Effect of Cetiedil on Cation and Water Movements in Erythrocytes

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ABSTRACT Cetiedil is a potential antisickling agent whose major effect appears to be at the erythrocyte membrane. To test the hypothesis that cetiedil alters cation transport, we studied the effect of the drug in promoting changes in cell water (W_c), cell sodium (Na_c), and cell potassium (K_c). Results are quite different depending on the presence or near absence of intracellular ATP. With fresh cells, 100 μM cetiedil causes little change in net cation or water movements compared with control cells incubated for 2 h. At cetiedil concentrations >100 μM, however, net movements of sodium and potassium increase considerably, and cell swelling results from a net Na_c gain that exceeds a net K_c loss. All water movements can be accounted for by cetiedil-induced net cation movements. When 100 μM ouabain is added along with cetiedil, net Na_c gain, net K_c loss, and net W_c gain are all increased compared with results obtained with cetiedil alone. External calcium inhibits cetiedil-induced changes in cation transport. With cells depleted of their ATP, cetiedil inhibits the typical potassium loss that occurs in the presence of external calcium; net sodium uptake changes little under these conditions, regardless of the presence or absence of external calcium. Our findings indicate a complex mode of action for cetiedil on the erythrocyte membrane, and support the hypothesis that the antisickling effect of the drug observed in vitro results from dilution of intracellular hemoglobin secondary to net salt and water gain.

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INTRODUCTION

Cetiedil, α-cyclohexyl-3-thiophenacetic acid 2-(hexahydro-1H-azepin-1-yl) ester, has aroused considerable interest recently as a potentially useful agent in the treatment of patients with sickle cell disease. The antisickling effect of cetiedil was first reported by Cabannes (1), who conducted a clinical study on the Ivory Coast that suggested that the drug has a beneficial effect on patients with acute vaso-occlusive crises. Currently, cetiedil is used in Europe for treatment of ischemic leg pain due to vascular disease. Known pharmacological properties of the drug include vascular smooth muscle relaxation, inhibition of phosphodiesterase, blockade of the effects of bradykinin and serotonin, analgesia, and inhibition of platelet aggregation (2). While many of these properties may benefit the patient with severe sickle cell crisis, the mechanism by which the drug acts on the erythrocyte to prevent sickling has not yet been elucidated.

The antisickling effect of cetiedil in vitro is evident at drug concentrations from 50 to 500 μM (3, 4). At the lowest effective concentration, this represents a drug-to-hemoglobin tetramer ratio of 1:100. The findings that cetiedil prevents sickling with only slight change in the minimal gelling concentration of deoxy hemoglobin S and with no change in the oxygen affinity of sickle cells (3, 4) seemed to rule out an inhibitory effect on the hemoglobin molecules as the main mode of action. A key observation by Asakura and co-workers (3) was that the hematocrit of sickle cells incubated overnight with 400 μM cetiedil increased by as much as 20%. They proposed that the primary site for drug action is at the cell membrane leading to cell swelling and a secondary dilution of hemoglobin. This hypothesis is attractive in explaining the drug's antisickling property since it is well known that small increases in cell water content can greatly delay cell sickling, the delay time for gelatin being

inversely proportional to the 30th power of the hemoglobin concentration (5). To test this hypothesis and evaluate the effect of cetiedil on cell membrane permeability, we undertook a systematic study of drug-induced net changes in cell water (W_c),[1] cell sodium (Na_c), and cell potassium (K_c) in both normal and sickle erythrocytes. Our results show that cetiedil causes marked increases in the net transport of both sodium and potassium. Such cetiedil-induced changes are enhanced by the cardiac glycoside ouabain and diminished by the presence of external calcium. When cells are depleted of their ATP, the effect of cetiedil is changed so that little potassium is lost and little sodium is gained. Ouabain is without effect and calcium exerts only small effect under these conditions.

