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Calcium enhances the hemolytic action of bile salts

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The lysis of human erythrocytes by bile salts in buffer containing isotonic saline was dramatically enhanced by the addition of 5–10 mM calcium chloride. All bile acids tested showed this effect, with a marked increase in lysis occurring at 0.75 mM for deoxycholate, 1 mM for chenodeoxycholate, 2.5 mM for ursodeoxycholate and 5.5 mM with cholate in the presence of 10 mM calcium chloride. The effect appeared to be specific for calcium; strontium chloride and magnesium chloride gave no stimulatory effect. The increased lysis of the erythrocytes in the presence of 1 mM deoxycholate and 1–10 mM calcium chloride was not associated with increased uptake of the bile salt by the cells (measured with [^{14}C]deoxycholate). Using erythrocytes previously labelled with [^{3}H]cholesterol, there was no evidence of an enhanced removal of that membrane component in the presence of calcium and deoxycholate, compared to deoxycholate alone. The sensitivity of the cells to the effect of calcium in the presence of 1 mM deoxycholate increased with the length of time of their storage at 4°C. The sensitivity returned to that of fresh cells after incubation at 37°C with 30 mM adenosine plus 25 mM glucose, but this treatment did not further diminish the lysis. Lysis in the presence of 10 mM calcium chloride and 1 mM deoxycholate was partially blocked by increasing the KCl concentration at the expense of NaCl. The maximum effect occurred with a buffer comprising 100 mM KCl/50 mM NaCl. A more dramatic reduction in the lysis followed the incorporation of the calcium chelator, quin2, into the cells. The lysis induced by 1 mM deoxycholate in the presence of calcium was reduced by 80% in quin2-loaded cells compared to controls. The data suggest that bile acids can promote the influx of calcium into erythrocytes, leading to lysis as a result of the efflux of intracellular potassium and/or the uptake of sodium from the incubation medium. The data further suggest that cellular effects may occur at lower bile acid concentrations than that thought to be required for detergent damage.

Introduction

Bile salts are potent in promoting damage to cell membranes and are lytic to many cell types, including erythrocytes [1–3], dispersed intestinal villus cells [4] and isolated hepatocytes [5]. At concentrations of 1 to 20 mM, solubilization of membrane lipid and protein is known to precede the destruction of the cell [2,3,5] but, with the exception of the intestine, few tissues are exposed to these levels of bile acids under normal conditions. Bile acids in blood at concentrations of less than 1 mM are not uncommon [6,7], however, and the cells of many tissues may encounter low levels of these detergents via that medium. Similarly, the colonic epithelium is continually exposed to low concentrations of bile acids in the aqueous phase of the colonic content [8].

The detergent activity of even the most potent

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bile acids is weak at concentrations less than 1 mM [2,3,5]. Many bile acids, however, have been shown to bind calcium at these levels [9–11] and facilitate its transfer across lipid bilayer membranes. This has been demonstrated using black lipid membranes [12], lipid vesicles [13] and purified jejunal brush-border membranes isolated from pig intestine [14]. In view of the sensitivity of cells to the levels of intracellular calcium, the ionophore activity of bile salts may be expected to influence cell behaviour or induce damage at concentrations much lower than those required for solubilization of membrane components.

The aim of this study, therefore, was to explore cellular effects which may occur as a result of the interaction of various bile salts with divalent cations. We have chosen to use human erythrocytes as a model system because of the simplicity of the membrane system and the ease of measurement of cell lysis. We report here that cell lysis induced by bile salts is markedly enhanced by 5–10 mM calcium, suggesting that, under some conditions, cellular damage may occur at lower bile salt concentrations than previously suspected.

Materials and Methods

All bile acids, quin2-AM, pyruvic acid and adenosine were purchased from Sigma Chemical Co. (St. Louis, MO) in the highest purity available and were used without further purification. The purity of the bile acids was confirmed by gas-liquid chromatography as the methyl ester acetates, as previously described [15]. The deoxycholic, chenodeoxycholic and cholic acid preparations contained no detectable impurities. The ursodeoxycholic acid contained less than 1% chenodeoxycholic acid, while the lithocholate preparation contained trace amounts of cholanic acid. Calcium chloride (dihydrate), magnesium chloride (hexahydrate) and strontium chloride (hexahydrate) were obtained from BDH Chemical Co. (Toronto, Canada) in the highest purity available. [^{14}C]Deoxycholic acid and [1,2(n)-^{3}H]cholesterol were purchased from Amersham International, U.K.

