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PASSAGE OF PYRIDINIUM OXIMES INTO HUMAN RED CELLS

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Abstract—Contrary to popular views, pralidoxime chloride, a positively charged oxime and a drug used to treat poisoning from organophosphorus insecticides, penetrates the red cell membrane. Two other therapeutically related drugs that have similar dissociation constants and chemical function groupings penetrate to negligible extents. Pralidoxime chloride did not bind to either red cell stroma or hemoglobin. The rate of entry into red cells by pralidoxime chloride did not change when the pH of blood was changed by ±0·6 pH unit. Since the rate of entry of pralidoxime chloride into red cells from plasma was identical to the rate at which oxime passed from red cells into plasma, the process was likely due to simple diffusion. In addition, the fraction of pralidoxime chloride entering the red cells was independent of drug concentration. The penetration was not affected by active transport inhibitors, such as ouabain or *N*-methyl maleimide. The selective penetration could not be related to lipid solubility.

CONSIDERING the therapeutic role of the pyridinium oximes in managing intoxication caused by agents which inhibit cholinesterase, we investigated their distribution in blood as separated plasma and red cells. Three oximes were used in the study: pralidoxime chloride (2-PAMCI; *N*-methylpyridinium-2-aldoxime chloride), *N,N'*-trimethylenebis(pyridinium-4-aldoxime)dibromide (TMB-4 bromide) and *N,N'*-oxydimethylenebis(pyridinium-4-aldoxime)dichloride (toxogonin chloride; LUH-6). Since the erythrocyte membrane behaves as a lipid-like barrier to the penetration of organic compounds, and positively charged, lipid-insoluble compounds like hexamethonium and tetramethylammonium enter the red cell slightly, if at all,[1,2] the positively charged oximes would not be expected to pass into the red cell. A recent study by Simon and Edery[3] indicated they do not. However, in our report, we show conclusively that only 2-PAMCI enters the red cell in therapeutic concentrations and that the other two positively charged oximes penetrate in negligible concentrations. This observation contradicts a report by Erdmann and Engelhard[4] indicating that toxogonin is found in erythrocytes in concentrations equal to that of plasma. In our work, in which oximes were measured in blood of human subjects* receiving the drugs, our findings *in vitro* were confirmed. Forces responsible for the selective penetration of red cells by 2-PAMCI were investigated.

METHODS

Studies in vivo *with human subjects—oral administration.* Six subjects were given oral doses of 2-PAMCI, ranging from 41 to 96 mg/kg. Heparinized whole blood samples, collected from each subject prior to testing, served as controls. Doses of 89–

* The subjects in these tests are enlisted U.S. Army personnel. These tests are governed by the principles, policies and rules for medical volunteers as established in AR 70–25, and the Declaration of Helsinki.

121 mg/kg of LUH-6, calculated to produce plasma levels equivalent to those produced by 2-PAMCI, were given to four other subjects.[5] Blood samples were withdrawn at timed intervals. Plasma and hemolyzed red cells were analyzed for respective oxime concentrations in an automated system.[6] TMB-4 was not administered to human subjects because of reported side effects caused by this oxime.

Entry of oximes added to whole blood into red blood cells in vitro. 2-PAMCI was added to fresh human heparinized whole blood to give final concentrations ranging from $6{\cdot}3 \times 10^{-6}$ to 2×10^{-4} M (1–35 μg/ml). The whole blood sample was immersed in a shaking bath at 37°. An aliquot was withdrawn and centrifuged for 10 min at 2500 rev/min. Hematocrits were taken of all samples prior to separation of the blood. The separated plasma was removed and saved for analysis, while the separated red cells were washed twice with equal volumes of isotonic saline. The washed red cells were hemolyzed with 0·01% saponin. Aliquots of plasma, hemolyzed red cell and whole blood were assayed for oxime concentration by the automated procedure.[6] At timed intervals additional aliquots were removed from the whole blood sample, the plasma and red cells were separated and the three samples assayed for oxime concentration. Concentrations of TMB-4 and LUH-6 were also added to whole blood. Separated plasma and red cell concentrations of each oxime were also measured by the automated method. Wavelengths of 336, 345 and 353 nm were used to determine 2-PAMCI, TMB-4 and LUH-6 respectively. Similar studies were carried out with 2-PAMCI in blood which had been adjusted with small volumes of acid and alkali to pH values 6·8 and 8·0. pH readings were taken throughout the experiment to ascertain that pH changes did not occur.

