THE TRANSPORT OF PHOSPHATE IONS ACROSS THE HUMAN RED CELL MEMBRANE

I. THE DISTRIBUTION OF PHOSPHATE IONS IN EQUILIBRIUM AT COMPARATIVELY HIGH PHOSPHATE CONCENTRATIONS

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SUMMARY

1. The concentrations of chloride and phosphate ions in the cellular and extracellular phases have been determined in suspensions of human red cells incubated for about 3 h in salt solutions containing various concentrations of chloride and phosphate ions.

2. The concentration ratio of primary phosphate ions between the cellular and extracellular phases was in all cases found to be related to that of the chloride ions by a constant factor.

3. The significance of this result and its possible consequences for the problem of the mechanism of the transport of phosphate ions across the human erythrocyte membrane are discussed.

INTRODUCTION

To the understanding and elucidation of the mechanism of the phosphate ion penetration of the red cell membrane, a knowledge of the equilibrium concentrations of the phosphate ions on both sides of the membrane is essential, provided an equilibrium is established at all.

As early as in 1921, IVERSEN[1] tried to solve this problem by means of the very primitive methods of phosphate determination then available. Later determinations of the phosphate concentrations in serum and cells have been carried out by HALPERN on freshly drawn blood[2], and by MUELLER AND HASTINGS on incubated whole blood[3]. None of these works have, however, settled the problem quite satisfactorily. Often it has been taken for granted that if the distribution of phosphate ions between cells and serum were defined by passive diffusion processes, then such an equilibrium distribution would invariably exist in freshly drawn blood. Furthermore, such a passive distribution, which is found, *e.g.*, for chloride ions, has been assumed to be

Abbreviations: ATP, adenosinetriphosphate; 2,3-DPGA, 2,3-diphosphoglyceric acid; TCA, trichloroacetic acid.

characterized by a constant concentration ratio cells/plasma. Consequently, it has not been deemed necessary to determine this ratio in each case.

During the last few years a great number of investigations on the $^{32}PO_4$ uptake of human erythrocytes have been performed. In some of these experiments optimal conditions were chosen for the uptake, and in others various enzyme poisons were added, notably fluoride. GOURLEY[4-6] and PRANKERD *et al.*[7], who have been particularly active in this field, have formulated a theory of the mechanism of uptake of phosphate ions in human erythrocytes. According to this theory the phosphate ions penetrate the membrane of the erythrocyte by an enzymic process in which extracellular inorganic phosphate is incorporated directly[5,6]—or through a few intermediate steps[7]—into the intracellular ATP or ATP located at the membrane.

This mechanism of penetration seems to make possible a thermodynamically active transport, as defined by ROSENBERG[8,9], so that Donnan distribution of the phosphate ions should be found only occasionally.

That the phosphate ions penetrate the red cell membrane or pores in it by diffusion has, however, been maintained by HAHN AND HEVESY[10] (working with rabbit red cells), by MUELLER AND HASTINGS[3], and by DUNKER AND PASSOW[11]. The findings of these authors seem to be irreconcilable with those of PRANKERD *et al.* and GOURLEY, although the last mentioned author will not exclude a small measure of diffusion in addition to the active uptake[5].

DUNKER AND PASSOW[11] have mainly been working with bovine and equine red cells. These cells are different from human red cells in several respects. In particular they have a very low glycolytic rate and a very low content of 2,3-DPGA[12], so conclusions regarding the mechanism of penetration into human red cells can hardly be drawn from experiments on bovine or equine red cells, or, for that matter, rabbit red cells.

In case the phosphate ions penetrate the membrane by diffusion, the membrane potential, which may be measured by the distribution of the chloride ions, determines the distribution of the phosphate ions between the cellular and extracellular phases.

In this paper are recorded our determinations of the equilibrium distribution of the monovalent phosphate ions at various concentrations, and since individual variations in pH, cell water content, and chloride ion distribution are recorded in the literature, we have measured these parameters in each experiment. Furthermore, we have endeavored to determine the distributions of phosphate and chloride ions under identical experimental conditions, in order to make errors originating in the determinations of cell volumes cancel out as far as possible.