Solutions of each bile acid were made up in a buffer comprising 150 mM NaCl/10 mM Tris-HCl (pH 7.4)/25 mM glucose (referred to as buffer throughout the manuscript unless otherwise indicated). These were then diluted 1 : 1 with buffer solutions containing the appropriate concentrations of divalent cation.

Human erythrocytes were obtained from fresh blood drawn by venipuncture and using EDTA as anticoagulant. The cells were washed twice with 5 vol. buffer before use. The cells were incubated at 37°C at 10% hematocrit in the solutions of bile salt and divalent cation. After 30 min of incubation, the samples were centrifuged 2 min at 8000 × *g* and the extent of lysis was determined by comparing the absorbance (418 nm) of a 100-fold diluted sample of the supernatant with that of red cells lysed totally in distilled water.

The uptake of radiolabelled deoxycholate and the release of [^{3}H]cholesterol by the erythrocytes was determined using a modification of a method previously described for cholesterol [16,17]. Erythrocyte cholesterol was labelled using heat-treated plasma containing trace amounts of [^{3}H]cholesterol as previously described [17]. Deoxycholate and [^{14}C]deoxycholate (0.5 μCi/mg) were mixed in a methanolic solution and the solvent was subsequently evaporated. Buffer was added to give a final concentration of 2 mM. The red cells were incubated as before in this solution and centrifuged after 30 min of incubation. The supernatant was transferred directly into a liquid scintillation vial followed by 10 ml Optifluor (United Technologies Packard). The cells were lysed with 2 vol. water and extracted with 50 vol. isopropanol. The extract was transferred directly into a scintillation vial followed by 10 ml of the scintillation cocktail. The number of counts was determined in a Packard liquid scintillation counter and the data were expressed as the ratio of counts present in the cell fraction vs. those in the total (cells plus supernatant) sample after an incubation of 30 min.

To measure the influence of intracellular calcium, populations of human red cells were loaded with quin2-AM according to the method of Tsien et al. [18] before the lysis induced by deoxycholate was measured. Quin2-AM suspensions were prepared by injecting 0–50 μl volumes of a 50 mg/ml solution of the compound in dimethylsulphoxide into rapidly stirred buffer comprising 100 mM KCl/50 mM NaCl/10 mM Tris/25 mM glucose/10 mM pyruvic acid/10 mM adenosine.

Freshly isolated erythrocytes were incubated in these solutions at 10% hematocrit for 2 h at 37°C. After the incubation the cells were centrifuged and washed several times in buffer. The entry of quin2-AM into the cells and its hydrolysis to the tetracarboxylate form was confirmed by measuring the fluorescence of a cell lysate with and without calcium chloride. The measurements were obtained by irradiating solutions containing 1 μl of packed erythrocytes per ml buffer with light having an excitation wavelength of 340 nm on a Cary 219 ultraviolet spectrophotometer (Varian Associates, Sunnyvale, CA, U.S.A.) modified to measure fluorescence.

Results

Erythrocytes incubated in buffer containing 1 mM deoxycholate show lysis that increases linearly with time and reaches about 5% after 30 min. Fig. 1 shows that the lysis occurring under these conditions was markedly stimulated by the addition of calcium chloride. The maximum effect occurred at calcium concentrations of 8–10 mM. Similar results are found whether the bile salt and divalent cation are pre-mixed as aqueous solutions or the bile salt is aliquotted as a lipid film and the buffer is added to it. In either case, there was no evidence of cloudiness in the solutions or precipitation of the detergent. There was no enhancement of the lysis when the cells were first treated with bile salt followed by calcium chloride after washing away the detergent, or vice versa. Calcium chloride alone had no measurable effect on the cell lysis. Strontium or magnesium chlorides had no detectable effect in the presence of 1 mM deoxycholate.

