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Antisickling activity of amino acid benzyl esters

(sickle cell disease/hydrophobic bonding/cell penetration/erythrocyte flexibility)

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ABSTRACT **The sickling of homozygous sickle cells upon deoxygenation is inhibited in the presence of 3 mM L-phenylalanine benzyl ester (Phe-OBzl) or benzyl esters of other aromatic or hydrophobic amino acids. Phe-OBzl was found to permeate into erythrocytes rapidly, and the deoxygenated cells maintained considerable flexibility as measured by their ability to pass through 3-μm pores. The osmotic fragility of the cells was unchanged and the oxygen dissociation curve was shifted slightly from a 50% saturation value of 28.5 mm Hg to 31.0 mm Hg. At lower concentrations of Phe-OBzl some antisickling activity was seen. The Phe-OBzl antisickling activity may involve both binding to deoxyhemoglobin S and modification of the erythrocyte membrane. This class of compounds has considerable potential as therapeutic agents for the treatment of sickle cell disease.**

In sickle cell disease, Hb S aggregates upon deoxygenation. This distorts the erythrocyte membrane and ultimately results in a clogging of capillaries. A wide variety of chemicals have been investigated in an attempt to find a suitable therapeutic agent. These agents either can react covalently or may work through noncovalent interactions. There are two general targets for these agents, Hb S or the erythrocyte membrane. On binding to Hb S, these substances delay or prevent intracellular Hb S gelation and thereby inhibit the sickling process (1–3). Intercalation into the membrane may increase sickle cell erythrocyte flexibility (4, 5) and thereby decrease the capillary clogging. Certain amino acids (6, 7) and small peptides (8, 9) have been shown to produce varying degrees of *in vitro* inhibition of deoxy-Hb S gelation. This activity is directly related to the hydrophobicity of the molecules (10). The importance of hydrophobic binding is not unexpected in view of the fact that the mutation in the β-globin chain that is responsible for sickle cell disease changes β6(Glu) to β6(Val). Nonpolar substances may alter intermolecular interactions involved with a complementary hydrophobic site for β6(Val) and, thereby, prevent or delay gelation of deoxy-Hb S.

Antisickling peptides can be put into erythrocytes artifically, and they inhibit sickling (8). By themselves, however, these peptides do not have antisickling activity. This stems from the fact that effective compounds have aromatic side chains which have a low solubility in aqueous media. Furthermore, relatively high concentrations (≥20 mM) were needed to show substantial antigelling activity under the oxygen concentrations found in venous blood (8). Even di- or tripeptides fail to penetrate the erythrocytes cell wall or else have a very slow rate of incorporation. In an attempt to overcome these negative features, we have investigated several derivatives and have discovered that various amino acid benzyl esters have significant antisickling activity. L-Phenylalanine benzyl ester (Phe-OBzl) has been studied in detail to evaluate not only its antisickling capacity but also its permeability and its effect on cell osmotic fragility and oxygen affinity. We conclude that this class of compounds may have considerable utility as therapeutic agents for sickle cell disease.

MATERIALS AND METHODS

Whole blood from homozygous sickle cell patients, usually young adults, was drawn into sterile heparinized tubes and used within a period of 96 hr. [^{14}C]Phenylalanine was purchased from New England Nuclear.

Chemical Synthesis. L-Phenylalanine benzyl ester (unlabeled and ^{14}C-labeled) was synthesized according to the method of Sokolovsky *et al.* (11). The other amino acid and peptide benzyl esters as well as other esters were purchased from YEDA Research and Development, Israel.

***In Vitro* Sickling Experiment.** Sickle cell erythrocytes were washed four times with buffer A (145.5 mM NaCl/40 mM KCl/1.65 mM Na_2HPO_4/0.16 mM KH_2PO_4/0.01 mM $MgCl_2$/1.1 mM glucose, pH 7.2). This removed the buffy coat and plasma. A 20% (wt/vol) suspension of washed cells in 300 μl of buffer A containing 3.0 mM of the compound was swirled at 37°C for 30 min. Then, 20 μl of the cell suspension was deoxygenated with 20 μl of 2% sodium metabisulfite ($Na_2S_2O_5$) for 15 min at 37°C. The cells were fixed in 1% formalin and placed under anaerobic conditions between siliconized glass cover slips; 300 cells were counted to determine the percentage of unsickled cells.

