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MODE OF TRANSPORT AND POSSIBLE MECHANISM OF ACTION OF L-PHENYLALANINE BENZYL ESTER AS AN ANTI-SICKLING AGENT

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L-Phenylalanine benzyl ester (Phe-Bz) and a number of ester analogues prevent sickling of erythrocytes from sickle cell disease patients. The compounds tested exhibit anti-sickling activity in the concentration range 0.5–3.0 mM. A general feature of these compounds is the presence of two aromatic rings in their molecular structure. The anti-sickling agents rapidly enter the erythrocyte and are hydrolysed to their component molecules. Incubation of human erythrocytes with 3.0 mM L-phenylalanine for 30 min at 37°C results in accumulation of 2.0 mmol L-phenyalanine/l cells, while incubation of erythrocytes with 3.0 mM Phe-Bz under similar conditions results in the production of 4.0 mmol L-phenylalanine/l cells and an equivalent amount of benzyl alcohol. Both L-phenylalanine and benzyl alcohol are inhibitors of the gelation of deoxyhaemoglobin S (deoxy-HbS) in vitro. Moreover, Phe-Bz and related anti-sickling agents fluidize the lipid bilayer of the erythrocyte membrane, inhibiting several transport systems, including those for L-phenylalanine, uridine and sulphate ions, as well as the Na^+ pump and the Na^+/K^+ cotransporter, but increasing the passive influx and efflux of both cations and anions. The accumulation of Phe-Bz hydrolysis products within the erythrocyte together with the effects of Phe-Bz on cation permeability result in the influx of water causing the cell to swell. Thus, treatment of erythrocytes with 3.0 mM Phe-Bz at 37°C for 30 min causes an increase in mean cell volume of 14.8%, decreasing the mean intracellular haemoglobin concentration from 34 to 29.6 g%. The increase in cell volume caused by Phe-Bz and its analogues together with the direct effects of their hydrolysis products on HbS probably act in concert to bring about the anti-sickling effect.

Introduction

Sickle cell disease causes considerable morbidity and, in a poor socio-economic environment, shortening of life. The primary cause of the disease is a mutation in the sixth position of the haemoglobin β-chain where an L-glutamic acid residue has been replaced by an L-valine. This substitution causes a drastic reduction in the solubility of sickle cell haemoglobin (HbS) when deoxygenated. Under these conditions, the HbS molecules polymerise to form intracellular fibres which are responsibe for the deformation of the disc biconcave erythrocyte into a sickle shape [1,2]. In vivo, sickled erythrocytes tend to block capillaries, causing stasis, and thereby starve organs of both nutrients and oxygen and eventually cause hypofunction or complete tissue destruction.

At the present time, the most popular approach to prevent or reverse sickling is to employ compounds which directly affect the haemoglobin

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molecule [3–6]. However, there are several other compounds which prevent sickling by affecting the erythrocyte membrane [7,8], causing an increase in the cell volume of the erythrocyte and thus reducing the intracellular haemoglobin concentration below its minimum gelling concentration [9].

Recent experiments from this laboratory have shown that amino acid benzyl esters have anti-sickling activity [10]. One of this class of compounds, L-phenylalanine benzyl ester (Phe-Bz), is effective at a low concentration and is therefore a potential therapeutic agent for the treatment of sickle cell disease. This report describes an investigation of its mechanism of action.

Materials and Methods

Materials. L-[U-^{14}C]Phenylalanine (500 mCi/mmol), ^{24}Na (2 m Ci/ml), ^{86}Rb (12 mCi/mg Rb) and $Na_2^{35}SO_4$ (276.6 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. L-[U-^{14}C]Phenylalanine benzyl ester was chemically synthesised from L-[U-^{14}C]phenylalanine and non-radioactive benzyl alcohol and purified as previously described [10]. All other chemicals were of analytical grade obtained from Frutarom Ltd. Haifa, Israel, British Drug House Ltd., Poole, U.K., Sigma (London) Chemical Co., U.K. and Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Preparation of erythrocytes. Whole blood samples from sickle cell disease patients and normal subjects were collected into heparinized tubes. Sheep blood samples were obtained from normal animals maintained by The A.R.C. Institute of Animal Physiology, Babraham, Cambridge, U.K. Cells were washed with 20 vol. of ice-cold incubation medium containing 15 mM Tris-HCl, 135 mM NaCl, 5 mM KCl, 3.1 mM $MgCl_2$, 5 mM glucose and 0.1 mM EDTA, pH 7.2. The buffy coat was discarded.

Anti-sickling and desickling experiments. To investigate anti-sickling activity, various amino acid esters at concentrations of up to 5 mM were incubated with 10% suspensions of sickle cell erythrocytes at 37°C. At various time intervals aliquots were removed and deoxygenated with a 2% sodium metabisulphite solution and examined under a Zeiss microscope. In desickling experiments the erythrocytes were deoxygenated by incubating the cells under a stream of nitrogen gas. A solution of amino acid ester was then added to give a final concentration of 3.0 mM. At various time intervals aliquots were removed and fixed with 1% (v/v) formalin solution. These cells were also examined under a Zeiss microscope.

