RESISTANCE TOWARDS CALCIUM INDUCED BILIRUBIN DEPENDENT HEMOLYSIS IN PORCINE ERYTHROCYTES

Boh Boon Kim and Saad Tayyab

Biomolecular Research Group, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

ABSTRACT

Hemolysis of erythrocytes from human and porcine blood was studied at different calcium chloride (CaCl_2) concentrations (0.04–1.83 mM) and at a constant bilirubin concentration (72 mM). Although, human erythrocytes showed significant hemolysis (64%) at the highest CaCl_2 concentration (1.83 mM) used in this study, remarkable resistance to this phenomenon was observed with porcine erythrocytes as only 11% hemolysis was observed. A similar pattern in the behavior of both human and porcine erythrocytes was observed when parameter such as bilirubin concentration or time of incubation with bilirubin was varied. Other divalent cations such as Mn^{2+} , Ba^{2+} and Mg^{2+} were either least effective or ineffective in inducing hemolysis in presence of bilirubin. Serum albumin played a protective role in this phenomenon in a concentration dependent manner, as no hemolysis was observed at a bilirubin/albumin molar ratio of 1:1 or less. Differences in the structural make-up of proteins and lipids in the erythrocyte membranes of the two species may account for their different behavior.

KEY WORDS

Bilirubin, Calcium, Erythrocytes, Hemolysis, Human, Mammalian species, Porcine, Serum albumin.

INTRODUCTION

Unconjugated bilirubin (UCB), a potentially toxic pigment produced by heme catabolism, occurs mainly as an electrically neutral diacid in the unbound form (1). Passive diffusion has been suggested for the transport of internally hydrogen bonded bilirubin (2) across any cell membrane (3). Various toxic effects of UCB including neurotoxicity have been described which include inhibition of various enzymes (4, 5), strand breakage in DNA (6), induction of apoptosis (7–9), inhibition of long-term potentiation of synaptic transmission (10) and toxicity to astrocytes and neurons (11) to name a few. UCB has been shown to cause perturbation of membrane dynamics in rat brain neurons (8), disruption of membrane lipid polarity and fluidity, modified protein mobility and increased production of reactive oxygen species in rat mitochondria (9). Oxidative injury, loss of membrane asymmetry and functionality and

calcium intrusion in synaptosomal membrane vesicles have also been implicated in the pathogenesis of encephalopathy by hyperbilirubinemia (12).

Like other cell types, erythrocytes are also capable of binding UCB to their membranes (13–15). Use of erythrocytes as a model system to study bilirubin binding and toxicity has been widely used by several groups (13, 15–19). Interaction of UCB with human erythrocytes has been shown to induce morphological alteration, membrane structural perturbation and loss of phospholipid asymmetry (20–22).

Calcium is known to play an important role in the maintenance of erythrocyte conformation (23, 24). Some of the calcium related phenomena reported are aggregation of erythrocyte membrane proteins (25), transbilayer diffusion of plasma membrane phospholipids (26) and transition in cell shape from the normal biconcave disk to rounded spherocyte (27).

Bilirubin has been shown to induce hemolysis of human erythrocytes in presence of calcium (18). Differences in the calcium induced bilirubin dependent hemolytic phenomenon have been found among different mammalian erythrocytes (28). This prompted us to study this phenomenon with the

Address for Correspondence :

Prof. Saad Tayyab

Biomolecular Research Group

Institute of Biological Sciences

University of Malaya, 50603 Kuala Lumpur, Malaysia

E-mail: saadtayyab2004@yahoo.com

erythrocytes obtained from porcine blood, a hitherto uninvestigated source. Studies on species differences in bilirubin related phenomena become important for selecting an animal model to develop preventive measures against fatal kernicterus. Here we report our results on the comparative studies of calcium-induced bilirubin-dependent hemolysis in both human and porcine erythrocytes.

MATERIALS AND METHODS

Human (*Homo sapiens*) blood was obtained from the Blood Bank of the University of Malaya Medical Centre, Kuala Lumpur. Porcine (*Sus scrofa*) blood was collected fresh in 1.32% (w/v) sodium citrate solution from a local slaughter house. Bilirubin (B-4126) and bovine serum albumin, fraction V (lot 015K0591) were purchased from Sigma Chemical Company, USA. Chloride salts of calcium, magnesium, barium and manganese were products of Systerm® Chemicals, Malaysia. Other reagents used were of analytical grade.

