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Behavior of Erythrocytes in Phosphate Buffer Systems

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Abstract □ Hemolytic behavior of human erythrocytes in sodium and potassium phosphate buffer systems was investigated. The data were used to calculate *hemolytic i* values for the buffer components at various pH values. The experimental *i* values were lower than those predicted by a theoretical equation, and the deviations were attributed to changes in pH causing alterations in the permeability of the red cell membrane to the anions and/or cations in solution or changes in the red cell contents. Alkaline solutions appear to be more favorable environments for the red blood cell under the conditions studied. The increased osmotic fragility (low *hemolytic i* values) at lower pH values was attributed to an increase in the osmotic activity of the cell contents and subsequent movement of water into the cell.

Keyphrases □ Erythrocytes, hemolysis—phosphate buffer solutions □ Isotonic coefficients, phosphate buffer solutions—erythrocytes hemolysis □ Hemolysis curves—phosphate buffer solutions □ Osmotic fragility, erythrocytes—low pH effect

Phosphate buffer systems are used in parenteral solutions primarily to stabilize the active ingredient against chemical degradation. Those buffer systems employed should normally have as low a buffer capacity as possible so that body buffer systems will not be significantly disturbed when the solution is injected. Phosphate buffers are routinely used in the compounding of intravenous solutions when adjustments between pH 6 and 8 are needed.

The purposes of this investigation were to study the effect of phosphate buffering agents on red blood cells and to determine tonicity values based on measurements of fragility of human red cells in various phosphate buffer systems. The hemolytic method was employed, and isotonic coefficients were calculated by comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium and potassium phosphate buffer solutions. Experiments were designed to determine isotonic coefficients of buffer components at various pH values.

EXPERIMENTAL

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of a 22-year-old male Caucasian donor. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donor and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min. and then the blood was decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Buffer Solutions and Determination of pH—All chemicals employed were reagent grade quality, and distilled water was used to prepare all solutions. Stock solutions were prepared (approximately 0.133 *M*); from these, quantitative amounts were taken in the desired ratios and diluted with water to produce 50-ml. samples. The pH was checked using a pH meter (Corning model 7).

Quantitative Determination of Percent Hemolysis—The hemolytic method was used in each experiment to determine the extent of hemolysis of erythrocytes in the phosphate buffer solutions. This method is a quantitative one, being based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes were transferred 5 ml. of the standard sodium chloride solution (0.06, 0.062 ... 0.07, 0.072 *M*) and 5 ml. of the buffer system being tested. After the test tubes were brought to a constant temperature by being placed in a water bath (37 ± 0.5°), 0.05 ml. of blood was pipeted into each tube. The tubes were then inverted several times to ensure thorough mixing and allowed to remain 45 min. at 37°. After centrifuging, the absorbance of the supernatant liquid was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter.

To find the percent hemolysis, the absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min. at 37° followed by centrifuging.

Calculation of *i* Values—Through the use of the hemolytic method, concentrations of sodium chloride and the buffer solutions giving the same degree of hemolysis could be determined. Knowledge of these concentrations made it possible to calculate isotonic coefficients (*i* values) through the use of the following equation:

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{molar concentration of} \\ \text{NaCl causing} \\ 25\% \text{ hemoly is} \end{pmatrix} = \begin{pmatrix} i \text{ value of} \\ \text{buffer components} \\ \text{in solution} \end{pmatrix} \begin{pmatrix} \text{molar concentration} \\ \text{of buffer solution} \\ \text{causing } 25\% \\ \text{hemolysis} \end{pmatrix} \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.154 *M* (0.9%) sodium chloride in water (1).

Curves showing the degree of hemolysis in sodium chloride-water solutions and phosphate buffer solutions were plotted on

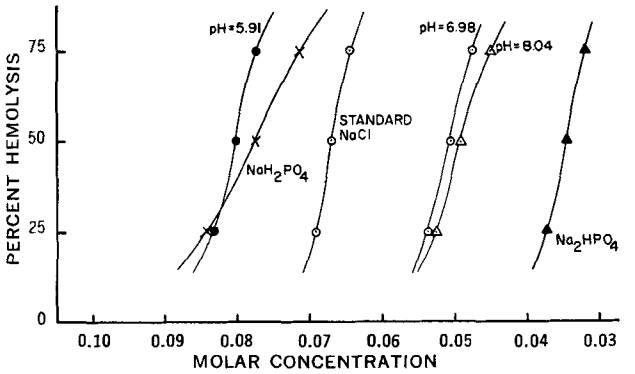

Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various NaH_2PO_4–Na_2HPO_4 buffer solutions.*

rectangular coordinate graph paper. From these curves, it was possible to determine the molar concentrations of sodium chloride and the buffer solutions causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of i for the particular phosphate buffer solution at concentrations producing 25, 50, and 75% hemolysis. The various i values for the buffer solutions tested in this study are shown in Tables I and II.