METHODS

In the experiments, freshly drawn human blood was used throughout. The blood was taken from healthy adults and from patients of both sexes in the adult medical wards of the Finsen Memorial Hospital. These patients did not suffer from diseases affecting the red blood cells, except one with hypochromic anemia, (donor No. 10). Heparin was used as anticoagulant.

Immediately after being drawn, the blood was centrifuged at 1000 $\times$ g for 10 min, and the serum and the buffy coat were removed. The cells were washed twice with Ringer solution. About 20 ml of cells were suspended in an equal volume of a salt

solution (see below) containing 200 mg glucose/100 ml. This suspension was incubated anaerobically in a 50-ml flask, which was gently rotated in a water bath for 2–5 h. The temperature of the bath was in all experiments 37.0 ± 0.2°. Generally the incubation was started 50–60 min after withdrawal of the blood.

The Ringer solution used for washing the cells had the following composition: 0.80 % NaCl, 0.037 % KCl, 0.014 % $CaCl_2$, 0.008 % $MgCl_2$, 0.01155 % KH_2PO_4, and 0.0634 % $Na_2HPO_4,2H_2O$, pH 7.4. The various salt solutions with different phosphate concentrations in which the cells were incubated, were prepared with the above solution as basis, given amounts of NaCl being replaced by $KH_2PO_4 + Na_2HPO_4$ so as to preserve a pH of 7.4 and isotonicity, and glucose being added. In all solutions for incubation, $CaCl_2$ was, however, omitted to prevent precipitation of calcium phosphate at higher phosphate concentrations.

After incubation, samples were taken from the suspension, and determinations of pH, chloride and inorganic phosphate concentrations, and dry weight were made. Another part of the suspension was centrifuged, and inorganic phosphate and chloride concentrations and dry weight were determined in cells and in extracellular phase.

Centrifugation was carried out in a centrifuge from Ole Dich, Denmark, holding twelve centrifuge tubes, each with a capacity of 400 µl. Suspensions were separated in the course of 45 sec at 13,000 × *g*. For the determination of chloride, inorganic phosphate, and dry weight, samples were taken with a 100 µl Carlsberg pipette from the supernatant and from the packed cell column. While the pipette used delivered 100.0 ± 1.0 µl of water, and presumably very closely the same volume of supernatant or TCA solution, it was found to deliver only 101.4 mg or 93.0 ± 1.5 µl of packed cells. Thus, the analytically determined quantities of chloride and phosphate ions refer to 100 µl of supernatant or to 93 µl of packed cells.

Trapped volume

The volume of extracellular phase between the cells in the packed column has been determined earlier with the aid of Evans Blue T 1824. The method is, as worked out by MAIZEL[13], a modification of a method used by SHOHL AND HUNTER[14] for determination of the percentage of serum in whole blood. In this determination even a slight degree of hemolysis will, however, cause serious disturbances in the colorimetric readings of the concentration of Evans Blue. We therefore chose to use the isotope ^{22}Na in the determination of the trapped volume in our experiments. The amount of ^{22}Na in the cell phase was determined 2–4 min and about 20 min after mixing the cells with a Ringer solution containing ^{22}Na. This procedure made it possible to correct for any uptake of ^{22}Na by the cells by extrapolation to zero time. However, this uptake proved to be of no significance at the prevailing temperature during the time intervals in question. As a mean of sixteen determinations we found the trapped volume to constitute 4.9 ± 0.2 µl or 5.2 ± 0.25 volume per cent of the cell phase samples. The experimental conditions in these determinations were in all respects identical with those of the normal experiments.

Chloride was determined mercurimetrically as follows: A quantity of 100 µl of extracellular phase or 93 µl of packed cells was mixed with 100 µl of 25 % (w/v) TCA by thorough stirring with a small glass rod. After 10 min the mixture was centrifuged for 2 min at 13000 × *g*, and 100 µl of supernatant was transferred to a tube containing 1 ml of water and 100 µl of 0.2 % diphenylcarbazone in 95 % ethanol.