Preparation of erythrocyte suspension : Erythrocytes were collected by centrifugation of blood at 1575xg for 15 min at 4°C after removing the upper 'buffy' coat by gentle decantation. The cells were washed three times with 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl and then collected by centrifugation at 1575xg for 15 min. Equal volume of the same buffer was added to the final packed cell volume to obtain a 50% hematocrit value.

Preparation of solutions : Bilirubin solution was prepared just before use by dissolving a few crystals of bilirubin in 38 mM sodium carbonate solution, pH 11.0 containing 0.15 NaCl and its concentration was determined by the method of Malloy and Evelyn (29). The bilirubin solution was kept in dark and used within 1 h of preparation. All the experiments were carried out under dim light. Salt solutions (1.83 mM) [calcium chloride (161.4 mg/100 ml), barium chloride (268.2 mg/100 ml),

magnesium chloride (223.2 mg/100 ml) and manganese chloride (217.3 mg/100 ml)] were prepared in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl.

Bilirubin toxicity assay : Bilirubin toxicity to erythrocytes was determined by using the incubation procedure as suggested by Bratlid (13) with slight modification and measuring hemolysis. The incubation mixture contained 1 ml of erythrocyte suspension, 1 ml of bilirubin solution, 0.1–1.0 ml divalent cations with the remaining volume of 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl in a total volume of 6.0 ml. Erythrocytes were incubated first with increasing concentrations of divalent cations for 30 min at 37°C followed by further incubation with 72 mM bilirubin for 30 min at 37°C. The contents were centrifuged at 1575xg for 15 min and the supernatant was collected to measure the absorbance at 540 nm. The percentage of hemolysis was calculated as $A_2/A_1 \times 100$ where A_2 and A_1 are the absorbance values of calcium/divalent ion-induced hemolysate in presence or absence of bilirubin and hemolysate of erythrocytes with water in 6.0 ml after 1 h of incubation.

In another experiment, bilirubin concentration was increased by taking different volumes from a stock bilirubin solution to obtain the desired concentration of bilirubin in the range of 18–180 mM, while the concentration of calcium was kept constant (1.83 mM).

In the time-course experiment, erythrocytes were first incubated with a constant amount of calcium (1.83 mM) for half an hour, followed by further incubation with 72 mM bilirubin for varying time periods up to 120 min.

In the albumin protection experiment, varying volumes of stock albumin solution were added to get the final albumin concentration in the range of 24–96 mM before the addition of bilirubin to obtain different bilirubin/albumin molar ratios.

RESULTS

Human erythrocytes treated with different concentrations of CaCl_2 in the range 0.04–1.83 mM for 30 min at 37°C showed significant hemolysis upon further incubation with bilirubin (Fig. 1). There was a linear increase in hemolysis reaching a value of 47.3% at 0.22 mM CaCl_2 , beyond which it sloped off. At the highest CaCl_2 concentration (1.83 mM), the percentage of hemolysis was 64%. Calcium and bilirubin blanks, where the erythrocytes were treated with either bilirubin or CaCl_2 , produced insignificant (~3%) hemolysis. Values of percentage hemolysis shown in Fig. 1 were determined after subtracting

Table 1: Hemolytic potential of various divalent cations on human and porcine erythrocytes in the absence of bilirubin. Erythrocytes were incubated with chloride salts of divalent cations (1.83 mM) for 1 h at 37°C. Values are expressed as Mean $\pm$ SE

Bivalent cations (1830 μM)	% Hemolysis	
	Human	Porcine
CaCl_2	4.60 $\pm$ 0.12	3.51 $\pm$ 0.41
MnCl_2	15.38 $\pm$ 0.48	0.39 $\pm$ 0.05
BaCl_2	3.06 $\pm$ 0.07	0.48 $\pm$ 0.09
MgCl_2	2.52 $\pm$ 0.12	0.52 $\pm$ 0.05

Fig 1 : Calcium-induced bilirubin-dependent hemolysis in human (●) and porcine (○) erythrocytes. Erythrocytes were first incubated with different concentrations of CaCl_2 (0.040-1.83 mM) in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl for 30 min at 37°C followed by further incubation with 72 mM bilirubin for 30 min at 37°C. Percentage hemolysis was calculated in the same way as described in Materials and methods. Each point is the mean of two independent experiments in duplicate.