METHODS

Heparinized blood obtained from normal controls or patients with homozygous sickle cell disease was used within 1 h of being drawn for the experiments requiring high ATP. After initial centrifugation and removal of the buffy coat, the cells were washed three times with physiological saline at 4°C. Cells were then test incubated at a 2% hematocrit using a shaker waterbath at 37°C. Incubation flasks were siliconized to minimize hemolysis at the higher cetiedil concentrations. At timed intervals, aliquots were withdrawn, and rapidly centrifuged at 4°C; sufficient supernatant fluid was then aspirated so that the resulting suspension had a hematocrit of ~30%. This new suspension was introduced into specially prepared nylon tubes (W. E. Doremus Co., Harwinton, Conn.) with long-stemmed pasteur pipettes, then immediately centrifuged at 10,000 *g* for 10 min. Packed cells were separated from their supernatant fluid by slicing the nylon tube 2 mm below the cell-fluid interface. Cells were then routinely analyzed for water, sodium, potassium, and ATP contents. Specific details of cell separation with these nylon tubes, as well as methods for measurements of cell water and intracellular cation contents, have been described previously (6). Sodium and potassium were analyzed with a Varian atomic absorption spectrophotometer (Varian Techtron Pty. Ltd., Melbourne, Australia). ATP was determined enzymatically on the perchloric acid extract of packed cells (7).

Cetiedil citrate monohydrate was kindly provided by Johnson & Johnson International (New Brunswick, N. J.). This compound is weakly soluble in water but dissolves readily in absolute ethanol. Aliquots from a 125-mM stock solution in ethanol were added to incubation flasks to give the desired final concentrations. A comparable volume of ethanol without cetiedil was added to the control incubations. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. Unless specified otherwise, incubation solutions contained: 5 mM KCl, 10 mM glucose, 10 mM Na-TES or Mg-TES [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid] adjusted to pH 7.4 at 37°C, and sufficient NaCl (~160 mM) to adjust osmolality to 300 mosmol. TES buffers were prepared as described previously (6).

To deplete intracellular ATP, cells were incubated for >24 h at a 5% hematocrit at 37°C in a buffer containing: 5 mM NaCl, 160 mM KCl and 5 mM Mg-TES, pH 7.4. Incubation of cells in such substrate-free solution for <24 h was not sufficient to result in net potassium loss when the cells were switched to buffer low in potassium and containing calcium.

Because cell water content changed rapidly during many of the incubations, cell ion contents and cell water are expressed in terms of the constant, kilograms cell solid. Millimoles per kilogram cell solid can be converted to millimoles per liter cell water by dividing by W_c, the cell water content (units = kilograms cell water/kilograms cell solid). Millimoles/liter erythrocytes are approximated by dividing the term millimoles per kilogram cell solid by $1 + W_c$. In our calculations, a correction factor of 2% for plasma trapping in the packed erythrocyte column is included. Trapping was determined independently with inulin. Normal values obtained in our laboratory for fresh erythrocytes in their own plasma are given in Table I.

RESULTS

To investigate the effect of cetiedil on erythrocyte membrane permeability to cations, we incubated fresh cells for 1 h in the absence and presence of cetiedil at concentrations ranging from 100 to 500 μM. The experiments were performed twice with cells from patients with homozygous sickle cell disease and twice with control cells. Values for Na_c and K_c are plotted in Fig. 1. Increasing concentrations of cetiedil cause the cells to become increasingly permeable to both sodium and potassium so that Na_c increases and K_c decreases. There was no measurable difference in the net cation movements observed when the effect of cetiedil on sickle erythrocytes was compared to the effect of the drug on normal erythrocytes. At 500 μM cetiedil, hemolysis was prominent with both cell types, approaching 50% in some experiments following 1 h of incubation.

The time-course of cetiedil's effect on net cation movements in normal erythrocytes is shown in Fig. 2. In the presence of 300 μM cetiedil, net sodium gain exceeds net potassium loss over the interval measured, and the cells swell. Cell swelling is so rapid with this concentration of cetiedil that a small, but measurable increase in cell water was observed even for the zero time samples.

TABLE I
Normal Values for Na_c, K_c, and W_c

	kg cell H_2O/kg cell solid	mmol/kg cell solid	mmol/liter cell H_2O	mmol/liter RBC
Na_c		23.2±2.3	12.6±2.0	9.1±1.5
K_c		236±6	129±2	82±2
W_c	1.873±0.017			

n = 25, ±SEM.
RBC, erythrocytes.

[1] *Abbreviations used in this paper*: K_c, cell potassium; Na_c, cell sodium; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; W_c, cell water.