Antigelation test. A tube gelation test using deoxy-HbS was conducted by a modification of the method of Bookchin et al. [11]. To 0.1 ml of HbS-inhibitor solution (containing 32.6 g% HbS) at 0°C under nitrogen gas were added 5 μl of 10% sodium dithionite in 0.05 M Tris-HCl buffer, pH 7.2. The deoxygenated solution was incubated at 37°C for 30 min followed by transfer to 0°C for 10 min and then returned to 37°C for a further 30 min. The reaction mixtures of both control and experimental tubes are normally in the fluid state at 0°C because the solubility of deoxy-HbS increases with decreasing temperature [1]. Artifactual gelation at 37°C due to concentration of deoxy-HbS as a result of evaporation may result in gelation even at 0°C and can therefore be detected by the present method.

Compounds were judged antigelation agents when the solutions of deoxy-HbS remained in the fluid state at 37°C. In the absence of inhibitor, controls gelled within 2 min of incubation at 37°C.

Transport studies. L-[U-^{14}C]Phenylalanine benzyl ester and L-[U-^{14}C]phenylalanine influx were measured at 37°C using the method of Young et al. [12] by mixing 0.2 ml of prewarmed washed cell suspension with an equal volume of prewarmed incubation medium containing the appropriate ^{14}C-labelled compound. The final haematocrit was in the range 10–30%. After a suitable incubation period, the cells were rapidly washed four times with 10 vol. of ice-cold incubation medium using an Eppendorf 3200 microcentrifuge (10 s, 15000 $\times g$). The washed, packed cells were processed for radioactivity determination as described previously [12].

In some uptake experiments, incubations were terminated by rapid centrifugation of cells (10 s, 15000 $\times g$) and careful aspiration of the cell free supernatants. The trapped extracellular space present in these pellets (typically 10–20% of cell volume) was estimated in parallel experiments where cells were mixed with the appropriate con-

centration of L-[U-^{14}C]phenylalanine at 0°C and immediately centrifuged. These blanks were subtracted from cell pellet values to allow calculation of cell-associated radioactivity. Duplicate samples were employed for each condition. The volumes of cells in flux tubes were calculated from the concentrations of haemoglobin in the cell suspensions added to the flux tubes.

Unidirectional potassium influx was measured using ^{86}Rb as a tracer for potassium. The potassium concentration in the flux medium was 5.0 mM and ouabain, when present, was also at a concentration of 0.1 mM. Incubations (10 min at 37°C) were terminated by washing as described above, and radioactivity present in protein-free cell extracts determined by Cerenkov counting. Fluxes were calculated according to the method of Sachs et al. [13]. Active or pump fluxes were taken as the difference between the total Rb influx and the influx in the presence of ouabain. The difference between the Rb influx in the presence of ouabain and the flux in the presence of both ouabain and bumetanide (0.25 mM) [14] was taken as influx by the Na^+/K^+ contransport system. Uptake in the presence of both ouabain and bumetanide was taken as the leak of cations into the erythrocyte.

$^{35}SO_4$ efflux and uridine influx were measured by the methods of Fortes and Ellory [15] and Jarvis et al. [16], respectively. For inhibition studies, Phe-Bz was added to the cells at the same time as the radioactive permeant.

Metabolism of Phe-Bz. Erythrocytes were incubated with [^{14}C]Phe-Bz as described above, and the cell pellets immediately lysed with ice-cold distilled water and protein depleted with trichloroacetic acid. Without further preparation, the trichloroacetic acid extracts were subjected to TLC on aluminium sheet silica gels (5 × 7.5 cm, thickness 0.2 mm: E. Merck, Darmstadt, F.R.G.). The solvent system consisted of 2 parts ethyl acetate and 1 part methyl alcohol. The chromatograms were processed for radioactive counting by cutting the aluminium sheet into 0.5 cm strips at right angles to the direction of the run. Each strip was cut into several small pieces and placed in a scintillation vial containing 1 ml distilled water. After vigorous shaking to leach out Phe-Bz and its metabolites from the silica gel, 8 ml of scintillation fluid were added. Samples were again shaken for several seconds and then counted for radioactivity. Details of the determination of the apparent half-life of Phe-Bz in concentrated haemolysates are given in the legend to Fig. 7.

Octanol / water partition coefficients. The method of Ross and Pfleger [17] was used to determine the octanol/water partition coefficients of Phe-Bz and L-phenylalanine. 2 ml of octanol at 25°C were added to an equal volume of Tris-HCl buffer (15 mM, pH 7.4) containing the appropriate ^{14}C-labelled compound at a concentration of 0.04 mM and the mixture vigorously shaken for 60 s. The aqueous and organic phases were separated by centrifugation at 1300 × *g* for 5 min. 1 ml of aqueous and oil layers was pipetted directly into scintillation fluid in separate vials and counted for radioactivity with quench correction.