Incorporation Measurement. Normal erythrocytes were used in the incorporation study. [^{14}C]Phe-OBzl (specific activity, 514 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to a 20% (wt/vol) suspension of erythrocytes in buffer A containing 3.0 mM unlabeled Phe-OBzl. The container was stoppered, incubated at 37°C, and swirled at 100 cycles/min. At each time point, 300 μl of the erythrocyte suspension was pipetted into an Eppendorf centrifuge tube and centrifuged (1800 × *g*). The supernatant was treated with 10 μl of 100% trichloroacetic acid to precipitate any protein that may have been present and preserved. The erythrocyte pellet was washed three times with buffer A containing 3.0 mM unlabeled Phe-OBzl in order to free the erythrocyte of unincorporated labeled Phe-OBzl and to reduce efflux of radioactive material from the erythrocytes to medium.

The erythrocyte pellet was weighed and then lysed with 500 μl of cold distilled water. The proteins were precipitated with 10 μl of 100% trichloroacetic acid. For each sample, the radioactivity was determined in 100 μl of supernatant solution, 100 μl of deproteinized hemolysate, and 100 μl of the last erythrocyte wash. The ratio of radioactive material inside the cell to that outside was calculated as follows, using a cell water content of 68%:

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Abbreviation: Phe-OBzl, L-phenylalanine benzyl ester.

$$\text{ratio} = \frac{C^s - C^b}{C^f} \times \frac{500 + (W \times 0.68)}{W \times 0.68}$$

in which C^s is cpm per μl of deproteinized hemolysate, C^f is cpm per μl of the incubation medium, C^b is the small background cpm per μl of the last wash of the erythrocyte pellet, and W is the weight in milligrams of the erythrocyte pellet.

Filterability Measurements. After incubation of a 5% (wt/vol) erythrocyte suspension in buffer A at 37°C for 0.5 hr, a 1.5-ml portion of the well-mixed suspension was transferred rapidly to the upper chamber of a filter assembly. The filter assembly, 47 mm in diameter, had a small void volume and was maintained at 37°C. The erythrocytes were stirred for 1 min under a continuous flow of N_2 gas at 75 ml/min. The cells were then rapidly deoxygenated by addition of a freshly prepared sodium dithionite solution to give a final concentration of 12 mM $Na_2S_2O_4$ with pH 7.0 ± 0.05. The cell suspension was continuously stirred for 2 additional min under N_2 gas and then was filtered through a 3.0-μm-pore-size Bio-Rad polycarbonate filter with a negative pressure of 1.0 cm Hg that was established instantaneously in the lower chamber of the assembly. The cells in the lower chamber were harvested, centrifuged (1800 × g) to remove any extraneous buffer, and then weighed.

Oxygen Dissociation Measurement. The oxygen dissociation curve was determined as described (8) with the exception that a 2.0% (wt/vol) cell suspension was used. Treated erythrocytes were exposed to 2.0 mM Phe-OBzl at 37°C for 30 min prior to deoxygenation for the measurements.

Osmotic Fragility. Treated normal and sickle cell erythrocytes were incubated in buffer A containing 2.0 mM Phe-OBzl at 37°C for 30 min. The cells were rapidly centrifuged, and the pellet from the initial cell suspension (20%, wt/vol) was diluted 1:60 in isotonic (24 mM) sodium barbiturate buffer at pH 7.2. A similar procedure was used for untreated cells. The percentage hemolysis was determined as a function of time by using the method of Danon (12) with the Fragilograph D3. The lysis occurred as a result of dialysing the erythrocytes against distilled water at 37°C; the released hemoglobin was measured at 540 nm.