Fig 2 : Bilirubin dependence of calcium-induced hemolysis of human (●) and porcine (○) erythrocytes. Erythrocytes were first incubated with CaCl_2 (1.83 mM) for 30 min at 37°C in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl followed by further incubation with increasing concentrations of bilirubin (18-180 mM) for 30 min at 37°C. Each point is the mean of two independent experiments in duplicate.

the contribution of calcium and bilirubin blanks. In contrast, when porcine erythrocytes were treated with CaCl_2 and bilirubin exactly in the same manner, only 11% hemolysis was noted at the highest CaCl_2 concentration (1.83 mM). The percentage of hemolysis was even lesser at lower CaCl_2 concentrations (Fig. 1).

Figure 2 shows dependence of calcium-induced hemolysis of both mammalian erythrocytes tested on bilirubin concentration. Incubation of these erythrocytes at fixed CaCl_2 concentration (1.83 mM) for 30 min at 37°C followed by further incubation with increasing concentrations of bilirubin (18–180 mM) for 30 min at 37°C led to a marked increase in percentage hemolysis of human erythrocytes up to 72 mM bilirubin concentration. Increasing the bilirubin concentration from 72 to 180 mM resulted in a 1.4 fold increase in percentage of hemolysis. Porcine erythrocytes showed less hemolysis compared to human erythrocytes even at the highest bilirubin concentration (180 mM).

Time-course of hemolysis in the presence of fixed concentrations of CaCl_2 (1.83 mM) and bilirubin (72 μM) is shown in Fig 3. In these experiments, the first incubation time of erythrocytes with CaCl_2 was 30 min, whereas the second incubation time after the addition of bilirubin was varied from 0–120 min. Results showed the dependence of hemolysis on

incubation time with bilirubin as marked hemolysis (72%) was observed upon incubating calcium loaded human erythrocytes with bilirubin for 45 min. Further increase in incubation time up to 120 min led to 91% hemolysis. A similar effect of incubation time was also observed with porcine erythrocytes. However, the increase in percentage hemolysis was slow and steady as only 33% hemolysis occurred upon 120 min incubation time with bilirubin.

Figure 4A shows the effect of different concentrations of Ca^{2+} , Ba^{2+} , Mg^{2+} and Mn^{2+} in inducing hemolysis of human erythrocytes in the presence of bilirubin. Ba^{2+} and Mg^{2+} ions were least effective in inducing hemolysis as only 4–5% hemolysis was observed within the whole range of these cations' concentration. Mn^{2+} induced significant hemolysis which increased slowly with increasing concentration and reached 20% at the highest concentration, but the extent of hemolysis was low compared to the one observed with CaCl_2 (62%).

In contrast, porcine erythrocytes showed remarkable resistance towards cation induced bilirubin dependent hemolysis compared to human erythrocytes (Fig 4B). A maximum of 10% hemolysis was observed with the highest concentration of CaCl_2 (1.83 mM) used, which was significantly lower than the extent of hemolysis (62%) observed with human

Fig 3 : Effect of time on calcium-induced bilirubin-dependent hemolysis of human (●-●) and porcine (○-○) erythrocytes. Erythrocytes were first incubated with 1.83 mM CaCl_2 for 30 min at 37°C in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl followed by further incubation with 72 mM bilirubin at 37°C for 0–120 min. Each point is the mean of two independent experiments in duplicate.

erythrocytes. The other divalent cations remained ineffective in inducing hemolysis as a maximum of 3% hemolysis was observed with these cations.

Table 1 shows the results of cation-induced hemolysis of both human and porcine erythrocytes in the absence of bilirubin. Mn^{2+} was most effective in inducing hemolysis of human erythrocytes as nearly 15% hemolysis was observed at 1.83 mM concentration. The percentage of hemolysis of human erythrocytes in the presence of Ca^{2+} , Ba^{2+} and Mg^{2+} was 5%, 3% and 3% respectively. In case of porcine erythrocytes, CaCl_2 induced only 4% hemolysis whereas other cations failed to produce any significant hemolysis (< 1%).