Results

Anti-sickling effects of Phe-Bz and related compounds

To test the structural features of Phe-Bz necessary for activity, a number of Phe-Bz analogues were investigated for their ability to prevent sickling compared with Phe-Bz and its parent compounds L-phenylalanine and benzyl alcohol. Common features of the effective anti-sickling agents listed in Table I are the presence of two aromatic rings and an amino group in their molecular structures. When one aromatic ring was replaced by an

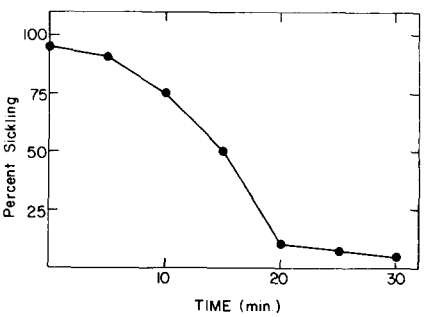

Fig. 1. Time course of anti-sickling effect of Phe-Bz. Sickle erythrocyte suspensions of haematocrit 20% were incubated with 3.0 mM Phe-Bz at 37°C. Cells were deoxygenated with 2% sodium metabisulphite at various times after Phe-Bz addition and the percentage of sickle cells determined.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON SICKLING OF HOMOZYGOUS SICKLE CELLS – STRUCTURE-ACTIVITY RELATIONSHIP

Except where indicated, compounds were present at 3.0 mM. Higher concentrations of compounds caused lysis of cells. The temperature of incubation was 37°C and cells (10% haematocrit) were deoxygenated with 2% sodium metabisulphite solution.

Compound	Percent of non-sickled cells
1. L-Phenylalanine (Phe)	0
2. Benzyl alcohol	0
3. L-Phe benzyl ester·HCl	90–100
4. L-Phe 2-phenylethyl ester·HCl (2.0 mM)	90–100
5. L-Phe 3-phenylpropyl ester·HCl (10 mM)	90–100
6. L-Phe 4-phenylbutyl ester·HCl (0.9 mM)	90–100
7. L-Phe ethyl ester·HCl	0
8. L-Cysteine	0
9. L-Cysteine benzyl ester·HCl	45– 50
10. *S*-Benzyl-L-cysteine benzyl ester·HCl (0.5 mM)	90–100
11. L-Phe thiamide	90–100
12. L-Phe thiophenemethyl ester·HCl	90–100
13. L-Phe 2-thiopheneethyl ester·HCl (2.0 mm)	90–100
14. L-Phe 2-phenoxyethyl ester·HCl (2.0 mM)	90–100
15. *S*-Benzyl-L-cysteine 2-phenoxyethyl ester·HCl (0.5 mM)	90–100
16. 1-*S*-Benzyl-2-amino-3-phenylpropane (2.0 mM)	90–100
17. Benzyl-1-sulphoxo-2-amino-3-phenylpropane (2.0 mM)	90–100