RESULTS

***In Vitro* Sickling.** Once the initial discovery was made that benzyl esters of aromatic amino acids would prevent sickling as observed microscopically, a survey was carried out to measure the effect of various esters and related compounds. Table 1 summarizes the percentage of unsickled deoxygenated homozygous cells after treatment with 1 of 14 different compounds, including 11 benzyl esters. An erythrocyte was considered to be unsickled if the cell was not in the characteristic sickle shape or in a crenated holly leaf pattern. The benzyl ester of L-phenylalanine produced 90% unsickled cells; the yields were 90% with that of tyrosine and 95% with that of tryptophan. All of these residues have high degrees of hydrophobicity, with somewhat more in the case of tryptophan. On the other hand, benzyl esters of an amino acid such as proline resulted in 60% unsickled cells and those of valine and leucine gave 50 and 55%, respectively. The latter amino acids have less hydrophobicity than the aromatic amino acids.

Table 1. Effect of various components on sickling of homozygous sickle cells

Compound*	% unsickled cells (visual assay)
L-Phe-OBzl	90
L-Tyr-OBzl	70
L-Trp-OBzl (0.5 mM)	95
L-Pro-OBzl	60
L-Ala-L-Phe-OBzl	90
Gly-L-Phe-OBzl	90
L-Val-OBzl	50
L-Leu-OBzl	55
L-Phe-L-Ala-OBzl	70
L-Trp-L-Lys-OBzl (0.5 mM)	70
D-Phe-OBzl	90
L-Phe-OMe	0
L-Tyr-octadecyl ester	10
L-Phe	0
Nothing added	0

* Present at 3.0 mM except where indicated.

Addition of an extra amino acid at the NH_2 terminus of Phe-OBzl had no appreciable effect on sickling activity. Both L-Ala-L-Phe-OBzl and Gly-L-Phe-OBzl inhibited sickling to the 90% level of the parent compound, Phe-OBzl. It is interesting that the benzyl ester of the dipeptide apparently was able to penetrate the erythrocyte as well as the amino acid benzyl ester did, as shown by the similarity in sickling inhibition. When benzyl esters were used with dipeptides in which the phenylalanine was one residue removed—for example, L-Phe-L-Ala-OBzl and L-Trp-L-Lys-OBzl—antisickling effect was slightly reduced to 70%.

The configuration of the amino acid did not seem to be important because the benzyl ester of D-phenylalanine was as effective as that of L-phenylalanine. However, the benzyl ester appears to be required; the methyl ester of phenylalanine was ineffective, as was the octadecyl ester of tyrosine. This suggests that the phenyl ring may be playing a role either in its interaction with the hemoglobin molecule or in its penetration into the erythrocyte.

The shapes of untreated deoxygenated sickle cells and of those treated with 3.0 mM Phe-OBzl are in marked contrast; untreated cells show the abnormal sickle and holly leaf shapes and those exposed to Phe-OBzl do not (Fig. 1). Protection of about 50% of the cells was observed within as little as 10 min of incubation. At a lower concentration of Phe-OBzl (1.0 mM), 60% of the cells appeared to be normal after a 30-min incubation. Some sphericity could be seen in the Phe-OBzl-treated cells compared to the biconcave shape of normal untreated cells (Fig. 1*B*). When normal cells were exposed to 1–5 mM Phe-OBzl, this same morphological feature was seen in varying degrees.

[^{14}C]Phe-OBzl Incorporation. The antisickling effect of the benzyl esters suggested that these compounds might be penetrating the erythrocyte cell wall. Accordingly, measurements were made of the incorporation of [^{14}C]Phe-OBzl into erythrocytes. The ratio of the intracellular to extracellular radioactivity was determined at 10-min intervals over a 1-hr period in the presence of 3.0 mM Phe-OBzl. There was a linear initial rate of uptake of slightly more than 3%/min for the first 30 min (Fig. 2). The rate of increase did not decrease to zero until slightly after 40 min, when full incorporation was seen. Afterward there was an apparent 10% decrease of the Phe-OBzl levels in the erythrocytes. This apparent efflux of Phe-OBzl may have its origin in a partial hemolysis of cells as a result of prolonged incubation or it may have been due to intracellular hydrolysis of Phe-OBzl or both. Thin-layer chromatography of a trichloroacetic acid deproteinized hemolysate of erythocytes incubated with ^{14}C-labeled Phe-OBzl for 1 hr revealed a weak band at the known phenylalanine position. The ratio of intensities of the Phe-OBzl and L-phenylalanine bands was approximately 4:1.

