

Intraerythrocyte pH and Physiochemical Homogeneity¹ (40299)

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Intraerythrocytic pH is a major determinant of glycolytic metabolism, membrane function, and oxygen dissociation. We have investigated the possibility that in a physiochemical sense these functions are controlled by separate pH environments within the red cell.

Caldwell (1), Adler *et al.* (2), and Waddell and Bates (3), have demonstrated that the results of the determination of intracellular pH (pH_i) by the distribution of a weak base differs from that determined by the distribution of a weak acid in a heterogeneous system. Using a weak acid, the pH_i closer to the highest pH of the various intracellular compartments is recorded, while a weak base reflects a value closer to the lowest pH of the compartments. If the pH_i as determined by a weak acid and a weak base are identical, then the cell interior is likely to be homogeneous (1). Using rat diaphragm muscle Adler (4) showed that the pH_i measured by the weak acid 5,5-dimethylloxazolidine-2,4-dione (DMO) was significantly higher than the pH_i measured by the weak base, nicotine. Physiochemical inhomogeneity, therefore, was demonstrated, as one might expect on morphological grounds. Using the same weak acid and weak base, we have investigated the pH_i of the human erythrocyte.

Methods. Venous blood was mixed with 14.3 μg of heparin per ml, centrifuged, and the buffy coat removed. The erythrocytes were resuspended in their own plasma.

Radioactive DMO, 5,5-dimethylloxazolidine-2,4-dione-[2-¹⁴C] (New England Nuclear, Boston, MA), specific activity 11

mCi/mmole, was added to give a final concentration of 0.00066 mg/ml of blood. Radioactive nicotine, nicotine-methyl-[¹⁴C] (Mallinckrodt Corp., St. Louis, MO), specific activity 2.41 mCi/mmole, was added to a second aliquot of blood to give a final concentration of 0.011 mg/ml. Both tubes of blood were incubated at 37° for 20 min, a time we determined experimentally as adequate for both DMO and nicotine to reach equilibrium. The pH, pH_e , of each aliquot was measured to the nearest 0.01 unit using a Corning model 165 pH blood gas analyzer (Corning Scientific Instruments, Medfield, MA). The aliquots were centrifuged, the plasma removed and a microhematocrit corrected for trapped plasma [1.31% (5)] determined on the red cell packs. One ml of packed cells or one ml of plasma containing either nicotine-¹⁴C or DMO-¹⁴C was added to 5 ml of deionized water. Each sample was prepared in quadruplicate. One ml of each mixture was pipetted into 2.0 ml of 10% trichloroacetic acid (TCA), centrifuged, and 1 ml of the supernatant counted in a Packard Tri-Carb Liquid Scintillation Counter Model 3320. Counts per minute were converted to disintegrations per minute by use of a quench curve.

Binding studies. To investigate the possible binding of DMO to human plasma, the Hummel-Dryer technique (6) was utilized. Four ml of normal heparinized (14.3 $\mu\text{g}/\text{ml}$) plasma were incubated with 0.01 ml of DMO-[¹⁴C] at 37° for 30 min and an aliquot layered on to a G-25 Sephadex column with a 75 ml void volume after balancing the column with DMO-[¹⁴C] in heparinized (15 $\mu\text{g}/\text{ml}$) phosphate buffered saline. Aliquots of each fraction were counted and the optical density determined at 280 nm.

Binding studies of nicotine to human plasma and erythrocytes were also conducted. Ten ml of heparinized venous blood were incubated with 0.3 mg of nicotine-[¹⁴C] in 0.1 ml of 0.9% sodium chloride while another 10

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ml were allowed to incubate with the same amount of radioactive nicotine plus 0.85 mg of cold nicotine contained in 50 μ l of 0.9% sodium chloride. Three determinations were made from each tube of the ratio of intracellular to extracellular dpm per g of water.

Calculations. The external dpm for both DMO and nicotine determinations were converted to dpm/g of plasma water using 0.94 as the fraction of solvent water in plasma (7).

The internal dpm for both DMO and nicotine determinations were converted to dpm/g of erythrocyte water after correction for the trapped plasma in the erythrocyte pack, utilizing 0.59 as the fraction of solvent water in the erythrocyte (8-11).

The dpm/g of plasma water and cell water are then entered into the appropriate formula (12).

$$pH_i = pK'_a + \log \left[\frac{(DMO)_i (10^{pH_e - pK'_a} + 1) - (DMO)_e}{(DMO)_e} \right]$$

The pK'_a of DMO is 6.13 (12).

$$pH_i = pK'_a - \log \left[\frac{(\text{nicotine})_i (10^{pK'_a - pH_e} + 1) - (\text{nicotine})_e}{(\text{nicotine})_e} \right]$$

The pK'_a of nicotine was chosen as 7.85, the value used by Effros and Chinard (13).

The pH_e used was the average of the two measured values, which never differed by more than 0.01 of a pH unit. There was no significant difference between the pH_e values measured in the tube containing DMO versus the tube containing nicotine.