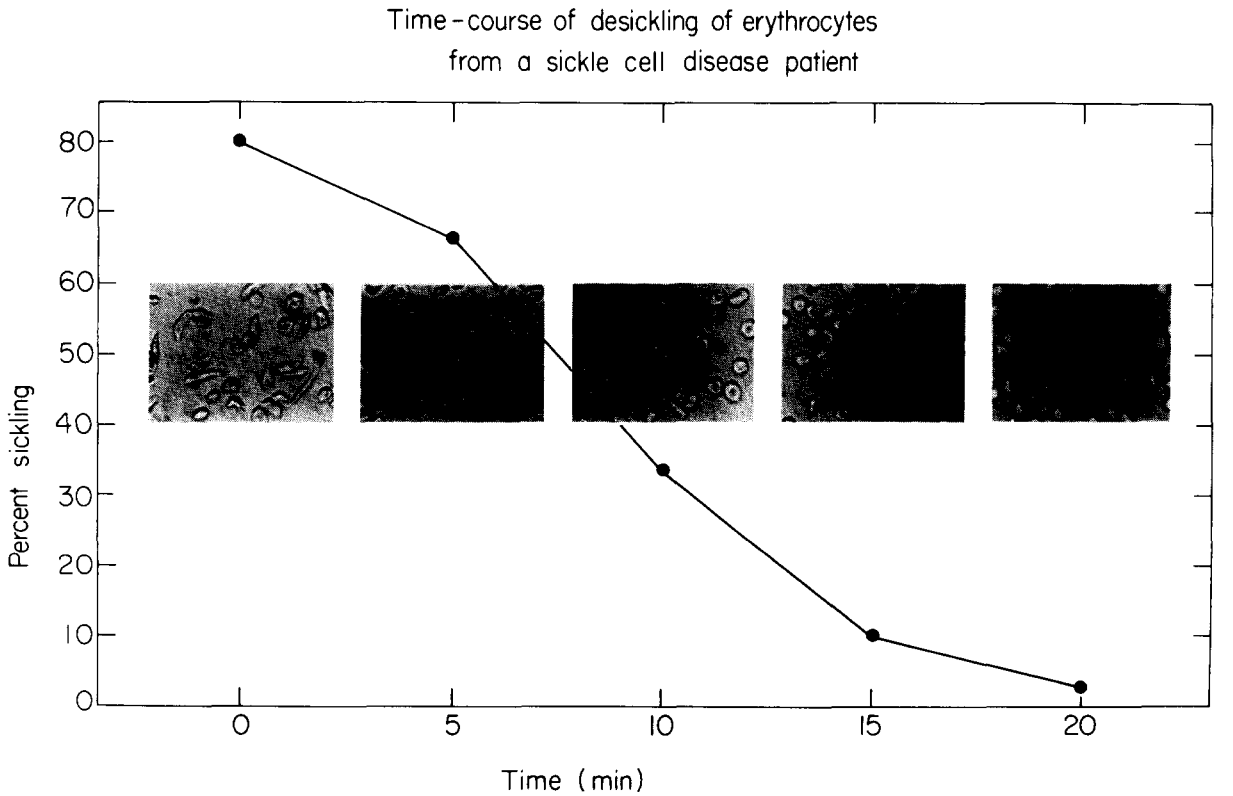

Fig. 2. Time course of desickling effect of Phe-Bz. Sickle cells were deoxygenated under a stream of nitrogen gas. The experiment was started by addition of 3.0 mM of Phe-Bz (final concentration) also under nitrogen. Aliquots of cell suspension were removed at predetermined time intervals, fixed in 1% formalin solution and examined under a Zeiss microscope.

TABLE II

EFFECT OF L-PHENYLALANINE BENZYL ESTER ON ERYTHROCYTE MEAN CELL VOLUME (MCV)

Erythrocytes were incubated at 37°C and a haematocrit of 10% with 3 mM L-phenylalanine benzyl ester (final concentration). At various time intervals, 0.2-ml aliquots were removed and pipetted into 8 ml Sigma-tone buffer, pH 7.0, and the mean cell volume measured in triplicate using a Coulter Counter. Sickle erythrocytes gave similar increases to those shown below for normal cells, while erythrocytes incubated in the absence of amino acid ester showed no significant change in cell volume. Essentially identical results were obtained when mean cell volume was determined using a microhaematocrit centrifuge.

Time (min)	MCV (μM^3)	% Increase
0	89.1 ± 0.4	-
5	96.5 ± 1.6	8.3
15	98.4 ± 0.8	10.4
30	102.3 ± 2.5	14.8

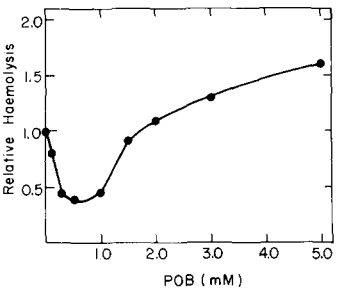

Fig. 3. Effect of Phe-Bz (POB) on hypotonic lysis of normal erythrocytes. Cells were preincubated at room temperature in 150 mM NaCl, 20 mM Tris-HCl (pH 7.2) containing the indicated concentrations of anti-sickling agent. After a period of 5 min, the cell suspensions were diluted with equal volumes of 20 mM Tris-HCl also containing the indicated concentrations of Phe-Bz. Haemolysis in the absence of anti-sickling agent was 40%.

aliphatic group, the anti-sickling effect was drastically reduced. The amino group gives a net positive charge to the compounds in solution. Fig. 1 shows the time course for the onset of Phe-Bz anti-sickling activity while Fig. 2 shows the time course of the desickling effect of Phe-Bz. Stomatocytes were formed on incubating erythrocytes with Phe-Bz for 5–10 min, while prolonged incubation (20–30 min) resulted in the formation of spherocytes. Some cells maintained their discbiconcave shape although they appeared swollen. By determining the mean cell volume of erythrocytes treated with Phe-Bz we were able to establish that Phe-Bz causes an increase in cell volume (Table II).

The formation of stomatocytes induced by Phe-Bz indicated that Phe-Bz distributes preferentially into the cytoplasmic half of the lipid bilayer, causing its expansion relative to the exterior half. This is due to the location of negatively charged phospholipids such as phosphatidylserine in the inner half of the lipid bilayer which attract cationic amphipathic molecules [18]. To test this prediction, the effect of Phe-Bz on hypotonic haemolysis was investigated. As predicted by Sheetz and Singer [18], low Phe-Bz concentrations protected the erythrocytes from haemolysis (maximum protection between 0.1 and 1.0 mM) while higher concentrations resulted in massive haemolysis (Fig. 3).

Using the modified gelation test described in Materials and Methods, we found that 50 mM Phe-Bz did not significantly retard gelation of purified HbS. Previous studies have found that Phe-Bz has no appreciable effect on the Hb oxygen dissociation curve (Acquaye, unpublished observation).