Sickle Cell Filterability. The visual assay monitors only a limited number of cells, but the filterability assay involves all

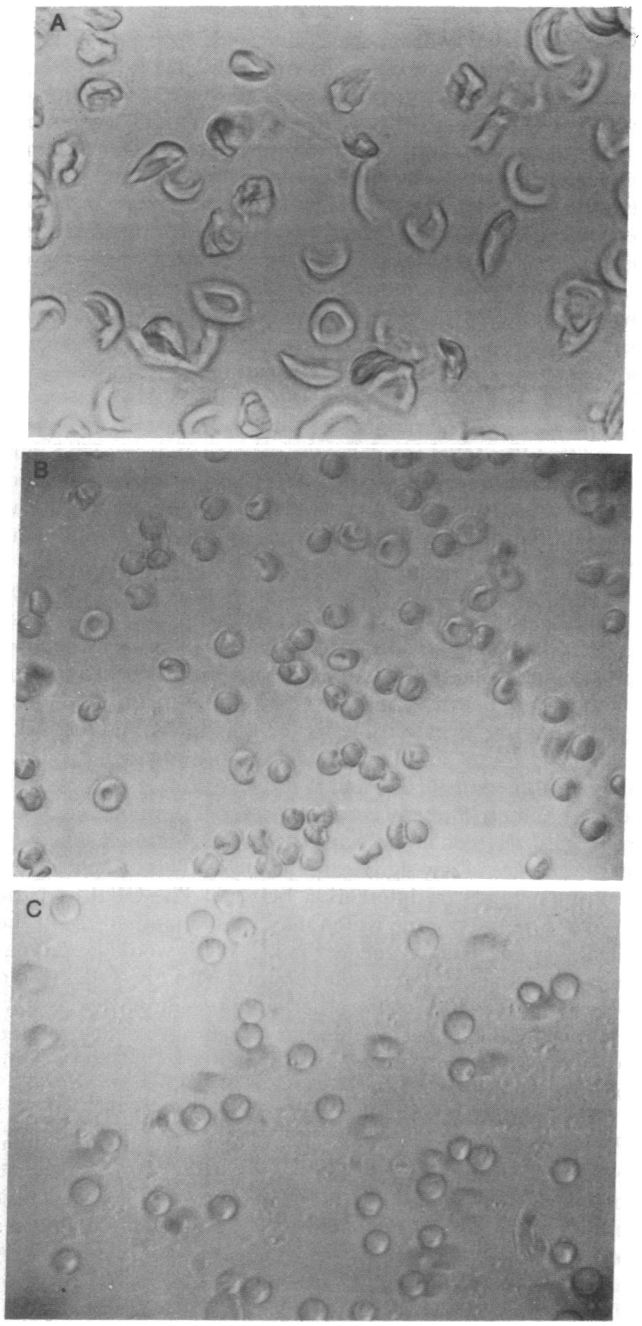

FIG. 1. Effect of 2.0 mM Phe-OBzl on erythrocytes from a homozygous sickle cell patient. Treated cells were incubated for 30 min with 2.0 mM Phe-OBzl in buffer. (*A*) Untreated deoxygenated sickle erythrocytes; (*B*) normal oxygenated cells; (*C*) treated deoxygenated sickle erythrocytes.

the cells. Here we measure the ability of the cells to maintain flexibility so that they can pass through a 3.0-μm pore. Filterability data for 5% (wt/vol) suspensions of sickle cell erythrocytes are presented in Table 2 for various Phe-OBzl concentrations. Filterability of deoxygenated Hb S cells was expressed relative to the fully oxygenated state by the average recovery ratio $\langle R \rangle$, the average weight of filtered deoxygenated cells to that of filtered untreated oxygenated cells, as determined for several filtration runs. The results are for partial deoxygenated conditions brought about rapidly by the addition, to an erythrocyte suspension, of sodium dithionite to a final concentration of 12 mM. Untreated cell samples that were fully deoxygenated by prolonged exposure to N_2 gas gave a recovery ratio of 0.13