The protective effect of albumin on calcium-induced bilirubin-dependent hemolysis of human and porcine erythrocytes was studied by adding different amounts of albumin to the mixture to get varying bilirubin/albumin (B/A) molar ratio in the range of 0.75–3.0. The concentrations of Ca^{2+} and bilirubin used in this experiment were 1.83 mM and 72 mM respectively, similar to those used in previous experiments. No significant hemolysis of both human and porcine erythrocytes was observed up to a B/A molar ratio 1:1 (Fig 5). In human erythrocytes there was a continuous increase in hemolysis on increasing the B/A molar ratio above 1:1. However, the maximum hemolysis which was observed above a B/A ratio of 3.0 was significantly lower than the one observed (~60%) in the experiment without albumin. In contrast, porcine

Fig 4 : Effect of various divalent cations on human (A) and porcine (B) erythrocytes in the presence of bilirubin. Erythrocytes were first incubated with chloride salts of divalent cations (0.183–1.83 mM) such as CaCl_2 (■-■), MnCl_2 (□-□), BaCl_2 (△-△) and MgCl_2 (▲-▲) for 30 min at 37°C followed by further incubation with 72 mM bilirubin for 30 min at 37°C. Each point is the mean of two independent experiments in duplicate.

erythrocytes were protected completely by albumin up to a B/A ratio of 2.0 as no hemolysis (< 1%) was observed under these conditions. Even at the highest B/A ratio (3.0), the percentage hemolysis was only about 3% which was much below the one observed with human erythrocytes.

DISCUSSION

Treatment of human erythrocytes with CaCl_2 followed by bilirubin resulted in significant hemolysis as 64% hemolysis was observed at 1.83 mM CaCl_2 and 72 mM bilirubin concentration. This was in accordance with a previous report showing 62% hemolysis under these conditions (28). Porcine erythrocytes showed significant difference from human erythrocytes in calcium-induced bilirubin-dependent hemolysis ($P < 0.0001$). Hemolysis observed with porcine erythrocytes was about 5.6 fold less than in human erythrocytes at the highest CaCl_2 concentration used in this

Fig 5 : Effect of albumin on calcium-induced bilirubin-dependent hemolysis of human (●-●) and porcine (○-○) erythrocytes.

Erythrocytes were first incubated with 1.83 mM CaCl_2 for 30 min at 37°C in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM NaCl followed by the addition of different concentrations of albumin and 72 mM bilirubin to get a desired bilirubin/albumin molar ratio in the range of 0.75-3.0 and incubated for 30 min at 37°C . Each point is the mean of two independent experiments in duplicate

study (Fig 1). Furthermore, increasing bilirubin concentration beyond 72 mM while keeping the CaCl_2 concentration constant or increasing the time of incubation with bilirubin beyond 30 min did not change the pattern of hemolysis in these erythrocytes. Porcine erythrocytes showed a greater resistance even at lower CaCl_2 concentration compared to human erythrocytes. Difference in the extent of hemolysis observed with these erythrocytes remained significant at higher bilirubin concentrations too (> 72 mM). While porcine erythrocytes showed 29% hemolysis at 180 mM bilirubin concentration, the hemolysis was about 51% higher with human erythrocytes at the same bilirubin concentration.

Similarly, when the time with bilirubin was increased up to 120 min, human erythrocytes showed 58% more hemolysis compared to porcine erythrocytes. All these results reveal that porcine erythrocytes display a greater resistance of calcium-induced bilirubin-dependent hemolysis. A comparison of the patterns obtained in Figs. 2 and 3 with that of Fig. 1 show that differences in the behavior of porcine and human erythrocytes towards calcium-induced bilirubin-dependent hemolysis were more oriented towards bilirubin toxicity rather than calcium-induced phenomenon. This can be inferred from the observation that hemolysis of porcine erythrocytes became more significant (29–33%) only upon increasing either the concentration of bilirubin or time of incubation with bilirubin.

Although Ba^{2+} , Mg^{2+} , and Ca^{2+} have been reported to induce membrane aggregation through intramolecular chelation of phospholipid molecules (30), both Ba^{2+} and Mg^{2+} failed to induce hemolysis of human and porcine erythrocytes in the presence of bilirubin. Taking into account the hemolytic potential of Ca^{2+} , Mn^{2+} , Ba^{2+} and Mg^{2+} in the absence of bilirubin (Table 1) and their comparison to the hemolysis induced by these cations in the presence of bilirubin (Figs. 4a and 4b), it can be said that bilirubin-dependent hemolysis of these erythrocytes is calcium ion specific. The prominent role played by bilirubin in this hemolytic phenomenon was clearly reflected from the results of the albumin protection experiment (Fig 5) where no hemolysis was observed at a B/A ratio of 1:1 or less.