Results. The DMO and plasma binding study showed no rise or subsequent fall in dpm occurring in association with the collection of the plasma protein peak. Thus, there was no evidence of binding of DMO to human plasma.

The studies done to evaluate the possibility of nicotine binding to human plasma or erythrocytes showed that the ratio of internal to external dpm per g of water was identical at the two widely different concentrations of nicotine. This is strong evidence against binding or active transport of nicotine as well as evidence against the permeability of the erythrocyte membrane to the nicotine ion (14).

Table I shows the results of the pH_i deter-

minations using both DMO and nicotine. Four determinations using each indicator were carried out for each normal sample. The range of the four values falls within 0.16 of a pH unit. There is no significant difference by analysis of the 5 pairs of DMO and nicotine results.

Discussion. Caldwell (1) stated that if the values for pH_i obtained from multiple different indicator methods are in agreement it is probable that the cell interior is "reasonably uniform". Waddell and Bates (3), using a current operational definition of pH, stated that in an inhomogenous system, pH_i calculated from the distribution of a weak acid yields a pH value closer to the higher value, and that pH_i calculated from the distribution of a weak base yields a result closer to the lower pH value in that inhomogenous system.

Accurate determinations of pH_i using such indicators depend upon the absence of binding, the absence of active transport, and the impermeability of the cell membrane to the ionic species of the weak acid or weak base used. Waddell and Butler (15) demonstrated that DMO is not significantly bound to bovine serum albumin. Calvey (16) showed that DMO is not bound to, or actively transported by, rabbit erythrocytes. Bromberg *et al.* (17) showed that human erythrocytes do not bind DMO. We have demonstrated that DMO is not bound to human plasma and that nicotine meets the three criteria for accurate indicator compounds set forth above.

We have chosen 0.59 gm $H_2O/100$ ml of

TABLE I. pH_i (AVERAGE APPEARS ABOVE THE RANGE) DETERMINED BY DMO AND NICOTINE IN HUMAN ERYTHROCYTES.

Sample	pH_e	DMO	Nicotine
A	7.325	7.06 6.98-7.14	6.99 6.98-7.03
B	7.360	7.12 7.07-7.17	7.04 7.01-7.08
C	7.335	7.15 7.08-7.20	7.09 7.07-7.12
D	7.355	7.09 7.06-7.12	7.11 7.09-7.14
E	7.325	7.09 7.07-7.12	7.05 6.99-7.14
No significant difference ^a			

^a Calculations based on paired data analysis on five pairs.

cells as the fraction of solvent water in the erythrocyte. This value is amply supported (10–13). However, values up to 0.72 gm H₂O/100 ml of cells (9) can be defended. Use of the latter value would assume no bound water and would produce a DMO value 0.1 of a pH unit lower and a nicotine value 0.1 of a pH unit higher than the average values we calculated. Such a result would be at variance with the theoretical considerations presented by Waddell and Bates (3).

In performing the above experiments we used erythrocytes of various ages. Assuming that each cell is homogeneous but that they vary somewhat in intracellular pH as a function of age, the pH_i determined by DMO should be equal to the pH_i measured by nicotine.

There are only two results possible if a weak acid and a weak base are used to determine the pH_i. Either the weak acid gives a higher pH_i reading than the weak base, in which case the system is heterogeneous, or the readings are equal, in which case the system is homogeneous. Our experiments utilized erythrocytes from four normal donors and the same indicator compounds, DMO and nicotine, that were used to demonstrate physiochemical heterogeneity of skeletal muscle cells (4). Our results show no significant difference between the pH_i value obtained by DMO and that obtained by nicotine. It is possible that in a heterogeneous system pH_i values determined by these compounds could, fortuitously, turn out identical, as has been suggested by Carter (18) in his report of equal pH_i values determined by DMO and nicotine in barnacle muscle, an apparently heterogeneous system. We have no proof that this has not occurred here but it would seem unlikely given the difference between the erythrocyte and barnacle muscle fiber microscopically. Further, Bone *et al.* (19) using a single donor showed no significant difference between the hydrogen-ion concentration in erythrocytes as determined by the weak acid DMO and the weak base ammonia.

We conclude that the internal pH of the normal human erythrocyte is uniform throughout the cell and that this cell is physiochemically homogeneous. This information supports the concept that in the human erythrocyte hydrogen-ion dependent processes

such as glycolytic metabolism, membrane function and oxygen dissociation are regulated by a single value for each cell.

Summary. In order to determine the physiochemical homogeneity of the human red cell, intraerythrocyte pH was simultaneously measured using the weak acid 5,5-dimethyl-oxazolidine-2,4-dione (DMO) and the weak base nicotine. If a cell is homogeneous, such measurements will yield the same result. If a cell is heterogeneous, the DMO reading will be closer to the highest pH in the cell system, while the nicotine will read closer to the lowest pH. The results show no significant difference between the intracellular pH obtained by either of these methods (average by DMO = 7.10, by nicotine = 7.06 at an average external pH of 7.33). We conclude that the human erythrocyte is physiochemically homogeneous.

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