Passage of 2-PAMCI from red blood cells into added plasma in vitro. A large sample of heparinized human whole blood was divided into two separate portions, A and B. 2-PAMCI was added to A and the mixture incubated in a water bath at 37° for 4 hr. Sample A was then centrifuged and the plasma discarded. The red blood cells were washed twice with isotonic saline and the oxime concentration in the hemolysate was measured. Sample B was centrifuged and the red cell portion discarded. A volume of plasma from B equal to that removed from A was added to the separated washed red cells containing 2-PAMCI. This simulated whole blood mixture, C, approximating the hematocrit of the original blood sample, was incubated at 37° for periods up to 10 hr. Oxime assays of separated plasma and hemolyzed red cells from sample C were performed at hourly intervals.

Preparation of erythrocyte stroma. A sample of whole blood was centrifuged for 20 min at 1200 rev/min at 4°. The plasma was removed and the cells were resuspended in 0·05 M Tris-buffered saline at pH 7·4. The mixture was centrifuged for 20 min at 2500 rev/min at 4°. The wash procedure was repeated three times. The absorbance of the last wash was measured at 280 nm to detect the presence of protein. The cells were hemolyzed with 2·5 times their volume of distilled water. The mixture was centrifuged for 30 min at 3000 rev/min at 4°. A pellet of stroma formed. The stroma was washed twice with the Tris-buffered saline. The washed stroma pellet was then resuspended in a measured volume of diluent. 2-PAMCI in a final concentration of 2–4 μg/ml was added to the stroma. The mixture was centrifuged and the supernatant measured for oxime concentration by the automated procedure. Similar experiments were performed on the hemoglobin fraction in which final concentrations of 1·5 to 10 μg/ml were prepared and then assayed.

Distribution of undissociated and dissociated forms of the oximes in octanol. 2-PAMCI, TMB-4 and LUH-6 have similar dissociation constants, p*K*a values of approximately 8.[7,8] Octanol was saturated with phosphate buffer, pH 7·4 (0·1 M) and with water at pH 2 and pH 11. The aqueous and nonaqueous phases were separated by centrifugation and used in corresponding distribution studies.

To determine ultraviolet spectra of the oximes in octanol at pH 2, 7·4 and 11, aqueous solutions were prepared and brought to the respective pH value with microliter volumes of 5 N acid or base. An appropriate dilution was made in ethanol which was then added to octanol to obtain final concentrations of 10 μg/ml.

To determine the solubility of the three pyridinium oximes in octanol at physiological pH, concentrations were prepared in pH 7·4 buffer that had been saturated with octanol. The pH, which dropped to 7·1, was readjusted to 7·4 with microliter amounts of 5 N alkali. One-half ml of aqueous solution at a final concentration of 15 mg/ml and 5·0 ml octanol were shaken for 1·5 hr at 25°. The phases were separated by centrifugation. The pH of the aqueous phase was redetermined and the u.v. spectrum of the octanol phase was obtained.

Similar partition studies at pH 7·4 were performed with octanol to which lecithin was added in concentrations of 1·25, 2·5, 5·0 and 10 mg/ml.

Influence of ouabain and N*-ethylmaleimide on the penetration of oxime into red cells.* A 50-ml sample of fresh heparinized human whole blood was divided into three equal portions. Each portion was placed in an Erlenmeyer flask containing a magnetic mixing bar. Ouabain and *N*-ethylmaleimide (NEM) were added to separate flasks to give final concentrations of 5×10^{-4} M. Nothing was added to the third (control) flask. All flasks were maintained at 37° with continuous agitation. After 15 min, 2-PAMCI was added to each of the three flasks to give an oxime concentration of 10 μg/ml. Three-ml aliquots were removed from each flask at 0·5, 1, 2 and 3 hr after the addition of the 2-PAMCI. The aliquots were immediately centrifuged for 10 min at 2500 rev/min. The plasma was separated from the red cells. The red cells were then washed twice with isotonic saline, centrifuged again for 10 min at 2500 rev/min and the wash solution was removed from the cells. Plasma and saponin hemolyzed red cells were then assayed separately for oxime content.