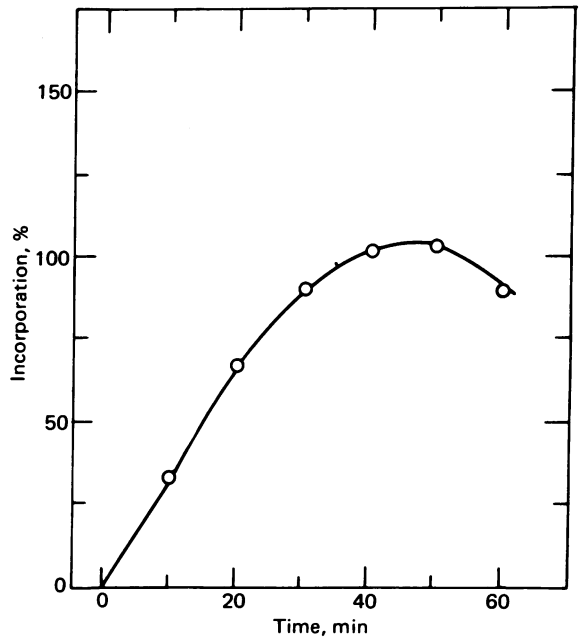

FIG. 2. The incorporation of [^{14}C]Phe-OBzl (3.0 mM in medium) into erythrocytes as a function of time.

in contrast with a value of 0.34 for partially deoxygenated control cells.

The filterability of partially deoxygenated treated cells changed appreciably upon exposure of the erythrocytes to low concentrations of Phe-OBzl. The average $\langle R \rangle$ increased to 0.48 at 1.0 mM Phe-OBzl and to 0.69 at 3.0 mM Phe-OBzl. A further increase in the Phe-OBzl concentration up to 5.0 mM did not appreciably alter sickle cell filterability. However, at slightly higher concentrations, hemolysis began to increase. L-Phenylalanine and benzyl alcohol, the hydrolysis products of Phe-OBzl, are known to possess some antigelling activity (7, 13). At a concentration of 3.0 mM, they did not affect sickle cell flexibility as measured by this assay. However, the possibility that they may act in concert with Phe-OBzl to produce an antisickling effect cannot be ruled out.

Oxygen Dissociation Curve and Osmotic Fragility Tests. Any potential therapeutic agent must not only prevent sickling but also have minimal adverse effects. Accordingly, we measured the effect of Phe-OBzl on both the oxygen dissociation curve and the osmotic fragility test. Fig. 3 presents the oxygen dissociation curves for treated (solid triangles) and untreated (solid circles) normal erythrocytes. A small shift in the oxygen dissociation curve to the right was seen for the cells treated with

Table 2. Effect of Phe-OBzl on filterability of sickle cell erythrocytes

Phe-OBzl, mM	$Na_2S_2O_4$, mM	$\langle R \rangle$*
0†	N_2 (g)	0.13
0	12	0.34
1	12	0.48
2	12	0.61
3	12	0.69

* $\langle R \rangle$ = average weight ratio of deoxygenated to oxygenated cells recovered after filtration through a 3.0-μm pore size Bio-Rad polycarbonate filter. Six measurements were made at each L-Phe-OBzl concentration using homozygous sickle cells from three patients (young adults).

† A stirred erythocyte suspension was deoxygenated by prolonged exposure (90 min) at 37°C to N_2 gas at a flow rate of 50 ml/min.