Preparation of Hemolysis Curves—Approximately 20 experiments employing human blood were performed. Standard hemolysis curves (Figs. 1 and 2) were constructed from the average readings of these experiments. Hemolysis curves for the various phosphate buffer solutions were constructed using the i values previously calculated (Tables I and II). Through a rearrangement of Eq. 1:

$$\begin{pmatrix}\text{molar concentration of}\\ \text{buffer components}\\ \text{in solution causing}\\ 25\%\text{ hemolysis}\end{pmatrix} = \frac{\begin{pmatrix}1.86\ i\text{ value for}\\ \text{NaCl in water}\end{pmatrix}\begin{pmatrix}\text{molar concentration of NaCl}\\ \text{causing 25\% hemolysis}\end{pmatrix}}{\begin{pmatrix}i\text{ value for buffer}\\ \text{components}\end{pmatrix}} \quad \text{(Eq. 2)}$$

the molar concentration of the buffer components in solution causing 25% hemolysis was calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various phosphate buffer solutions were constructed (Figs. 1 and 2).

THEORETICAL

The van't Hoff i is a correction factor used to account for the deviations of electrolytes and nonelectrolytes in solution from the laws of ideal solution. The isotonic coefficient, van't Hoff i, may be defined as the ratio of the colligative effect produced by a given molality of an electrolyte to the effect observed for the same molality of a nonelectrolyte (2). The term i as used for nonelectrolytes and electrolytes in solution indicates the degree of dissociation. In solutions of electrolytes, the value of i is determined principally by the

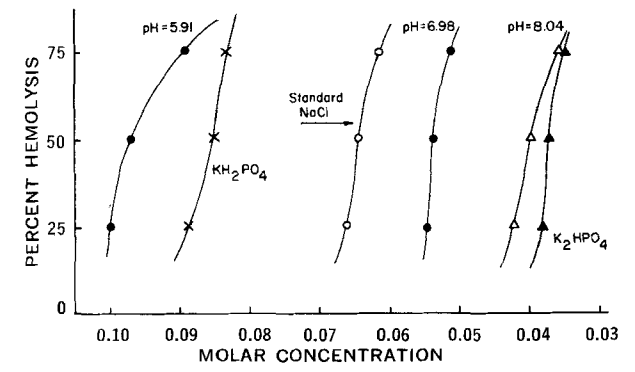

Figure 2—*Hemolysis of human erythrocytes after 45 min. at 37° in various KH_2PO_4–K_2HPO_4 buffer solutions.*

Table I—Values of i for Sodium Phosphate–Sodium Acid Phosphate Buffer Components, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Buffer Solution	Hemolysis, % 25	50	75	Average
Sodium phosphate:				
pH = 8.8	3.42	3.43	3.52	3.46
pH = 8.04	2.54	2.40	2.56	2.50
pH = 6.98	2.38	2.38	2.48	2.41
pH = 5.91	1.48	1.43	1.53	1.48
Sodium acid phosphate:				
pH = 4.8	1.46	1.52	1.58	1.52

[a] Each i value represents an average of at least two blood samples.

number of ions into which the molecule can dissociate and the charge on these ions. At infinite dilution, the value of i is identical with the number of ions into which a molecule of the solute can dissociate.