Transport and metabolism of Phe-Bz

In initial studies, the uptake of Phe-Bz by human erythrocytes was compared with that of L-phenylalanine using an established method which involves washing cells in ice-cold medium to remove extracellular radioactive material prior to estimation of intracellular radioactivity. Fig. 4 shows results from such an experiment which suggest that Phe-Bz entry is only slightly faster than that of L-phenylalanine. Another experiment showed that Phe-Bz was an effective inhibitor of L-phenylalanine entry, raising the possibility that the two compounds might share a common entry mechanism (Fig. 5). However, L-phenylalanine was found to have no significant inhibitory effect on Phe-Bz uptake (data not shown). Previous studies have shown that L-phenylalanine entry into human erythrocytes is mediated by the L- and T- amino acid transporters [19,20]. To investigate Phe-Bz transport in more detail, we measured the uptake of Phe-Bz by sheep erythrocytes, cells which lack both the L and T systems [20,21]. Radioactivity from Phe-Bz entered this cell type rapidly, and

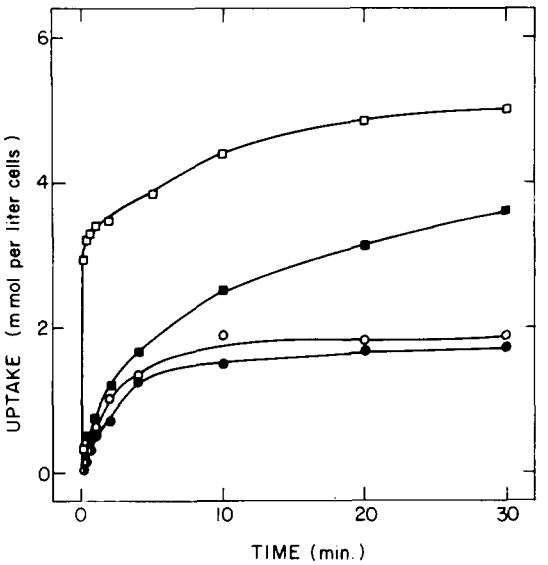

Fig. 4. Comparison of uptake of L-phenylalanine and Phe-Bz by normal human erythrocytes. Flux determinations involving washing of cells to remove extracellular radioactivity: L-phenylalanine (●———●), Phe-Bz (■———■). Flux determinations without washing of cells: L-phenylalanine (○———○) and Phe-Bz (□———□). Final haematocrit was 18% and substrate concentrations were 3.0 mM. Fluxes were performed at 37°C.

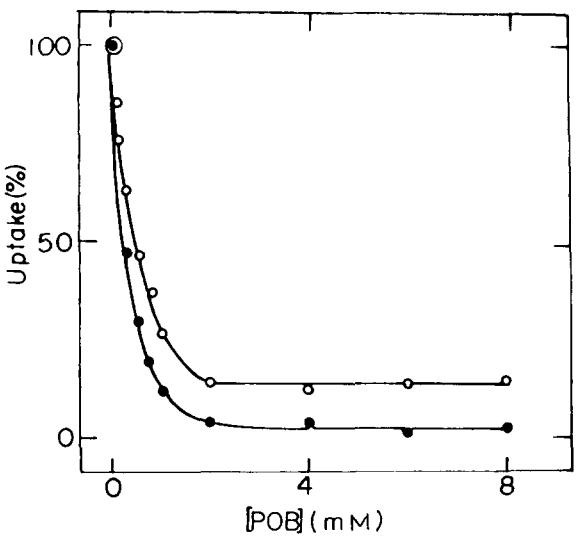

Fig. 5. Effect of Phe-Bz (POB) on L-phenylalanine (○———○) and uridine (●———●) uptake by human erythrocytes. Concentrations of substrates were 200 μM for L-phenylalanine and 1 mM for uridine. Incubation times were L-phenylalanine, 30 s, and uridine, 4 s. Haematocrit of cells suspension was 16%. See refs. 12 and 16 for details of the methods used.

accumulated (Fig. 6). As expected, L-phenylalanine entry was slow (see also Fig. 6). Thus, Phe-Bz entry into cells is not mediated by these carriers. The high octanol/water partition coefficient of Phe-Bz (22.1) compared to that of L-phenylalanine (0.03) suggests that Phe-Bz enters erythrocytes by simple diffusion through the lipid bilayer.