The greater extent of hemolysis observed with human erythrocytes compared to porcine erythrocytes can be attributed to various factors. Differences in the phospholipid and protein make-up (31–33) of human and porcine erythrocytes may account for the differences in hemolysis exhibited by these erythrocytes. As regards the phospholipid composition of the outer surface of these erythrocytes, porcine erythrocytes have been reported to possess a higher sphingomyelin (Sph) to phosphatidylcholine (PC) ratio (Sph/PC), i.e. (1.13) compared to the value (0.64) reported for human erythrocytes (32). In an earlier study, it has been shown that erythrocytes with high molar fraction of PC are more susceptible to lysis by bile salts than those with the high molar fraction of Sph in the total cholinephospholipids (34). In view of this, it appears that human erythrocyte membranes are more fluid compared to porcine erythrocytes and therefore the differences in the susceptibility to bilirubin-induced lysis may be ascribed to the differences in membrane fluidity.

Ca^{2+} -induced phase separation (30, 35) may also account for the differences in hemolysis observed with these erythrocytes in presence of bilirubin. Porcine erythrocytes are less sensitive than human erythrocytes towards various Ca^{2+} evoked effects (36). Porcine erythrocytes also differ markedly from human erythrocytes in the protein make-up of their membranes especially in the spectrin (bands 1 and 2), which is more abundant in porcine erythrocytes (33). Furthermore, several additional proteins such as band 2.3 and band 8 have been reported in porcine erythrocyte membranes (33). Increase in these cytoskeletal components in porcine erythrocyte membranes may provide greater protection to porcine erythrocytes towards calcium-induced bilirubin-dependent changes. More studies are required to explain the mechanism of calcium-induced bilirubin-dependent hemolysis and the differences shown by different erythrocytes towards

this phenomenon. Based on the above results, porcine can not be selected as a suitable animal model for developing preventive measures against bilirubin encephalopathy.

ACKNOWLEDGEMENTS

The facilities provided by the University of Malaya are gratefully acknowledged. The kind support of Tip-Top Meat Sdn. Bhd. in allowing us to collect fresh porcine blood is also acknowledged.