The Debye–Hückel limiting law states that the departure from ideal behavior in a given solvent is governed by the ionic strength of the medium and the valences of the ions of the electrolyte but is independent of their chemical nature (3). The valence type of the electrolyte should thus be the essential factor in determining the activity coefficient at a given ionic strength. For a solution containing more than one electrolyte, the overall i value of the components in solution should be governed by the proportional amount of each individual electrolyte. The overall i value (i_{sum}) of two components (electrolytes A and B) in solution would be described by the equation:

$$i_{sum} = i_A f_A + i_B f_B \quad \text{(Eq. 3)}$$

where i_A and i_B represent the i values of A and B determined in solutions having approximately the same molar strength as the mixture of electrolytes; f_A and f_B are the fractional amounts of A and B, respectively, of the total molar concentration and $f_A + f_B = 1$. Then:

$$i_{sum} = f_A(i_A - i_B) + i_B \quad \text{(Eq. 4)}$$

and a straight line should result when the i_{sum} values of the components in solution are plotted against the fraction of one of the components. For Eqs. 3 and 4 to be valid, there must be no chemical reaction between the electrolytes and no additional significant ionic interactions between the ions of the individual components.

Using vapor-pressure osmometer data, Cutie and Sciarrone (4) determined the NaCl equivalents of sodium acid phosphate and sodium phosphate and tonicity values of phosphate buffer mixtures (Sorensen buffer) at 37°. When these values were converted to their corresponding i values and plotted against the proportional amounts of salts in solution (NaH_2PO_4/Na_2HPO_4), a straight line resulted, showing excellent agreement with Eq. 4 (Fig. 3).

Equation 3 would be valid for activity coefficients calculated from physicochemical data such as vapor-pressure changes and freezing-

Table II—Values of i for Potassium Phosphate–Potassium Acid Phosphate Buffer Components, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Buffer Solution	Hemolysis, % 25	50	75	Average
Potassium phosphate:				
pH = 8.6	3.33	3.42	3.32	3.35
pH = 8.04	3.07	2.89	2.96	2.97
pH = 6.98	2.20	2.16	2.08	2.15
pH = 5.91	1.38	1.43	1.38	1.40
Potassium acid phosphate:				
pH = 4.5	1.24	1.25	1.24	1.24

[a] Each i value represents an average of at least two blood samples.

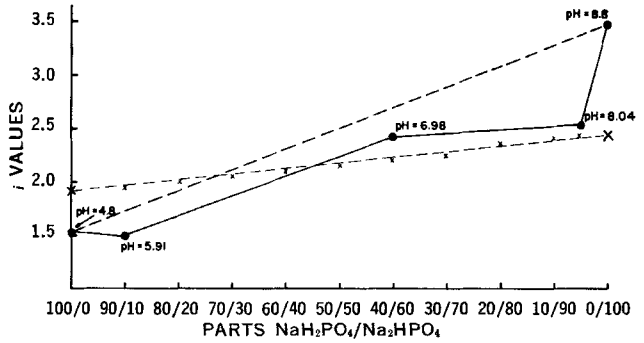

Figure 3—*Relationship between buffer composition and* i *values of* NaH_2PO_4–Na_2HPO_4 *buffer solutions. Key: - - -, theoretical line between* hem-i *values of* NaH_2PO_4 *and* Na_2HPO_4*; ——, experimental* hem-i *values; and* ×- -×*, theoretical line between* i *values of* NaH_2PO_4 *and* Na_2HPO_4 [i *values calculated from sodium chloride equivalents determined from vapor-pressure osmometer data (4)]. The small* ×*'s represent points calculated from tonicity values determined from vapor-pressure osmometer data (4).*

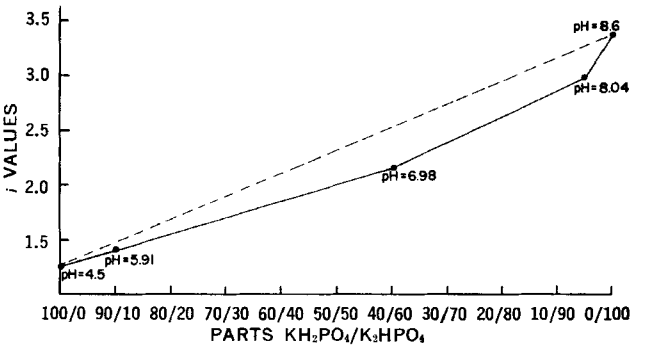

Figure 4—*Relationship between buffer composition and* i *values of* KH_2PO_4–K_2HPO_4 *buffer solutions. Key: - - -, theoretical line between* hem-i *values of* KH_2PO_4 *and* K_2HPO_4*; and ——, experimental* hem-i *values.*