Thin-layer chromatography analysis of intracellular radioactive material following Phe-Bz uptake revealed only the presence of L-phenylalanine in both human and sheep erythrocytes with no detectable Phe-Bz. Thus, Phe-Bz is rapidly hydrolysed within erythrocytes, raising the possibility that Phe-Bz 'uptake' under the conditions of these and earlier experiments by Gorecki et al. [10] might reflect the kinetics of intracellular Phe-Bz metabolism (enzymes, probably esterases) rather than transport. Subsequent influx experiments, performed without washing the cells, established that human erythrocytes were highly permeable to Phe-Bz (see Fig. 4). Both methods of measuring influx (with or without washing) gave similar relatively low estimates of L-phenylalanine entry into cells (see also Fig. 4), while other experiments established that Phe-Bz entered human and sheep cells rapidly even at 0°C (data not shown). Therefore, it would seem that Phe-Bz enters erythrocytes rapidly, but diffuses out again during the washing procedure used in earlier experiments. Under these conditions, the remaining cell-associated radioactivity is that fraction of intracellular Phe-Bz which has been hydrolysed to L-phenylalanine and which

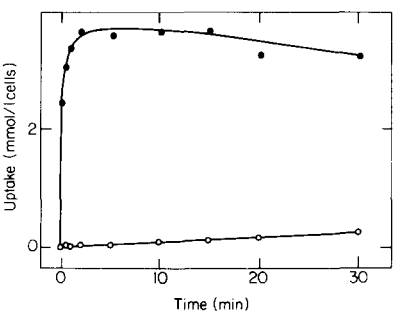

Fig. 6. Comparison of uptake of L-phenylalanine (○———○) and Phe-Bz (●———●) by sheep erythrocytes at 37°C. Substrate concentrations were 0.5 mM, haematocrit 16%. Extracellular radioactivity was removed by washing the cells with ice-cold medium [12].

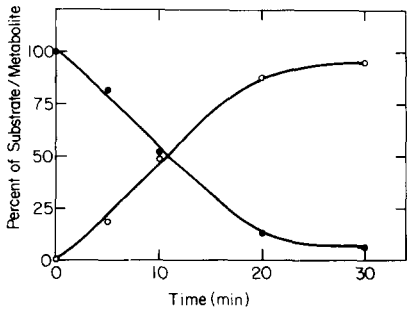

Fig. 7. Time course of metabolism of Phe-Bz in human erythrocytes. Disappearance of Phe-Bz (●———●) and the appearance of L-phenylalanine (○———○). Packed cells were lysed by freeze-thawing, twice. 200-μl aliquots of undiluted haemolysate at 37°C were mixed with 20-μl portions of pre-warmed 33.0 mM L-[^{14}C]phenylalanine benzyl ester (1.7 μCi/μmol) in microcentrifuge tubes. The mixtures were incubated at 37°C. At predetermined time intervals, incubations were diluted with 100 μl of ice-cold distilled water and immediately protein depleted with 50 μl of 100% (w/v) trichloroacetic acid and centrifuged using an Eppendorf microcentrifuge (Model 5412) (15000 × g, 10 min). Thin layer chromatograms of the supernatants were run on aluminium sheet silica gels. The R_f values of L-phenylalanine and Phe-Bz were 0.08 and 0.74, respectively.

has not been transported out of the cell. The absence of L-phenylalanine-transport systems in sheep explains the high level of L-phenylalanine accumulation following addition of Phe-Bz.

Fig. 7 shows the time course of Phe-Bz metabolism by human erythrocyte lysates prepared by freeze-thawing packed cells and demonstrates that Phe-Bz has an apparent half-life within human cells of about 10 min at 37°C, a result consistent with the uptake experiments. Erythrocyte membranes prepared by the method of Dodge et al. [22] did not seem to have any appreciable esterase activity because no hydrolysis of Phe-Bz occurred when the membranes were incubated with Phe-Bz at 37°C. This observation confirms that Phe-Bz hydrolysis is largely intracellular (see above). There were no significant differences in the uptake of L-phenylalanine and Phe-Bz by sickle and normal erythrocytes.

Effects of Phe-Bz on active and passive cation transport

Shape changes caused by amphipathic molecules such as 1-anilino-8-naphthalene-1-sulphonate (ANS) [15] and promazine [18] are often accompanied by alterations in the anion and cation transport in cells. Therefore, the effect of Phe-Bz on anion and cation transport in human erythrocytes was investigated. In a preliminary experiment it was established that the effects of Phe-Bz on cation transport were haematocrit dependent. For example, using erythrocyte suspensions of haematocrit 6.9 and 10%, 2.0 mM Phe-Bz caused the passive influx of ^{86}Rb into erythrocytes to increase by 7- and 5-fold, respectively, while no significant effect was observed using a cell suspension of 25.0%. Similarly, we found haematocrit-dependent inhibitory effects of Phe-Bz on ouabain-sensitive ^{86}Rb influx as well as on the Na^+/K^+ cotransport system. This haematocrit dependence of Phe-Bz action presumably reflects a progressive decrease in the effective concentration of Phe-Bz within the