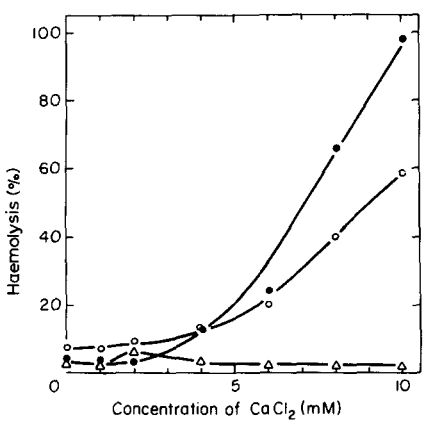

Fig. 1. Erythrocyte lysis in the presence of bile acids and calcium. Human erythrocytes were incubated for 30 min at 37°C in buffer (150 mM NaCl/10 mM Tris/25 mM glucose (pH 7.4)) containing the indicated amounts of calcium chloride and 1 mM concentrations of sodium deoxycholate (●), sodium taurodeoxycholate (○) or sodium taurocholate (△). The haematocrit was 10%. Haemolysis was determined as described in Materials and Methods.

Fig. 1 shows that at bile acid concentrations of 1 mM, the stimulatory effect of calcium was evident with both deoxycholate and taurodeoxycholate. The effect was less marked with the taurine-conjugated salt. At the 1 mM level the lysis induced by taurocholate, cholate or ursodeoxycholate was not stimulated by calcium ion. Lithocholate similarly gave no effect, but the difficulty in solubilizing this species prevented the drawing of firm conclusions.

To study the concentration of bile acid at which the maximum lytic effect occurred in the presence of 10 mM calcium chloride, erythrocytes were incubated with either deoxycholate, chenodeoxycholate, ursodeoxycholate or cholate over a wide range of concentrations. The data presented in Fig. 2A show that in the absence of calcium, the three dihydroxy bile salts tested lysed the cells at much lower concentrations than the trihydroxy, cholate. Chenodeoxycholate and deoxycholate behaved similarly and induced cell lysis at lower concentrations than ursodeoxycholate. With the addition of 10 mM calcium chloride, shown in Fig. 2B, the concentration of bile salt at which the lysis occurs was reduced in each case. The most dramatic enhancement occurred with cholate, but it is interesting that maximal lysis occurred with deoxycholate at concentrations as low as 1 mM.

We reasoned that one rationale for the enhanced lysis was the stimulation of bile acid uptake or a greater release of membrane components such as cholesterol in the presence of calcium. To test this, we measured directly the uptake of [^{14}C]deoxycholate by cells prelabelled with [^{3}H]-cholesterol. The results, shown in Fig. 3, demonstrate that the uptake of the labelled bile salt was linearly dependent on the concentration in the absence of divalent cation. At no concentration was the uptake enhanced by the inclusion of 10 mM calcium in the incubation fluid. The apparent

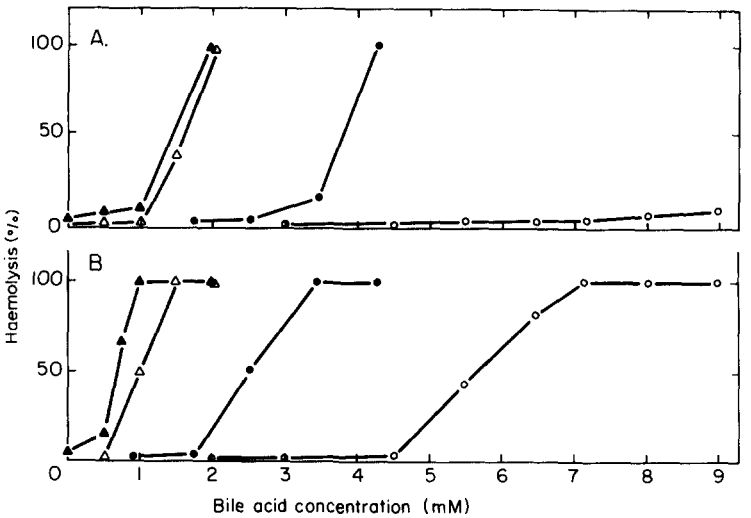

Fig. 2. Erythrocyte lysis vs. bile acid concentration in the presence or absence of calcium chloride. Erythrocytes were incubated for 30 min at 37°C in buffer containing the indicated amounts of deoxycholate (▲), chenodeoxycholate (△), ursodeoxycholate (●) or cholate (○) with no calcium chloride (A) or with 10 mM calcium chloride (B). Other conditions were as in Fig. 1.

reduction in the uptake at higher concentrations is likely to be the result of the onset of haemolysis, which can be seen in Fig. 3C, and a smaller resultant cell pellet. Fig. 3B shows the fraction of labelled cholesterol that is present in the cell pellet after the incubation. These data indicate that the cell lysis is not preceded by a greater efflux of cholesterol from the membrane in the presence of calcium. As before, the greater efflux apparent at 0.6 and 0.8 mM deoxycholate concentrations in the presence of the divalent cation may be explained by the onset of cell disintegration.