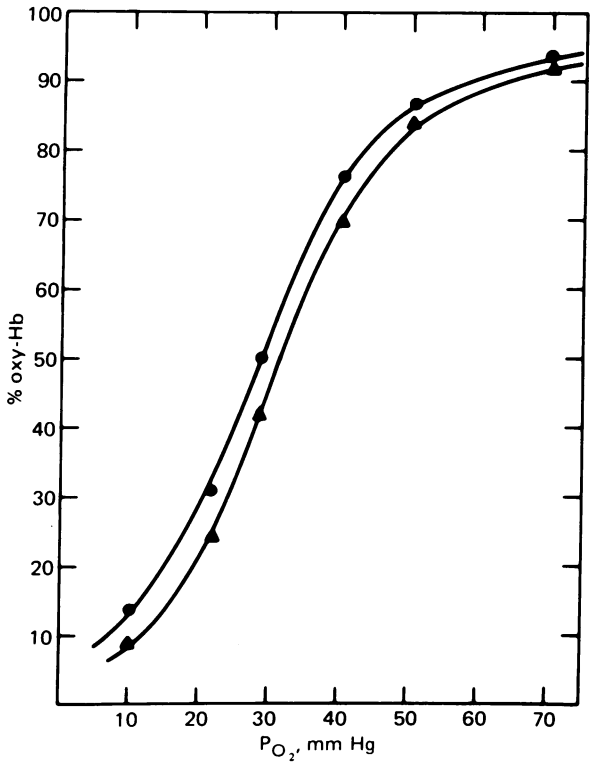

FIG. 3. Oxygen dissociation curve in the absence (●) and presence (▲) of 2 mM Phe-OBzl for a 2% (wt/vol) suspension of normal erythrocytes in phosphate-buffered saline.

2.0 mM Phe-OBzl (Fig. 3). This results in a P_{50} (50% O_2 saturation) of 31.0 mm Hg as opposed to 28.5 mm Hg for the untreated case.

The fragility of cells to osmotic stress is shown in Fig. 4. Normal cells treated with 2.0 mM Phe-OBzl for 30 min and untreated cells showed almost identical percentage hemolysis as a function of time. Similar experiments with sickle cell erythrocytes give a small but consistent difference between the untreated and treated cells (Fig. 4*B*). It appears from these results that 2.0 mM Phe-OBzl does not perturb the erythrocyte membrane to a significant extent. This is apparent from the small difference in hemolysis in Fig. 4*B* and, more importantly, from the observed difference in the osmotic fragility between the normal and sickle cell erythrocytes. Even after Phe-OBzl treatment, the Hb S cells still maintain their increased osmotic resistance which is characteristic of these erythrocytes (14). In contrast, normal cells show rapid lysis whether Phe-OBzl is present or absent.

DISCUSSION

Benzyl esters of hydrophobic amino acids, such as Phe-OBzl, are typical amphipathic molecules with a charged α-NH_2 group at one end and a hydrophobic portion for the rest of the molecule. Phe-OBzl has significant antisickling activity as demonstrated by the visual and filterability assays which complement each other. Exactly why it has this activity is not totally clear. Its amphipathic nature plus low molecular weight are probably responsible for its easy passage through the mosaic lipid–protein bilayer of the erythrocyte (15). This is reinforced by the report (16) that the benzyl ester of L-3,4-dihydroxyphenylalanine is much more easily transported across cell membranes than is the parent amino acid. However, permeability by itself does not appear to be the only factor in the antisickling activity of Phe-OBzl. The inhibition of gelation of deoxy-Hb S has been used to measure potential antisickling activity (10). We have found that Phe-OBzl can delay the gelation of deoxy-Hb S, but it requires significantly higher concentrations than those used in the visual or filterability assay. This suggests an additional role outside of the antigelling activity of Phe-OBzl at low concentrations.

A review (17) of the properties of amphipathic molecules reveals that many of them bind in a biphasic manner to intact erythrocytes: at low concentrations they give linear binding and promote resistance to hypotonic lysis; at higher concentrations they induce sphericity followed by hemolysis. There are also exceptions to this pattern, however. The tertiary amine lidocaine was found to alter the membrane, producing a cup-shaped appearance (18), and it did not protect erythrocytes against hemolysis at any concentration but promoted it. Some sphericity was seen in the shape of Phe-OBzl-treated cells even in the absence of any significant hemolysis. This morphological feature suggests an interaction between Phe-OBzl and the erythrocyte membrane which may lead to an increase in the flexibility of the deoxygenated sickle cell. It would be premature to assume that the results obtained with Phe-OBzl would necessarily be valid for the other effective amino acid benzyl esters, but is likely because similar effects have been reported on erythrocyte morphology and flexibility for aromatic compounds, steroids (19), and procaine hydrochloride (20).