The titration was performed with 0.002 M $Hg(NO_3)_2$ solution containing nitric acid in a concentration sufficient to prevent precipitation of basic salts. The amount of reagent consumed in a titration was 1–2 ml. Experiments showed that variations of 50 % in the TCA concentration had no influence on the color change or on the end-point of the titration. The standard error on the average of four samples was ± 0.46 % (70 quadruplicates).

pH values of the cell suspensions were always determined about 2 min after removal of the flask from the water bath. About 0.5 ml of the suspension was then transferred to a small glass vessel, and the pH value was read at once and after about 1 min. The temperature of the sample, measured with the aid of a thermocouple, was between 20 and 25°, the temperature of the room being 18 to 22°. To estimate the pH of the suspension at 37° the temperature coefficient of ROSENTHAL[15] was used:

$$pH_{37} = pH_t - 0.0147\,(37 - t)$$

where t = the temperature at which the determination is made. The standard error can be estimated to be less than 0.05 pH unit. A semimicro glass electrode (Radiometer GA 222) and a semimicro calomel electrode (Radiometer K 401) were used.

Dry weight was determined in duplicate. Samples of about 100 μl were pipetted onto tared aluminium dishes and weighed. The samples were dried at 12–15 mm Hg and 60° for 16–20 h in a desiccator containing silica gel. This treatment proved to give constant weights. The relative standard error on the average of two determinations was ± 2.8 % (42 duplicates).

The volume of intracellular water, V_c, in the sample of packed cells was calculated from the percentage, t_c, (w/w), of cell dry matter as follows:

$$V_c = \left[101.4\left[\frac{100 - tc}{100}\right] - 4.9\right]\mu l \qquad (1)$$

The first term in the right-hand member represents the total amount of water in the 93-μl sample (the density of the cell water being taken as one), while the second term represents the water content of the trapped extracellular phase. For the sake of convenience the above equation may be simplified to

$$V_c = (96.5 - 1.01\,t_c)\,\mu l \qquad (2)$$

Inorganic phosphorus

The procedure was developed on the basis of TAUSSKY AND SHORR's method[16]. The following molybdate stock solution was used: 50 g of ammonium molybdate is dissolved in about 400 ml 10 N sulfuric acid and diluted to 1000 ml with 10 N sulfuric acid. The reagent for analysis was prepared every day as follows: 10 ml stock solution was transferred to a 250-ml volumetric flask; about 150 ml water and 5 g $FeSO_4$, $7H_2O$ were added, and after dissolution the flask was filled up to its mark with water.

Procedure

A volume of 93 μl packed cells or 100 μl extracellular phase was added to 100 μl 25 % (w/v) TCA and thoroughly stirred with a small glass rod. After about 10 min the mixture was centrifuged for 2 min at 13,000 × g, and 100 μl supernatant was transferred to a tube containing 5 ml reagent. 15–20 min later the extinction was

read at 740 mμ on a Beckman spectrophotometer, model DU. The phosphate content in μmoles was calculated from a standard curve, each value calculated as the average of four determinations. The relative standard error of this average was 0.48 % (70 quadruplicates).

It is well known that this and similar methods always yield high values of the inorganic phosphate concentrations in solutions containing easily hydrolyzable organic phosphate. Since they are hydrolyzed, not only when protein is precipitated with TCA, but also in the strongly acid molybdenum reagent only insufficient protection of the organic phosphate is attained by precipitation at 0°.

LOWRY AND LOPEZ[17] and MARTIN AND DOTY[18] have developed methods which make it possible to determine inorganic phosphate in the presence of labile organic phosphates without causing the latter to hydrolyze. None of these methods could, however, be used directly to determine inorganic phosphate in samples of packed cells. When the method of LOWRY AND LOPEZ is applied to animal tissues, inhibition of the development of the molybdenum blue color often occurs[17]. This inhibition may be avoided by dilution, but in our case the dilution required would reduce the phosphate concentration to such a low value that it could not be determined with sufficient accuracy. If the method of MARTIN AND DOTY is applied directly, we find that a very stable emulsion is formed because of the high protein content of the erythrocytes. This emulsification takes place when the water phase containing molybdic and silicotungstic acids is extracted with an isobutanol–benzene mixture. While satisfactory separation is usually obtained within 30 sec, several minutes are required in the case of the erythrocytes.