Fig. 4 shows the deoxycholate/calcium-induced lysis in cells stored for different times at 4°C in plasma containing EDTA. It is evident that the sensitivity of the cells to the lytic effect of 1 mM deoxycholate in the presence of calcium becomes greater with the length of time they are stored. This increased sensitivity corresponded to an increased cellular osmotic fragility (data not shown). There was no significant difference in the bile acid-induced lysis occurring in the absence of calcium under these conditions.

As one of the cellular changes known to accompany storage of erythrocytes is the depletion of their ATP supply [19], we wished to know whether replenishment of cellular ATP would prevent the lysis. Fig. 4 shows that treatment of 15-day-old cells with 30 mM adenosine and 25 mM glucose restored their sensitivity to that of fresh cells but did not completely inhibit the lysis. Treatment of erythrocytes for 3 h in the presence of adenosine

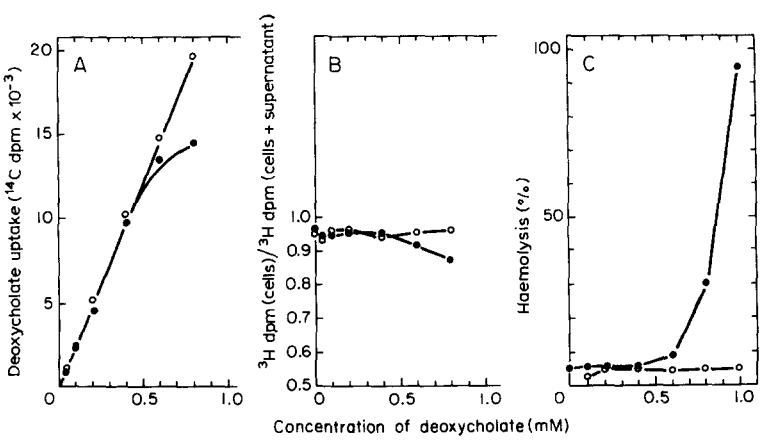

Fig. 3. Uptake of deoxycholate and release of membrane cholesterol from erythrocytes in the presence and absence of calcium. Erythrocytes possessing [^{3}H]cholesterol were incubated for 30 min at 37°C with the indicated concentrations of [^{14}C]deoxycholate in buffer with (●) or without (○) 10 mM calcium chloride. ^{3}H-labelled cells were prepared as described in Materials and Methods. The haematocrit was 10%. All other analytical manipulations are described in Materials and Methods. (A) Uptake of [^{14}C]deoxycholate by erythrocytes. (B) Fraction of erythrocyte membrane cholesterol remaining in the cells after incubation with deoxycholate. (C) Cell lysis.

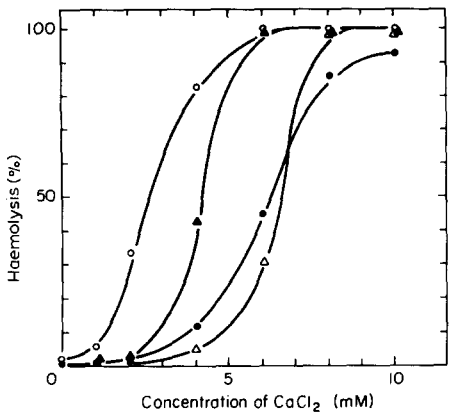

Fig. 4. Effect of cell storage on sensitivity to lysis by bile acids in the presence of calcium chloride. Incubations were carried out with 1 mM sodium deoxycholate as described in the legend to Fig. 1, using either freshly obtained erythrocytes (△), the same cells after storage for 10 days at 4°C with EDTA-containing plasma (▲), the same cells stored for 15 days at 4°C (○), or 15-day-old cells previously treated for 3 h in buffer at 37°C with 30 mM adenosine (●).