It is interesting to compare the activity of the dipeptide L-Phe-L-Phe with that of Phe-OBzl. The molecules are similar except that the dipeptide has lost its -COOH group and its peptide backbone NHCH has been exchanged for the O of the ester linkage. The negative charge on the dipeptide has been lost. The Phe-OBzl molecule now shows a dramatic antisickling activity in contrast to no observable effect for the dipeptide. The nonpolar nature of these two compounds is almost identical,

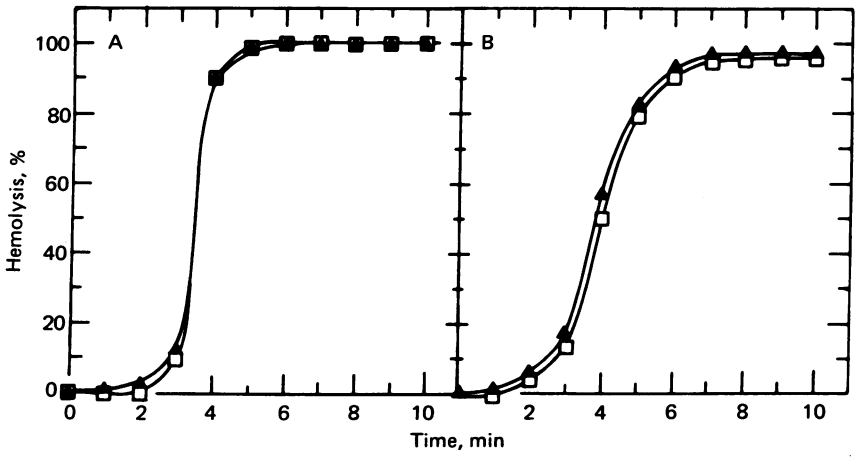

FIG. 4. Effect of Phe-OBzl in osmotic fragility tests with normal erythrocytes (*A*) and homozygous sickle erythrocytes (*B*) that were previously incubated at 37°C for 30 min without (□) or with (▲) 2 mM Phe-OBlz.

based on Nozaki and Tanford's hydrophobicity scale (21). It has been shown (10) that the hydrophobicity of compounds containing adjacent aromatic groups is linearly correlated to their antigelling activity based on delay times for deoxy-Hb S gelation of 20 min or more. These facts also suggest that the Phe-OBzl–membrane interaction may be a significant aspect of its antisickling nature. However, the valine benzyl ester, which is only 14% less hydrophobic than Phe-OBzl (21), has a substantially decreased antigelation activity as compared to Phe-OBzl. It shows a diminished antisickling activity in the visual assay, suggesting that it is necessary to have sufficient antigelation activity. We are thus left with two factors that may be important: antigelling activity due to binding to deoxy-Hb S and increased flexibility caused by membrane changes. It is not clear which is the major contributor to Phe-OBzl's antisickling activity, or whether they may be acting synergistically.

The brief survey of compounds in Table 1 shows that there are significant variations in the effectiveness of antisickling agent associated with changes in the nature of the substituents. Accordingly, further variation in the chemical architecture of the inhibitors can be explored in order to optimize their effectiveness.

A therapeutic agent for the treatment of sickle cell disease needs to satisfy many criteria. It must prevent sickling, act at low concentrations, not interfere with essential erythrocytic functions, and, of course, have a low toxicity. Preliminary toxicity studies of Phe-OBzl in mice indicate that it has a low toxicity value, with an LD_{50} near 1000 mg/kg. From the material presented here, we suggest that benzyl esters of hydrophobic amino acids and related compounds may prove to be useful in the treatment of sickle cell disease.

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