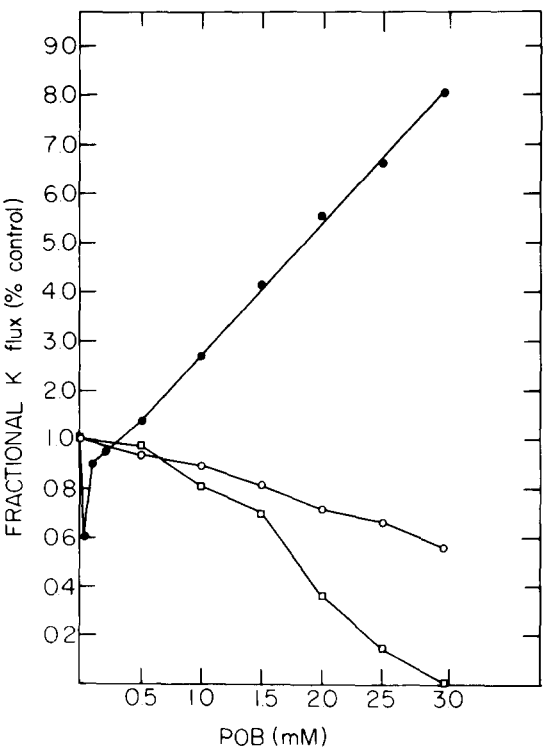

Fig. 8, Effects of Phe-Bz (POB) on active and passive cation transport in human erythrocytes. Cells were incubated at 37°C and a haematocrit of 5% for 10 min in incubation medium containing ^{86}Rb as a tracer for K. Ouabain-sensitive ^{86}Rb influx (□———□), ^{86}Rb uptake by the Na^+/K^+ cotransport system (○———○), and passive cation transport (●———●).

cell membrane both because the amount of membrane lipid increases with increasing haematocrit (erythrocyte concentration) and because higher cell densities will result in more rapid hydrolysis of the available Phe-Bz.

Fig. 8 shows the effect of Phe-Bz on the active and passive ^{86}Rb fluxes in human erythrocytes as a function of Phe-Bz concentration using a cell suspension of haematocrit 5%. At low Phe-Bz concentrations (below 1.5 mM), there was a gradual inhibition of the ouabain-sensitive ^{86}Rb flux with increasing concentration of Phe-Bz (30% inhibition at 1.5 mM Phe-Bz). Above this concentration there was a further progressive inhibition of the ouabain-sensitive ^{86}Rb influx. At 3.0 mM Phe-Bz, uptake by this route was completely inhibited. The Na^+/K^+ cotransporter was inhibited to a lesser extent (44% inhibition at 3.0 mM Phe-Bz). Thus, the ouabain-sensitive Na^+ pump is more sensitive to Phe-Bz inhibition than the Na^+/K^+ cotransport system. The effect of Phe-Bz on the passive influx (leak) of cations into human erythrocytes is more complex. At concentrations of Phe-Bz between 0.03 and 0.1 mM there was inhibition of the leak. Above this concentration there was a dramatic increase in the flux. At 1.0, 2.0 and 3.0 mM Phe-Bz the leaks were 254, 516 and 753% of the control, respectively (Fig. 8).

The increase in leak flux induced by Phe-Bz was not specific for Rb. Experiments with ^{24}Na revealed that the passive influx of ^{24}Na was also dramatically increased. When passive ^{24}Na influx was determined using an erythrocyte suspension of haematocrit 12%, 2.0 mM Phe-Bz caused the influx rate to increase from $13.0 \pm 0.8(3)$ μmol/l cells per h (control) to $431.4 \pm 25.8(3)$ μmol/l cells per h. Measurements of rate constants of ^{24}Na efflux in human erythrocytes in the presence of increasing concentrations of Phe-Bz confirmed that Phe-Bz increases movement of cations across the cell membrane (data not shown). Interestingly, 0.5 mM Phe-Bz caused a significant reduction in ^{24}Na efflux.

Effect of Phe-Bz on anion and nucleoside transport

Fig. 9 shows the effect of increasing Phe-Bz concentration in the incubation medium on SO_4^{2-} efflux from preloaded cells in the presence and absence of the anion transport inhibitor, 4-acetamido-4-'-isothiocyanostilbene-2,2'-disulphonic acid (SITS). Increasing Phe-Bz concentrations in the medium progressively inhibited SITS-sensitive SO_4^{2-} efflux mediated by the Band 3 anion-exchange transporter [23], which in the present experiment accounted for 97% of the total SO_4^{2-} efflux from the control cells. In contrast, SITS-insensitive SO_4^{2-} efflux was substantially increased in the presence of Phe-Bz (see also Fig. 9). These results parallel the effects of Phe-Bz on cation fluxes. Finally, Phe-Bz was also found to have a marked inhibitory effect on [^{14}C]uridine uptake by the erythrocyte nucleoside transporter (see Fig. 6), results similar to those observed with L-phenylalanine influx.