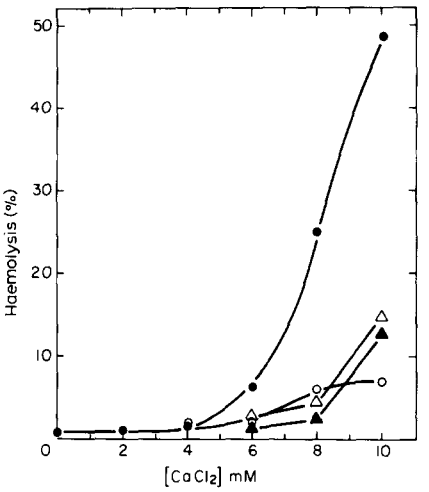

Fig. 5. The influence of intracellular quin2 on cell lysis induced by deoxycholate and calcium chloride. Fresh human erythrocytes were loaded with the chelator by incubating in buffer containing 1.0 mM (△), 0.5 mM (○), 0.25 mM (▲) or no quin2-AM (●) as described in Materials and Methods. The lysis induced by 1 mM deoxycholate in the presence of calcium chloride was determined as described in the legend to Fig. 1.

and glucose has been shown [19] to restore their ATP stores to the level found in freshly-isolated cells.

To determine whether the calcium-stimulated lysis required the entry of the cation into the erythyrocytes, we tested whether the intracellular chelator, quin2, would block the effect. Under the loading conditions used (0–1.0 mM quin2-AM, 10% v/v cells), intracellular levels of 0–10 mM quin2 were theoretically possible and we were able to demonstrate a calcium-sensitive fluorescence in the cells that responded to an excitation wavelength of 340 nm as reported [18]. This confirmed that the chelator had entered the cells and had been converted to the tetracarboxylate form. We did not attempt to quantitate further the amount of quin2 inside the cell.

The results obtained upon incubating these cells with 1 mM deoxycholate and calcium chloride, shown in Fig. 5, demonstrate that the lysis of the cells containing the chelator is much less than that of the control erythrocytes. It is evident from a comparison of these data and those in Figs. 1 and 4 that the degree of lysis in control cells, not loaded with quin2-AM but incubated in the same buffer, was reduced by 50% compared to freshly isolated cells. We attribute this to a protective effect of the pyruvate and adenosine that were added to the loading buffer to counteract the toxicity of formaldehyde that is released during the intracellular hydrolysis of quin2-AM [20].

The data shown in Fig. 5 demonstrate that the haemolysis is probably the result of the influx of calcium into the cell. One known effect of high

TABLE I

EFFECT OF BUFFER COMPOSITION ON ERYTHROCYTE LYSIS INDUCED BY BILE ACIDS AND CALCIUM CHLORIDE

Incubation solutions contained 1 mM sodium deoxycholate, 10 mM Tris, 25 mM glucose, 10 mM calcium chloride and KCl and NaCl at the concentrations indicated. The lysis of fresh human erythrocytes was determined after 30-min incubations at 37°C. All other conditions were as described in the legend to Fig. 1.

Salt concentration (mM)		Haemolysis (%)
NaCl	KCl	
150	0	100
100	50	88.8
50	100	23.6
25	125	64.7
0	150	78.3

intracellular calcium concentrations in erythrocytes is the stimulation of potassium efflux and sodium uptake [21], which may lead to cell lysis. If this were true in our case, then the lysis should be sensitive to the concentration of potassium in the incubation buffers. Table I shows that increasing the KCl content of the buffer at the expense of NaCl does decrease the haemolysis induced by 1 mM deoxycholate in the presence of 10 mM calcium chloride. A minimum was reached when the buffer comprised 100 mM KCl/50 mM NaCl. The buffer composition had no effect on the bile-salt-induced lysis in the absence of calcium chloride.

Discussion

In this report we demonstrate that the lysis of erythrocytes by bile acids is enhanced by the presence of calcium chloride in the incubation fluid. Strontium chloride and magnesium chloride did not stimulate cell lysis under the same conditions. The effect is manifest at calcium concentrations of greater than 5 mM (Fig. 1), while the concentration of bile acid required for half-maximal lysis depends on the structure: 0.7 mM for deoxycholate; 1.0 mM for chenodeoxycholate; 2.5 mM for ursodeoxycholate and 5.7 mM for cholate. This compares with 1.5, 1.5, 3.8 and over 10 mM for half-maximal lysis induced by deoxycholate, chenodeoxycholate, ursodeoxycholate and cholate, respectively, without calcium. Erythrocytes were not lysed by 1 mM solutions of lithocholate with or without calcium. Oelberg and co-workers [11] reported preliminary observations that 2 mM lithocholate in the presence of calcium ion promotes lysis in human erythrocytes. We were unable to achieve optically clear solutions containing 2 mM levels of this bile acid, perhaps accounting for the difference in our results.