RESULTS

Studies in vivo *with human subjects—oral administration.* Maximum concentrations of 2-PAMCI appeared in the plasma of most subjects after approximately 2 hr, while maximum levels appeared in the red cells after 3 hr (Table 1 is representative of four similar tables). Even after 6 hr, a therapeutic concentration was found in the erythrocytes. After 5 hr, the 2-PAMCI concentration in the red cell was equal to or greater than the oxime concentration in plasma. Only traces of LUH-6 appeared in the red cell after 3 hr (less than 0·2 μg/ml). TMB-4 was not administered because of concern for its toxicity.

Entry of oxime added to whole blood into red blood cells. 2-PAMCI initially distributes exclusively in the plasma portion of whole blood. The concentration of 2-PAMCI in separated plasma decreases with time, while the concentration of oxime in red cells increases (Table 2). The first red cell saline wash contains some oxime; the second, negligible amounts. The sum of the concentrations of 2-PAMCI in the plasma, red cell saline washes, and hemolyzed red cells was found to be nearly equal

TABLE 1. CONCENTRATIONS OF OXIMES IN THE PLASMA AND RED CELL IN HUMAN SUBJECTS

	Oral			
	Pralidoxime*		Toxogonin†	
Time (hr)	Plasma (μg/ml)	Red cell (μg/ml)	Plasma (μg/ml)	Red cell (μg/ml)
0·5	4·6	1·5	2·4	0
1	6·6	1·9	5·1	0
2	7·9	3·8	6·5	0
3	6·7	4·6	6·5	<1
4	4·8	4·3	4·9	<1
5	3·4	4·2	3·6	<1
6	2·3	4·0	2·8	<1

* Dose = 56·2 mg/kg.
† Dose = 120·7 mg/kg.

to the initial concentration of oxime in the whole blood. The rate of decrease of 2-PAMCI in concentrations ranging from $6{\cdot}3 \times 10^{-6}$ M to $2{\cdot}4 \times 10^{-4}$ M during the first 4 hr follows a pseudo first-order process (Fig. 1); that is, at all oxime concentrations, 0·26 per cent (k^1 equals $2{\cdot}6 \times 10^{-3}$ min^{-1}) of the oxime in the plasma enters into the red cell per min. The rate decreases until an equilibrium is reached. In similar studies in which TMB-4 and LUH-6 were added to whole blood, the plasma and red cell levels of the two oximes changed by small amounts (Table 2). Whereas 46 per cent of 2-PAMCI which was added to whole blood entered the red cell after 7 hr, less than 2 per cent of either TMB-4 or LUH-6 entered the red cell after a similar period of time.

The pH of the whole blood was adjusted to 6·8 and 8·0 to determine whether the dissociated or undissociated form of 2-PAMCI (p*K*a of 7·8)[7] had entered the red cell selectively. The oxime molecules are positively charged at all pH values. At pH values below 7·8, 2-PAMCI is relatively undissociated so that the total charge on the oxime molecule becomes +1. At pH values above 7·8, the oxime moiety becomes dissociated (negatively charged) so that the net charge in the molecule is zero.

The dissociated or oximate (zwitterion) form would be expected to be more lipid soluble and to penetrate the red cell membrane to a greater extent than the undissociated form; however, the rate of penetration into red cells at these pH values was identical to the rate of physiological pH (Fig. 2).

TABLE 2. DISTRIBUTION OF 2-PAMCI, TMB-4 AND LUH-6 ADDED TO WHOLE BLOOD*

	Plasma			Saline wash			Hemolyzed RBC		
Time (hr)	2-PAMCI	TMB-4	LUH-6	2-PAMCI	TMB-4	LUH-6	2-PAMCI	TMB-4	LUH-6
0·5	4·8	11·5	12·6	0·3	0·7	0·7	0·6	0·0	0·2
1	4·6	11·5	12·2	0·3	0·8	0·7	0·9	0·0	0·2
2	4·2	11·7	12·3	0·4	1·0	0·7	1·2	0·0	0·1
4	3·6	11·9	12·3	0·3	1·0	0·5	1·7	0·0	0·1
7	3·4	11·4	12·3	0·3	1·1	0·6	2·3	0·3	0·3

* All values are expressed in μg/ml and are corrected for hematocrit. Initial concentrations of 2-PAMCI, TMB-4 and LUH-6 were $2{\cdot}9 \times 10^{-5}$ M.