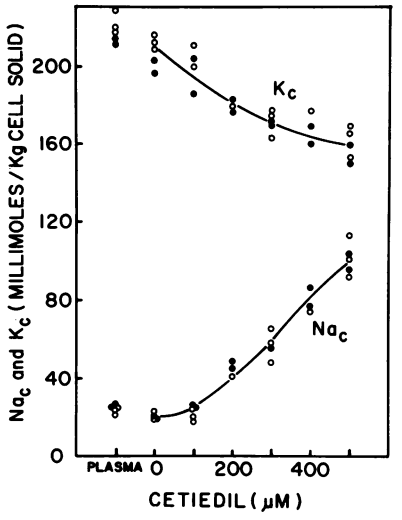

FIGURE 1 The effect of increasing concentrations of cetiedil on net changes in Na_c and K_c. Homozygous sickle cells (●) and control erythrocytes (○) were incubated in solutions containing 0–500 μM cetiedil as noted on the abscissa. After 1 h of incubation, cells were removed and assayed for Na_c and K_c contents, which are plotted on the ordinate. For comparison, values of Na_c and K_c obtained for fresh cells in their own plasma are also given. The curves are drawn by eye.

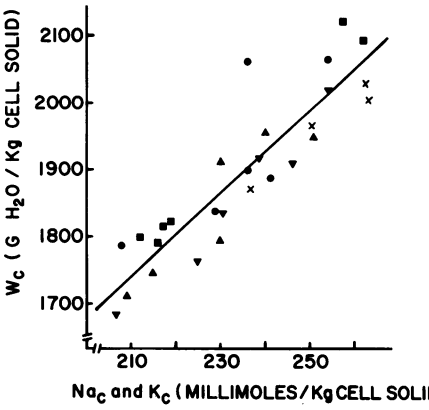

FIGURE 3 Cell water content, W_c, as a function of total cell cation content (Na_c plus K_c) after incubation with cetiedil. Results were taken from five separate experiments where cetiedil was present in different concentrations up to 500 μM and samples were taken at different intervals up to 60 min. Both normal and sickle cells were used for these experiments. The line was derived by linear regression analysis and is described by the equation: $W_c = 5.7\ (Na_c + K_c) + 560$. The reciprocal of the slope shows that a 175-mM salt solution is taken up by the cells as they swell. The regression coefficient is $r = 0.943$.

Fig. 3 illustrates that cell swelling with cetiedil is a direct consequence of net salt gain. Cell water content increases in a linear fashion as the sum content of Na_c plus K_c increases. The concentration of the salt solution taken up, derived from the reciprocal of the slope of the curve in Fig. 3, is 175 mmol/liter cell water. This is very nearly an isosmotic accumulation. The cation directly responsible for cell swelling under these circumstances is sodium, since the cells are simultaneously losing potassium (Figs. 1 and 2).

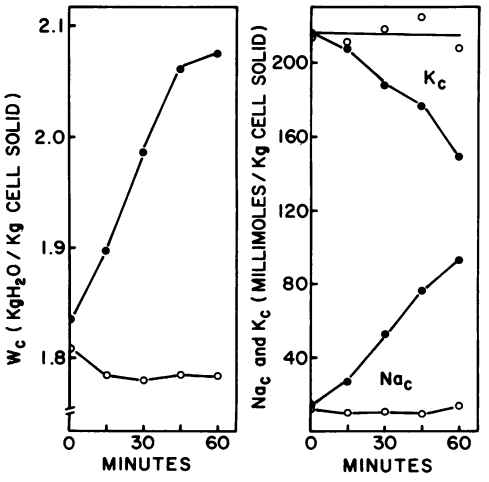

FIGURE 2 Time-course of the response to cetiedil. Normal erythrocytes were incubated in the absence (○) and presence (●) of 300 μM cetiedil. Mean cellular contents of W_c, Na_c, and K_c for cells in their own plasma were, respectively, 1.818 kg/kg cell solid, 18.9 mmol/kg cell solid, and 232 mmol/kg cell solid

We next investigated the effect of ouabain, an inhibitor of active Na-K transport (8) on the net cation changes that occur with cetiedil. Results from experiments with fresh erythrocytes containing normal ATP are illustrated in Fig. 4. With 100 μM ouabain present along with 300 μM cetiedil, cells accumulate sodium and lose potassium at rates that are significantly greater than those observed with cetiedil alone. In both instances, Na_c gain exceeds K_c loss, and the cells swell increasing their cell volume by 9% in the absence of ouabain and 15% in the presence of ouabain after 30 min. In the absence of cetiedil, ouabain-treated cells were stable with respect to Na_c, K_c, and W_c over the time period investigated. Similar results were obtained with sickle erythrocytes.