In order to ascertain how much organic phosphate is hydrolyzed when we use our modification of the method of TAUSSKY AND SHORR, we modified the method of MARTIN AND DOTY and compared the results obtained with the two methods. Our modification is: One volume of cells is added to one volume of ice-cold TCA and stirred thoroughly. After storage for 10 min in an ice bath, the mixture is centrifuged at 0°, and 100 or 200 μl of supernatant is treated according to MARTIN AND DOTY, except that silicotungstic acid is omitted. The volumes were changed to give greater sensitivity. By means of ^{32}P phosphate we examined the efficiency of the extraction of the phosphomolybdic acid under these somewhat changed conditions; we found that it was still excellent with fifteen seconds' shaking.

This modification of the method of MARTIN AND DOTY could not be used as a routine procedure for technical reasons, but it was useful for checking the method generally used. Erythrocytes from freshly drawn blood were washed with isotonic sodium chloride solution, and phosphate was determined by both methods. A series of comparative experiments showed that, on an average of six determinations, 0.30 μg P ± 30 % was split/93-μl sample of cells in our routine method, the values obtained with the modified method of MARTIN AND DOTY being taken as true. The lowest intracellular level of inorganic phosphate we found in this series of experiments was 0.272 μmoles/93 μl packed cells. Hence the numerically greatest correction for hydrolyzed organic phosphate was — 3.7 ± 1.1 %.

The recovery factor

As stated above, the concentration of chloride and phosphate ions in the intracellular phase were determined in the following way: A volume of 93 μl of packed

cells was transferred by means of a Carlsberg pipette to a micro centrifuge tube containing 100 μl 25 % (w/v) TCA. After stirring, standing, and collection of the protein trichloroacetate precipitate by centrifugation, 100 μl of the clear supernatant was withdrawn with a Carlsberg pipette pending, further analysis.

When the data obtained with the clear supernatant are to be interpreted, a number of phenomena must be taken into consideration. In the first place, mixing of 93 μl of cells with 100 μl of 25 % TCA will lead to a volume change so that the volume after mixing will not be 193 μl. In the second place, the protein trichloracetate precipitate will occupy a considerable proportion (about 20 %) of the total volume, so the volume available to the chloride and phosphate ions is considerably smaller than the total volume. Finally, the precipitate may bind greater or smaller amounts of anions and cations by occlusion and adsorption. The amounts of the individual ions thus bound may depend, *inter alia*, on the signs and magnitudes of their charges.

The proportions of the quantities of chloride and phosphate ions present in the intracellular phase that are removed with the 100 μl of supernatant must therefore be determined experimentally for each individual ion. We attempted in a special series of experiments to determine this recovery factor for chloride and for phosphate ions. This was done in the way that the data obtained for each individual blood sample were related to the result of a dry matter determination carried out at the same time.

Outline of the method

Volumes of 93 μl of packed cells and 100 μl of 25 % (w/v) TCA to which sodium chloride or [^{32}P]phosphate had been added (TCA*), were mixed with vigorous stirring, allowed to stand for 10 min, and centrifuged for 2 min at 13000 $\times$ g. Then the relative contents of chloride ions and of ^{32}P in 100 μl TCA* and in 100 μl supernatant were determined. Chloride was determined by the mercurimetric method described and ^{32}P was determined by measurement of bremsstrahlung in a well-type scintillation crystal. In the case of the chloride ions it was necessary to carry out a blank determination to make it possible to introduce a correction for the quantity of chloride ion already present in the cells.

As a result of this investigation it was found that the recovery factor, R_F, was, in terms of per cent, (1.75 $\pm$ 0.02) t_c (25 determinations). This was true of phosphate as well as of chloride ions. Thus, the quantity of, *e.g.*, chloride ions in a sample of packed cells is given by

$$M = \frac{100\ \mathrm{Cl}_c}{1.75\ t_c}\ \mu\text{moles} \qquad (3)$$

where Cl_c is the number of μmoles determined in 100 μl supernatant by the mercurimetric titration. For determination of extracellular concentrations, the recovery percentage was found to be exactly 50.0 for phosphate as well as chloride ions.