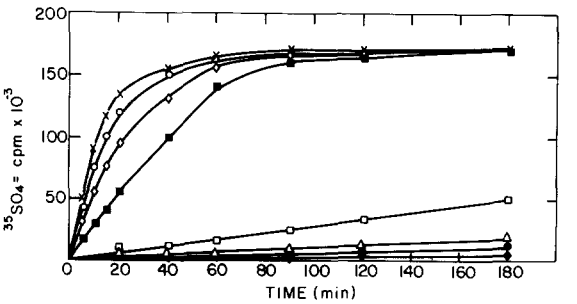

Fig. 9. Effect of Phe-Bz on sulphate efflux from preloaded human erythrocytes. Cells were equilibrated with a medium containing 180 mM sucrose, 40 mM NaCl, 20 mM $Na_2^{35}SO_4$, and 20 mM Tris-HCl pH 7.5 and then washed four times in ice-cold Tris-free Na/phosphate-buffered medium to remove extracellular radioactivity. Control erythrocytes and cells treated with excess SITS were then incubated at 37°C and a haematocrit of 10% with varying concentrations of Phe-Bz. 1-ml aliquots were removed at pre-determined time intervals, rapidly centrifuged (15 s, 15000 × *g*), and the supernatants counted for radioactivity. Control cells; no Phe-Bz added (×——×), 0.5 mM Phe-Bz (○——○), 1.5 mM Phe-Bz (◇——◇), 3.0 mM Phe-Bz (■——■). SITS-treated cells; no Phe-Bz added (◆——◆), 0.5 mM Phe-Bz (●——●), 1.5 mM Phe-Bz (△——△), and 3.0 mM Phe-Bz (□——□).

Discussion

The data presented here show that Phe-Bz enters the erythrocyte at a fast rate. The rapidity of the flux and subsequent intracellular metabolism of Phe-Bz make the kinetics of its transport difficult to analyse. However, by comparing the uptake characteristics of human and sheep cells, it is

possible to eliminate the involvement of mediated transport by the L- and T-amino acid transporters, the carriers responsible for the entry of aromatic amino acids into human erythrocytes. The rapidity of Phe-Bz entry into erythrocytes even at 0°C and the high octanol/water partition coefficient of Phe-Bz suggest that it enters cells mainly by diffusion through the lipid bilayer. Our results also show that Phe-Bz acts as a general inhibitor of erythrocyte membrane transport systems, while at the same time increasing the basal permeability of the cell membrane to both cations and anions. These effects together with the expansion of the membrane by Phe-Bz, indicated by cell-shape changes and hypotonic haemolysis protection, are likely to be functional consequences of the binding of Phe-Bz to membrane phospholipids and proteins. Experiments (unpublished observations) show that Phe-Bz and related anti-sickling agents fluidize the erythrocyte membrane. Phe-Bz partitions into the cell membrane and equilibrates with intracellular water in less than 1 min (see Figs. 4–6) while the onset of the anti-sickling effect occurs within 5–10 min of incubation of cells with Phe-Bz (Figs. 1 and 2). Therefore, the anti-sickling effect of Phe-Bz requires more than the simple presence of Phe-Bz within the cell, cell membrane or both. In this context it is important to note that Phe-Bz has no detectable effect on purified HbS.

The progressive increase in cell volume due to the increase in the passive influx of cations into the erythrocyte and the progressive intracellular production of L-phenylalanine and benzyl alcohol from Phe-Bz both correlate wih the time course for the onset of protection. L-Phenylalanine [24] and benzyl alcohol [25] have been found to have anti-gelling effects on isolated HbS. By combining the two compounds to form the highly permeable Phe-Bz we have been able to produce higher intracellular concentrations of L-phenylalanine (and possibly benzyl alcohol) than can be obtained by incubating cells with equivalent concentrations of the constituent molecules. Nevertheless, the intracellular levels of L-phenylalanine and benzyl alcohol we achieved in our experiments are still substantially less than those required to prevent gelation of deoxy-HbS [24]. It is therefore likely that the increase in mean cell volume induced by Phe-Bz also contributes to the anti-sickling effect. The volume change cannot be the only factor involved in the anti-sickling effect of Phe-Bz, since sickling is prevented before the normal minimum gelling concentration of HbS (24 g%) is reached.

In summary, therefore, it is likely that the anti-sickling action of Phe-Bz initially involves insertion of Phe-Bz into the cytoplasmic half of the cell membrane lipid bilayer, resulting in marked inhibition of mediated cation transport and a substantial increase in passive cation fluxes. This in turn leads to an increase in mean cell volume which results in a significant decrease in the intracellular HbS concentration. At the same time, Phe-Bz rapidly enters the cell where it is hydrolysed to L-phenylalanine and benzyl alcohol which probably interact directly with HbS. It is also possible that degradation of Phe-Bz and the concentration of its products within the erythrocyte contribute significantly to the increase in cell volume induced by Phe-Bz. These three separate components of Phe-Bz action act synergistically to prevent (or reverse) sickling of erythrocytes.

The progressive increase in cell volume induced by Phe-Bz eventually leads to lysis of a substantial percentage of treated cells. An analogue of Phe-Bz capable of bringing about controlled cell swelling without cell lysis and able to increase the solubility of deoxy HbS without undergoing hydrolysis would be clinically useful.

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