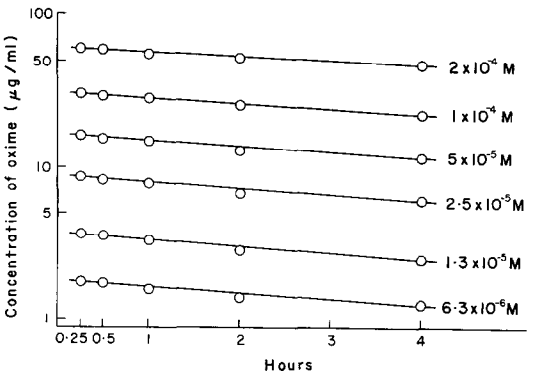

FIG. 1. Decrease in concentrations of 2-PAMCI from the plasma of whole blood into the red cells.

Passage of 2-PAMCI from red cells into added plasma. Prior to assay, the red cells were washed twice with saline. The second wash contained 0·1 μg/ml or less of oxime. The oxime in the red cell passes into the plasma. The rate constant for the efflux of 2-PAMCI from the red cell into the plasma is identical to the rate constant for the uptake of oxime by red cell from the plasma during the first 4 hr. The rate decreases until an equilibrium is reached.

Stroma and hemoglobin binding. The concentration of 2-PAMCI, as determined by the automated oxime assay,[6] in the supernatant of both stroma and hemoglobin solutions was identical to the concentration originally added. In this analytical system, protein or protein-bound material is excluded from analysis by a dialysis system. Therefore, one may conclude that 2-PAMCI did not bind either to red cell membrane or to hemoglobin.

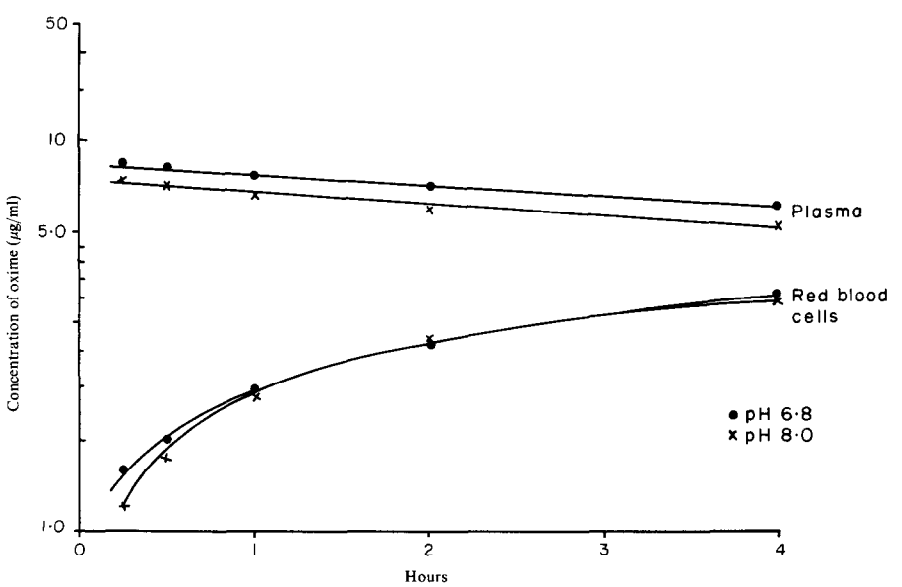

FIG. 2. Effect of pH on the disappearance of 2-PAMCI ($5{\cdot}6 \times 10^{-5}$ M) from plasma into erythrocytes.

TABLE 3. MOLAR ABSORPTIVITIES OF PYRIDINIUM OXIMES

Oxime	Form*	Wavelength max. (nm)	Absorptivity ($\epsilon \times 10^{-4}$)
		Octanol	
2-PAMCI	U	300	1·35
	D	358	1·97
TMB-4	U	290	3·43
	D	358	2·54
LUH-6	U	292	3·41
	D	362	2·55
		Water	
2-PAMCI	U	292	1·24
	D	336	1·83
TMB-4	U	281	3·39
	D	345	4·82
LUH-6	U	283	3·30
	D	353	5·05

* U = undissociated form; D = dissociated form.

Distribution of pyridinium oximes in octanol. Data on molar absorptivity of the undissociated and dissociated forms of the three oximes in water and octanol are shown in Table 3. In octanol, bathochromic shifts to 300 and 362 nm occur. Because equal concentrations of the undissociated and dissociated forms of 2-PAMCI are found at pH 7·4, the p*K*a of 2-PAMCI in octanol must have decreased from 7·8 in water to 7·4.