REFERENCES

1. Ostrow JD, Pascolo L, Shapiro SM, Tiribelli C. New concepts in bilirubin encephalopathy. *Eur J Clin Invest* 2003; 33: 988–97.
2. Ostrow JD, Mukerjee P, Tiribelli C. Structure and binding of unconjugated bilirubin: Relevance for physiological and pathophysiological function. *J Lipid Res* 1994; 35: 1715–37.
3. Zucker SD, Goessling W, Hoppin AG. Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and native hepatocyte membranes. *J Biol Chem* 1999; 274: 10852–62.
4. Karp WB. Biochemical alterations in neonatal hyperbilirubinemia and bilirubin encephalopathy: A review. *Pediatrics* 1979; 64: 361–8.
5. Lanone S, Bloc S, Foresti R, Almolki A, Taille C, Callebort J, Conti M, Goven D, Aubier M, Dureuil B, El-Benna J, Motterlini R, Boczkowski J. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB J* 2005; 19: 1890–2.
6. Rosenstein BS, Ducore JM, Cummings SW. The mechanism of bilirubin–photosensitized DNA strand breakage in human cells exposed to phototherapy light. *Mutat Res* 1983; 112: 397–406.
7. Grojean S, Koziel V, Vert P, Daval J. Bilirubin induces apoptosis via activation of NMDA receptors in developing rat brain neurons. *Exp Neurol* 2000; 166: 334–41.
8. Rodrigues CMP, Solá S, Castro RE, Laires PA, Brites D, Moura JJG. Perturbation of membrane dynamics in nerve cells as an early event during bilirubin induced apoptosis. *J Lipid Res* 2002a; 43: 885–94.
9. Rodrigues CMP, Sola S, Brito MA, Brites D, Moura JJG. Bilirubin directly disrupts membrane lipid polarity and fluidity, protein order and redox status in rat mitochondria. *J Hepatol* 2002b; 36: 335–41.
10. Zhang L, Liu W, Tanswell AK, Luo X. The effects of bilirubin on evoked potentials and long-term potentiation in rat hippocampus *in vivo*. *Pediatr Res* 2003; 53: 939–44.
11. Ostrow JD, Pascolo L, Brites D, Tiribelli C. Molecular basis of bilirubin–induced neurotoxicity. *Trends in Mol Med* 2004; 10: 65–70.
12. Brito MA, Brites D, Butterfield DA. A link between hyperbilirubinemia, oxidative stress and injury to neocortical synaptosomes. *Brain Res* 2004; 1026: 33–43.
13. Bratlid D. Bilirubin binding by human erythrocytes. *Scand J Clin Lab Invest* 1972; 29: 91–7.
14. Sato H, Aono S, Semba R, Kashiwamata S. Interaction of bilirubin with human erythrocyte membranes. Bilirubin binding to neuraminidase– and phospholipase– treated membranes. *Biochem J* 1987; 248: 21–6.
15. Hayer M, Piva MT, Sieso V, de Bornier BM. Experimental studies on unconjugated bilirubin binding by human erythrocytes. *Clin Chim Acta* 1989; 186: 345–50.
16. Roll EB, Christensen T, Gederaas OA. Effects of bilirubin and phototherapy on osmotic fragility and haematoporphyrin-induced photohemolysis of normal erythrocytes and spherocytes. *Acta Paediatr* 2005; 94: 1360–2.
17. Ali MK, Tayyab S. Binding of bilirubin to erythrocytes from different mammalian species. *Comp Biochem Physiol A* 1995; 111: 507–9.
18. Ali MK, Tayyab S. Calcium–induced bilirubin–dependent hemolysis of human erythrocytes. *Biochim Biophys Acta* 1997; 1326: 124–30.
19. Ali MK, Tayyab S. Effect of phospholipase C, trypsin and neuraminidase on binding of bilirubin to mammalian erythrocyte membranes. *Comp Biochem Physiol A* 2001; 129: 355–62.
20. Brito MA, Silva RFM, Matos DC, da Silva AT, Brites DT. Alteration of erythrocyte morphology and lipid composition by hyperbilirubinemia. *Clin Chim Acta* 1996; 249: 149–65.
21. Brito MA, Silva RFM, Brites D. Bilirubin induces loss of membrane lipids and exposure of phosphatidylserine in human erythrocytes. *Cell Biol Toxicol* 2002; 18: 181–92.
22. Brito, MA, Silva RFM, Brites D. Bilirubin toxicity to human erythrocytes: A review. *Clin Chim Acta* 2006; 374: 46–56.
23. LaCelle PL. Alteration of membrane deformability in hemolytic anemias. *Semin Hematol* 1970; 7: 355–71.
24. Palek J, Stewart G, Lionetti FJ. The dependence of shape of human erythrocyte ghosts on calcium, magnesium and, adenosine triphosphate. *Blood* 1974; 44: 583–97.
25. Carraway KL, Triplett RB, Anderson DR. Calcium–promoted aggregation of erythrocyte membrane proteins. *Biochim Biophys Acta* 1975; 379: 571–81.
26. Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF. Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. *Biochemistry* 1992; 31: 6355–60.

27. Hoffman JF. On the mechanism and measurement of shape transformations of constant volume of human red blood cells. *Blood Cells* 1987; 12: 565–88.
28. Ali MK, Tayyab S. Differential resistance to calcium–induced bilirubin–dependent hemolysis in mammalian erythrocytes. *Comp Biochem Physiol C* 1999; 122: 109–13.
29. Malloy HT, Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. *J Biol Chem* 1937; 119: 481–90.
30. Ito T, Ohnishi S, Ishinaga M, Kito M. Synthesis of a new phosphatidylserine spin–label and calcium–induced lateral phase separation in phosphatidylserine–phosphatidylcholine membranes. *Biochem* 1975; 14: 3064–9.
31. Hamazaki H, Hotta K, Konishi K. Comparative studies of human, equine, porcine and bovine erythrocyte membrane sialoglycoproteins. *Comp Biochem Physiol B* 1976; 55: 37–44.
32. Barenholz Y, Thompson TE. Sphingomyelins in bilayers and biological membranes. *Biochim Biophys Acta* 1980; 604: 129–58.
33. Bewaji CO, Olorunsogo OO, Bababunmi EA. Comparison of the membrane–bound (Ca^{2+} – Mg^{2+})–ATPase in erythrocyte ghost from some mammalian species. *Comp Biochem Physiol B* 1985; 82: 117–22.
34. Lowe PJ, Coleman R. Membrane fluidity and bile salt damage. *Biochim Biophys Acta* 1981; 640: 55–65.
35. Ohnishi S, Ito T. Calcium–induced phase separation in phosphatidylserine–phosphatidylcholine membranes. *Biochem* 1974; 13: 881–7.
36. Allan D, Michell RH. Calcium ion–dependent diacylglycerol accumulation in erythrocytes is associated with microvesiculation but not with efflux of potassium ions. *Biochem J* 1977; 166: 495–9.