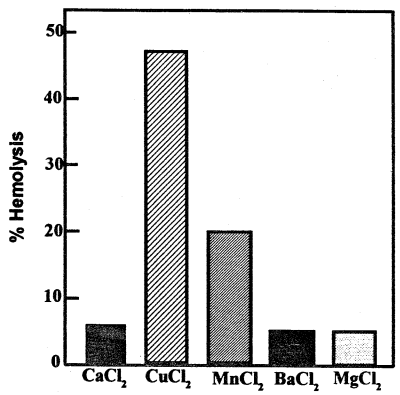

Fig. 10. Effect of various bivalent cations on human erythrocytes in the absence of bilirubin. Erythrocytes were incubated with different salts of bivalent cations (1.67 mM) for 1 h at 37°C.

percentage hemolysis was 22%. From these results it is clear that hemolysis is induced by Mn^{2+} alone, unlike bilirubin-dependent calcium-induced hemolysis of human erythrocytes. Similarly, in the absence of bilirubin, Cu^{2+} (1.67 mM) alone can induce about 47% hemolysis (Fig. 10), whereas in the presence of bilirubin, about 69% hemolysis was observed. This is because Cu^{2+} alone or complexed with bilirubin can act as a potential oxidative agent [11], thereby causing changes in membrane permeability. It can also cross-link various proteins in the membrane. Therefore, hemolysis induced by Cu^{2+} is different from that of bilirubin-dependent calcium-induced hemolysis of human erythrocytes.

The possible reason for this phenomena of bilirubin-dependent calcium-induced hemolysis of human erythrocytes can be explained in the following way. Since human erythrocyte membranes have a high ratio of phosphatidylcholine and phosphatidylserine [12], they are, therefore, highly sensitive to calcium to form hydrophobic aggregates of phospholipid molecules in erythrocyte membrane. Furthermore, the potentiality of bilirubin to remain in hydrophobic medium [8] increases the binding of bilirubin to these hydrophobic phospholipid aggregates of membranes. Since calcium-bound human erythrocytes are unstable due to transformation of the normal cells to echinocytes [4], the binding of bilirubin to the Ca^{2+}-chelated hydrophobic phospholipid aggregates of such transformed cells may cause further imbalance which results in hemolysis.

Although calcium-induced hemolysis of erythrocytes has been reported in the presence of other ligands such as toxin [13] and complement components [14], the mechanism of calcium-induced bilirubin-dependent hemolysis may or may not be similar to the mechanism of calcium-induced hemolysis in the presence of other ligands and thus requires further investigation.

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