At pH 7·4, the dissociated form of 2-PAMCI partitions in octanol, while the oximate forms of TMB-4 or LUH-6 do not. The undissociated forms of all three oximes distribute in octanol at physiological pH (Table 4).

In order to determine further whether 2-PAMCI was transported across the cell membrane, the distribution of the three oximes was studied at pH 7·4 in octanol and in octanol containing added amounts of the phospholipid, lecithin. Quaternary ammonium ions can form ion pairs with organic anions,[9] lecithin with the oximate form of 2-PAMCI. Possibly this type of complex would enhance the transfer of 2-PAMCI, but not the other oximes. All concentrations of lecithin in octanol caused

TABLE 4. PARTITION COEFFICIENTS OF PYRIDINIUM OXIMES IN OCTANOL AND pH 7·4 BUFFER*

Lecithin (mg/ml)	2-PAMCI		TMB-4		LUH-6†	
	U	D	U	D	U	D
	Octanol/buffer ($\times 10^{-3}$)					
	2·28	2·30	1·96	0	0·56	0
	Octanol with lecithin/buffer ($\times 10^{-3}$)					
1·25	2·30	2·68				
2·5	2·39	2·68				
5·0	2·55	2·47	2·38	0	0·50	0
10·0	3·64	2·90				

* U = undissociated form; D = dissociated form.
† The dissociated form is unstable at pH 7·4.

a 17 per cent increase in the distribution of the dissociated form of 2-PAMCI. However, the distribution of the undissociated form of 2-PAMCI increased with increasing concentration of phospholipid.

The rates of penetration of red cell membrane by 2-PAMCI which was added to whole blood and to whole blood containing 5×10^{-4} M ouabain and NEM were identical.

DISCUSSION

The ability of three functionally and structurally related compounds to penetrate the erythrocyte membrane was investigated. All have, in addition to a quaternary pyridinium ion, a dissociable oxime with nearly identical p*K*a values, and all are excellent reactivators of inhibited cholinesterase.[10] Of the three, only 2-PAMCI penetrates the red cell to an appreciable extent. The investigations *in vitro* were corroborated by studies in which two of the three oximes were administered orally to human subjects. Appreciable concentrations of 2-PAMCI, but only trace amounts of LUH-6, were found in red cells of the subjects.

After 2-PAMCI was added to whole blood, essentially all the oxime was concentrated in the plasma. The initial rate of decrease of 2-PAMCI from plasma into red cells was found to be equal to the rate of decrease of 2-PAMCI from red cells into plasma. The exchange is pseudo first-order: at any selected time interval and oxime concentration, the fraction of drug in the red cell to that in the plasma is constant. The 2-PAMCI did not bind either to red cell membrane or to hemoglobin.

The effect of active transport inhibitors such as ouabain and *N*-ethylmaleimide (NEM) were also studied. Ouabain, a specific inhibitor of active cation transport into and out of the cell, did not affect the rate of 2-PAMCI penetration. NEM, a compound which interacts with sulfhydryl compounds that could facilitate active transport, also had no effect on the rate of 2-PAMCI penetration.

In order to assign some type of mechanism to the selective penetration of 2-PAMCI, the solubilities and distribution of the dissociated and undissociated forms of the three oximes were studied in octanol and in octanol to which selected concentrations of lecithin were added. Lecithin, being a phospholipid with a quaternary ammonium group, might form ion pairs with the anionic species of 2-PAMCI at physiological pH.

Of the three oximes, only the dissociated form of 2-PAMCI was soluble in octanol. However, the undissociated form of all three pyridinium oximes was distributed in the octanol phase. All concentrations of lecithin added to octanol equally enhanced the solubility of the dissociated form of 2-PAMCI. The distribution of the undissociated form of 2-PAMCI increased with increasing concentrations of phospholipid. The distribution data, although interesting, do not indicate the reason why only 2-PAMCI singularly penetrates red cell *in vivo* and *in vitro*.

Our studies also show that only 2-PAMCI penetrates red blood cells at identical rates at pH values of 6·8, 7·4 and 8·0. These data negate the widely accepted concept that the penetration of red cell membrane by a drug is related to its dissociation constant. The hypothesis that the erythrocyte membrane acts as a selective lipid-like barrier to the penetration of organic compounds is also not supported by this study.

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