Results presented thus far demonstrating the effect of cetiedil on fresh erythrocytes are nearly opposite to those reported for cells depleted of their ATP by pretreatment with inosine and iodoacetic acid (9). To investigate this phenomenum further, we chose the alternative method of depleting cells of their ATP by prolonged incubation in substrate-free solutions containing high potassium. Next, we incubated the cells in solutions containing low potassium, 2 mM calcium, and different concentrations of cetiedil. Under these conditions, cells not exposed to cetiedil lose potassium

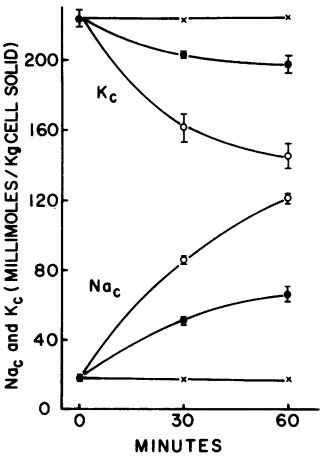

FIGURE 4 The effect of ouabain on cetiedil-induced changes in Na_c and K_c in normal erythrocytes. Incubations include 100 μM ouabain (X), 300 μM cetiedil (●), and ouabain plus cetiedil (O). After 1 h incubation, cells with cetiedil alone gained 167±11 g H_2O/kg cell solid while cells with cetiedil plus ouabain gained 282±8 g H_2O/kg cell solid. Cell volume was unchanged with ouabain alone. Results shown were obtained with cells from a single normal donor (n = 3±SEM). Comparable results were obtained with cells from a patient with sickle cell disease.

at a rapid rate (Fig. 5). This immediate potassium efflux is slowed dramatically by increasing concentrations of cetiedil, confirming an earlier report (9) that cetiedil inhibits the so-called "Gardos effect" (10). Under these conditions of nearly absent intracellular ATP, ouabain was without effect (data not shown).

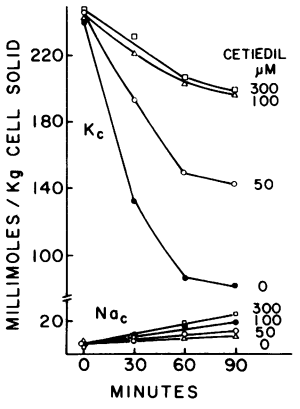

FIGURE 5 Effect of cetiedil on Na_c and K_c for normal erythrocytes depleted of ATP. Cells were incubated 30 h in substrate-free solutions containing high potassium, as described in Methods. Cells were then washed twice with physiological saline and test incubated in solutions containing: 5 mM KCl, 2 mM $CaCl_2$, 5 mM Mg-TES pH 7.4 at 37°C, 160 mM NaCl and different cetiedil concentrations. At the start of the test incubation, cells contained 133 μmol ATP/kg cell solid, or ~4% of their initial ATP.

Two additional points are apparent from consideration of Fig. 5. First, although increasing concentrations of cetiedil promote net Na_c gain, the effect is trivial compared to the striking net Na_c gain observed when cetiedil is added to fresh erythrocytes (compare Fig. 2). Second, maximal inhibition of net K_c loss in ATP-depleted cells occurs by 100 μM cetiedil, a drug concentration that has minimal effect on fresh erythrocytes after 1 h incubation (compare Fig. 1).

One major difference in the experimental design for fresh and ATP-depleted erythrocytes is that incubations with energy-depleted cells contained calcium to facilitate the Gardos effect (10) while those with fresh cells contained no calcium. To explore a possible role for external calcium in preventing permeability increases to sodium caused by cetiedil, experiments with ATP-depleted erythrocytes were repeated in the presence and absence of calcium. Results of a representative experiment are illustrated by Fig. 6. In the absence of external calcium, cells exposed to cetiedil (open squares) lose little potassium, and net sodium gain is only slightly increased over that observed with calcium present. Although such stimulation of net sodium gain is reproducible upon removing calcium from the incubation, it is much smaller than that observed when fresh erythrocytes are treated with cetiedil (open triangles).