RESULTS

Table I presents the results of thirteen experiments with blood from ten donors. Not included are several preliminary, less accurate experiments, which have, however, given very similar results. The concentrations of chloride and phosphate ions in the

TABLE I

RESULTS OF THIRTEEN EXPERIMENTS WITH BLOOD FROM TEN DONORS

P_t = total inorganic phosphate, ex = extracellular phase, c = cellular phase, $R_{c_2}\gamma_{c_2}$ and $R_{P^-}\gamma_{P^-}$ are defined in eqns. 6 and 14, $Q = R_{P^-}\gamma_{P^-}/R_{c_2}\gamma_{c_2}$.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Expt.	*Donor No.*	*Incubation medium Concn. in mmol/l of:*		*µmole phosphate in 100 µl supernatant from precipitation of:*		*µmole chloride in 100 µl supernatant from precipitation of:*		*Time of incubation in min.*	*pH corr. to 37°*	*Dry weight per cent*				*Average of Q for each conc. interval*
		Cl	P_t	*ex*	*c*	*ex*	*c*			t_c	$R_{Cl}\gamma_{Cl}$	$R_{p^-}\gamma_{p^-}$	*Q*	
1	1	95.1	40.0	1.44	0.778	5.72	3.68	170	7.11	34.4	0.785	0.752	0.96	
2	2	95.1	40.0	1.27	0.663	5.52	3.52	185	7.08	35.7	0.764	0.721	0.94	
3	3	95.1	40.0	1.45	0.791	5.72	3.72	215	7.05	35.8	0.780	0.744	0.95	0.98
4	4	95.1	40.0	1.29	0.627	6.96	4.08	150	7.19	38.4	0.671	0.709	1.06	
5	5	95.1	40.0	1.30	0.766	5.60	3.80	285	7.12	35.2	0.824	0.795	0.97	
6	6	119.3	22.2	0.769	0.442	6.32	4.04	200	7.00	34.7	0.776	0.798	1.01	
7	7	119.3	22.2	0.729	0.392	6.48	4.16	240	7.13	35.9	0.767	0.756	0.97	0.99
8	8	119.3	22.2	0.800	0.466	6.48	4.20	240	7.13	34.3	0.793	0.816	1.01	
9	9	119.3	22.2	0.785	0.465	6.52	4.44	255	7.09	35.2	0.827	0.795	0.95	
10	7	131.5	13.3	0.483	0.262	6.92	4.44	190	7.19	35.0	0.777	0.762	0.96	
11	10	131.5	13.3	0.520	0.356	7.08	5.08	210	6.98	29.9	0.960	0.924	0.95	1.00
12	8	131.5	13.3	0.505	0.277	7.00	4.40	195	7.18	33.5	0.777	0.789	1.00	
13	9	131.5	13.3	0.515	0.305	7.24	4.64	220	7.12	35.3	0.773	0.834	1.06	

salt solution in which the twice washed cells are incubated are stated in Columns 2 and 3. Attention is called to the fact that before incubation the cells are always washed with the salt solution mentioned under METHODS. In the following columns are listed the final quantities of phosphate and chloride ions found in 100 μl supernatant from precipitated extra- and intracellular samples, the time of incubation being given in Column 8. The phosphate quantities from the samples of intracellular phase have been corrected for organic phosphate hydrolyzed, as discussed under METHODS.

The absence of any correlation between the values of Q (see page 19) and the corresponding incubation times as well as experience from [^{32}P]phosphate exchange experiments have convinced us that incubation periods of about 3 h are sufficient for establishment of equilibrium. This is in accord with the result of IVERSEN[1], who added isotonic Na_2HPO_4 solution to defibrinated blood (rabbit blood, to be sure). His data show that the added phosphate distributed itself uniformly in the course of 2–4 h.