The results suggest that cell lysis is the result of the influx of large amounts of calcium mediated by the bile acid. The lysis is probably not due to an enhancement of the detergent action of the bile acid brought about by the divalent cation. There are three reasons for this belief: firstly, we found no evidence for a greater uptake of labelled bile acid in the presence of calcium (Fig. 3); secondly, there was no evidence of a greater removal of membrane cholesterol prior to lysis in the calcium/bile acid-containing solutions; thirdly, the cell lysis was not enhanced by calcium when the erythrocytes were treated first with bile acid; then subsequently with calcium chloride after washing the detergent away. The events leading to cell lysis occurred only when the detergent and the divalent cation were present together. Thus, removal of membrane protein or phospholipid which may occur at these concentrations of bile acid [2,3] is probably not responsible for the effect of calcium in this case. Alteration of the cholesterol/phospholipid ratio has been shown to influence the rate of influx of calcium into erythrocytes [22], but by similar reasoning an effect of this parameter in the present experiments should have been evident when the cells were first treated with bile acid, then with calcium.

It has been shown by many workers that bile acids possess the ability to bind calcium [9–11] and facilitate the transport of that ion across lipid membranes [12–14]. A positive indication that bile salts promoted the cellular uptake of calcium in our experiments was the blockage of the lysis by the intracellular calcium chelator, quin2. At the highest calcium concentration used, the lysis induced by 1 mM deoxycholate was reduced from 50% to about 10%. Evidently, even at the lowest quin2 loading, which could have theoretically reached 2.5 mM inside the cells, the chelator provided enough intracellular buffering capacity to reduce the lysis significantly. At the low levels of quin2 used, it is unlikely that the reduced lysis is the result of the immobilization of extracellular calcium by quin2 released from damaged cells.

The intracellular quin2 did not completely block the haemolysis. This was probably not due to formaldehyde generated during the hydrolysis of the acetoxymethyl ester, since pyruvate, which has been shown to block the toxic effects of the aldehyde [23], was included in the buffers used during the loading of the cells. Furthermore, damage induced by formaldehyde would be expected to have been greater with the higher quin2 loadings, and that was not the case. It may be, however, that the chelator itself or its calcium complex are toxic to the cells. Tiffert et al. [20] have shown that human erythrocytes loaded with quin2 accumulate much higher concentrations of

calcium in the presence of an ionophore than cells not containing the chelator. The loaded cells also showed an increased passive permeability to calcium [20]. The inability of quin2 to completely block the lysis may be a result of the accumulation of these abnormally high concentrations of calcium. Tsien [24] proposed a similar explanation to account for the ability of an intracellular chelator similar to quin2 to delay, but not completely block, the efflux of potassium from human erythrocytes after their exposure to the calcium ionophore, A23187. Increased intracellular calcium levels are known to lead to the activation of channels allowing the efflux of potassium and the influx of sodium into erythrocytes [21].

The latter phenomenon may underlie the calcium-stimulated haemolysis that occurs in our experiments. The evidence for this supposition is the marked diminution of cell lysis that accompanies the introduction of KCl into the incubation buffer (Table I). Increasing the concentration of KCl outside the cell would be expected to minimize the effects of a depletion of cytosolic potassium. Davson [25] reported that high levels of intracellular calcium increased the efflux of potassium from human erythrocytes and led to cell lysis. Strontium and magnesium, however, did not enhance potassium efflux. This finding may explain why these cations did not lead to cell lysis in the presence of bile acids in our experiments. It is probable that the uptake of strontium and magnesium was also stimulated by the detergents, but their influence on the erythrocytes was less damaging.

The relevance of these findings to cells in contact with bile salts and calcium in vivo remains to be established. Under normal conditions, erythrocytes in the blood stream are likely to be protected against the lytic effects of elevated bile salt levels by albumin. There is typically 5 mM calcium present in human serum, however, and it is of interest to note that morphological changes in erythrocytes have been noted with serum sodium taurocholate concentrations as low as 0.2 mg/ml (approx. 0.4 mM) after long term exposures in vitro [6] and with levels as low as 15 μM lithocholate in monkeys [26]. The findings may also bear on the situation in the colon, where epithelial cells are exposed continuously to aqueous phases which contain up to 30 mM calcium and magnesium [27] but which rarely contain greater than 1 mM total bile acid [8], mostly deoxycholate. The results presented here suggest that cellular damage may occur under these conditions.

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