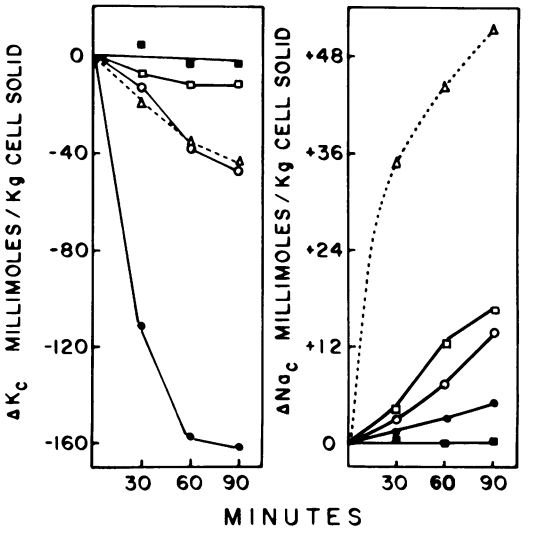

FIGURE 6 Effect of calcium (2 mM) on net changes in Na_c and K_c in the presence of cetiedil (300 μM). A portion of the normal cells was preincubated 32 h (●, O, □) as described in Methods to reduce intracellular ATP to a mean level of 82 μmol ATP/kg cell solid. Fresh cells (■, △) contained a mean level of 3,080 μmol ATP/kg cell solid. Legend: ■, high ATP control; ●, low ATP + Ca; O, low ATP + Ca + cetiedil; □, low ATP + cetiedil (absent Ca); and △, high ATP + cetiedil (absent Ca).

The inhibitory effect of calcium on cetiedil-induced changes in net sodium influx is much more apparent in fresh erythrocytes (Fig. 7). Cells were incubated 1 h with 300 μM cetiedil and different concentrations of calcium. As external calcium is increased, cetiedil-induced net Na_c gain and K_c loss are both inhibited. With 5 mM external calcium, there is little net change in cell cation content, and cell volume is stable.

DISCUSSION

Since formulation of the equations of Hofrichter and co-workers (5), it has become evident that one way to slow the rate of sickling is to dilute the intracellular hemoglobin. Under normal circumstances, the erythrocyte maintains a constant volume, and thus constant hemoglobin concentration, by balancing the passive movements of sodium into and potassium out of the cell with the active extrusion of sodium and active uptake of potassium via the ATP-dependent, Na-K coupled pump (11). Water and small anions are, for practical considerations, at equilibrium. In theory, intracellular hemoglobin concentration can be altered by three different general mechanisms involving the cell membrane. These are: (*a*) changes in plasma osmolality, (*b*) changes in membrane permeability to cations, and (*c*) changes in the efficiency of the pump in maintaining the erythrocyte at a steady state.

Several clinical trials of sickle cell disease have been carried out that were designed to lower intracellular hemoglobin concentration by lowering plasma osmolality. In one such trial, intravenous administration of distilled water to two patients with sickle cell trait and gross hematuria successfully stopped the hematuria, presumably by reversing sickling in the hypertonic renal medulla (12). In another trial, vigorous intravenous hydration with hypotonic fluids was claimed to shorten the recovery phase from acute vaso-occlusive crises (13). More recently, the chronic administration of 1-desamino-8-D-arginine vasopressin, which lowers plasma sodium and thus osmolality, was shown to decrease mean hemoglobin concentration and to protect susceptible patients from vaso-occlusive crises (14).