point depression. For Eq. 3 to be extended to *i* values determined from data based on the ability of a substance to hemolyze red blood cells, an additional necessary requirement would be that the components in solution have no additional effect on the red blood cell than either of them alone in solution. Then Eq. 3 could be written:

$$hem\text{-}i_{sum} = hem\text{-}i_A f_A + hem\text{-}i_B f_B \qquad \text{(Eq. 5)}$$

where $hem\text{-}i_{sum}$ is the overall *hemolytic i* value for electrolytes *A* and *B*, and $hem\text{-}i_A$ and $hem\text{-}i_B$ are the *hemolytic i* values of *A* and *B* determined in solutions having approximately the same molar strength as the mixture of electrolytes. If $hem\text{-}i_A$ and $hem\text{-}i_B$ are determined for *A* and *B*, then Eq. 5 predicts that a straight line can be drawn between these *hem-i* values as shown in Fig. 3, and that any point on this line relates the $hem\text{-}i_{sum}$ to the proportional amounts of *A* and *B* in solution. If this line is the same as the line obtained using *i* values (determined from vapor-pressure or freezing-point data), then it could be assumed that the red cell membrane is acting as a perfect osmometer and the components in solution do not affect the membrane structure or the cell's contents.

A different straight line would result if either or both of the *hem-i* values were appreciably different than the *i values* (from colligative properties). Higher or lower *hem-i* values could be attributed to either the selective permeability of the red cell membrane to the anions and/or cations in solution, or to the alteration of integrity of the cell by some action of the substance on the erythrocyte membrane. If the experimental data do not fall on the predicted straight line drawn between $hem\text{-}i_A$ and $hem\text{-}i_B$, then the irrational behavior can be attributed to some change in the solvent environment brought about by mixing the electrolytes or to some unusual effect on the cell membrane and/or cell contents by the mixture. In the case where the electrolytes are buffer components, the main environmental change would be one of pH.

RESULTS AND DISCUSSION

When blood was added to 0.09–0.03 *M* phosphate buffer solutions, typical sigmoidal hemolysis curves resulted (Figs. 1 and 2). These curves were constructed in the manner described in the *Experimental* section of this report, utilizing the data presented in Tables I and II.

Tables I and II list the calculated values of *hem-i* for sodium and potassium phosphate buffer systems at various pH values. The *hem-i* values for sodium and potassium phosphates *per se* were higher than expected, whereas the *hem-i* values for the sodium and potassium acid phosphates were lower than their theoretical value of 2. As expected, the *hem-i* values of the various buffer solutions were between the values of their acid and basic components. Sodium phosphate had an unusually higher *hem-i* value (3.46) compared to the *i* value determined from vapor-pressure data (2.43). When the pH was changed from 8.8 to 8.04 by a small change in the NaH_2PO_4/Na_2HPO_4 ratio from 0/100 to 5/95, there was a dramatic decrease in the *hem-i* value. A similar type of behavior was not exhibited by the potassium salts, although there was some decrease in the *hem-i* value.

The experimental *hem-i* values were lower than those predicted by Eq. 5 for both sodium and potassium phosphate buffer systems (Figs. 3 and 4). Apparently the changes in pH cause alteration in the permeability of the red cell membrane to the anions and/or cations in solution or alter the red cell contents in some way. At alkaline pH values, both sodium and potassium buffer systems exhibited higher *hem-i* values than the corresponding physicochemical *i* values; at acid pH values, the *hem-i* values were lower (Figs. 3 and 4). The alkaline solutions appear to be more favorable environments for the red blood cell under the conditions studied.

The osmotic fragility of erythrocytes is known to be altered by changes in pH (5, 6). Parpart *et al.* (6) showed that changes in pH, from 7.8 to 6.4, of hypotonic solutions resulted in an increase in osmotic fragility. The changes in volume and, thus, water of cells suspended in isotonic media at different pH values have been well documented (7). Murphy (8, 9) recently showed that decreasing pH, from 8.0 to 6.2, increases cell water and osmotic fragility. His data indicated that the influence of pH on osmotic fragility was through changes in both the amount of cell water and the osmotic behavior of cell water. The increase in cell water, with the decrease in pH, probably results from an increase in the osmotic activity of the cell contents and subsequent movement of water into the cell. In the present studies, the increased osmotic fragility at lower pH values was manifested in lower *hem-i* values than *i* values predicted by colligative property data.

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