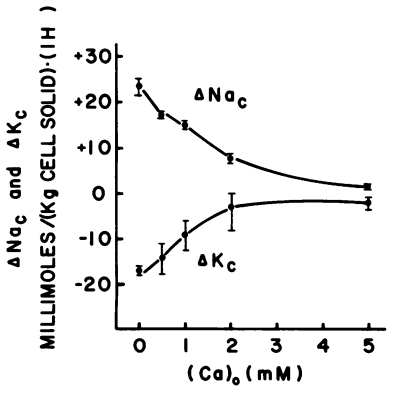

FIGURE 7 Effect of calcium on net changes in Na_c and K_c in normal fresh erythrocytes incubated with cetiedil (300 μM) for 1 h. At zero time, cells contained: 12.6±0.5 mmol Na_c/kg cell solid, and 226±5 mmol K_c/kg cell solid. Error bars denote SEM, $n = 4$.

The manipulation of the Na-K pump or of cell membrane cation permeability specifically to prevent sickling represents an area that to date remains largely unexplored. Cetiedil is the first drug to be reported that inhibits erythrocyte sickling by acting directly on the cell membrane, causing cell swelling (3). The results of this investigation show that cetiedil increases cell volume in fresh erythrocytes by promoting net salt gain. Although cetiedil causes increased fluxes of both sodium and potassium, net Na_c gain exceeds net K_c loss, and rapid cell swelling results (Fig. 2). Such excess of Na_c gain over K_c loss occurs over the entire range of cetiedil concentrations tested (Fig. 1). In all cases, sufficient salt is accumulated to account for net water gain (Fig. 3), and there is no need to invoke alternative mechanisms for cell volume increases. The concentration of cetiedil required to produce measurable changes in membrane permeability (>100 μM, Fig. 1) in fresh cells compares favorably with the drug concentration required for inhibition of sickling in vitro (3, 4). Approximately 2 mg cetiedil/kg body wt would be required to achieve intravascular concentrations of 100 μM in vivo. If administered as a single intravenous dose, this would require 10 times more drug than is given typically to human subjects (1). However, little is known about the metabolism and excretion of cetiedil, and blood levels have not been reported. Single intravenous injections of 2 mg cetiedil/kg body wt have been administered successfully to animals (2).

Studies performed to help clarify the mechanism of cetiedil's action on normal erythrocytes are thus far inconclusive. Both sodium uptake and potassium loss are further enhanced when ouabain is added to fresh cell suspensions containing cetiedil (Fig. 4). This finding is consistent with a hypothesis that cetiedil acts at some membrane locus separate from that occupied by the Na-K pump. Thus, with ouabain absent and the pump functioning, the cell extrudes some of the sodium gained and takes up some of the potassium lost in the presence of cetiedil, similar to what would be expected if cetiedil were simply acting like an ionophore, making the membrane leaky to sodium and potassium. This hypothesis becomes inadequate however, when the effects of calcium and removal of ATP are considered. In the absence of intracellular ATP, cetiedil has surprisingly little effect on net cation fluxes as long as extracellular calcium is omitted from

the incubation (Fig. 6). Whether this lack of effect is due to a nonfunctioning pump, substrate depletion, or inhibitor accumulation remains to be determined. Normal erythrocytes depleted of their ATP undergo a well-described loss of KCl and water that depends on extracellular calcium (10). The efficacy of cetiedil in blocking net KCl loss that we observe under such conditions (Fig. 5) confirms an earlier report of the inhibitory nature of the drug (9). The physiological significance of such inhibition in the absence of ATP remains to be determined. In our experiments, it was necessary to deplete the cells of >90% of their ATP before inhibition, rather than stimulation, of net fluxes by cetiedil was achieved. Inhibition of the calcium-induced potassium fluxes by cetiedil in ATP-depleted cells (Figs. 5, 6), and reciprocal inhibition of cetiedil-induced net cation fluxes by calcium in ATP-replete cells (Fig. 7), may suggest a common membrane site for the action of calcium and cetiedil. Experiments are now underway to determine whether extracellular or intracellular calcium is the important variable.

Clearly, the mechanism of action for cetiedil's effect on cation transport remains unknown. Before any definitive statements can be made about the site of action of the drug, unidirectional flux studies must be carried out with and without ouabain and with and without intracellular ATP. In addition, interpretations of mechanism of action obtained with normal erythrocytes will need to be extended to sickle cells. The effect of the drug on leukocytes and platelets remains to be elucidated. We believe that further studies with cetiedil and related compounds will not only prove useful in the search for anti-sickling agents, but will also help clarify the basic physiology of the erythrocyte membrane transport system.

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