The percentage of dry matter in the packed cells is listed in Column 10. As a mean of all determinations (except the one on a patient with hypochromic anemia, donor No. 10), we have found this percentage to be 35.3 % $\pm$ 1 % relative. However, it must be borne in mind that the experimentally determined values for the cell dry matter ought to be corrected for the volume of trapped extracellular phase for calculation of the percentage of dry matter in the cells themselves.

In their work on the distribution of ^{32}P-labelled phosphate ions between cells and serum in human blood, EISENMAN, OTT, SMITH AND WINKLER[19] used a value for cell dry matter of 28 %. This value, the mean of ninety-four analyses, was taken from the work of EISENMAN, MACKENZIE AND PETERS[20]. Here, however, the percentage of dry matter in the cells was computed from measurements of dry matter in serum and whole blood and of hematocrit. The very low figures may thus be explained by a high percentage of trapped plasma in the packed cell column of the hematocrit capillary. In addition to the results listed in the table the following values have been determined in each single experiment: the percentage of dry matter in the extracellular phase and the percentages of dry matter and of cell volume in the cell suspension. In the thirteen experiments, the dry matter in the extracellular phase has varied between 1.2 and 1.8 % (w/w), most of which, about 1 %, has been inorganic salts. Consequently, no correction for extracellular dry matter has been introduced. This result further shows that if any hemolysis has taken place, it must have been very slight. For the cell suspension as a whole, the content of dry matter has been 16.0 $\pm$ 1.4 % (w/w) (not including donor No. 10, the one with hypochromic anemia). This result as well as the cell volume of 41.6 $\pm$ 1.9 % may be considered an expression of the uniformity of the suspensions incubated. The cell volumes have been determined in quadruplicate by centrifugation in short capillary tubes for 2 min at 13,000 $\times$ g. As will be seen from the pH values listed in Column 9 of the table, the pH has fallen during the incubation to a value 0.2 to 0.4 unit lower than the initial value, pH 7.4, of the salt solution incubated.

Calculations

In the interpretation of these analytical data we have made the assumption that all the water inside the cells is free and osmotically available so that the total volume,

V_c, should be used in the calculation of the thermodynamic activities of the dissolved ions. HUTCHINSON[21] has found in experiments with ^{14}C-tagged alcohols and $H_2{}^{18}O$ that these substances seem to mix into all the water of human erythrocytes, indicating that as far as these substances are concerned practically all the water inside the cell is free.

On the basis of the assumption that all the intracellular water is available, the ratio of the intracellular to the extracellular chloride ion activity, $R_{Cl} = {}^aCl_c/{}^aCl_{ex}$, is calculated in the following manner. Since the R_F for determination of extracellular matter is 50 %, the concentration of chloride ion in the extracellular phase is given by eqn. 4.

$$[Cl_{ex}] = \frac{2\ Cl_{ex}}{100} \text{ moles/l} \tag{4}$$

where Cl_{ex} is the number of μmoles of chloride ion/100 μl of supernatant as determined by the mercurimetric titration. The chloride concentration in the intracellular phase is obtained from eqn. 5:

$$[Cl_c] = \frac{\left(\frac{100\ Cl_c}{1.75\ t_c} - 4.9\ \frac{2\ Cl_{ex}}{100}\right)}{96.5 - 1.01\ t_c} \text{ moles/l cell water} \tag{5}$$

In this expression, the first term of the numerator represents the total quantity of chloride ions, in terms of μmoles, contained in the 93-μl sample of packed cells; *cf.* eqn. 3. The second term of the numerator represents the amount of chloride ion contained in the 4.9 μl trapped extracellular phase. Finally, the denominator is the expression of eqn. 2 for the volume of the intracellular aqueous phase contained in the 93 μl packed cells.

Now R_{Cl} is found by division of eqns. 4 and 5:

$$R_{Cl}\gamma_{Cl} = \frac{[Cl_c]}{[Cl_{ex}]} = \frac{\left(\frac{100\ Cl_c}{1.75\ t_c} - 0.098\ Cl_{ex}\right) 50}{96.5 - 1.01\ t_c)\ Cl_{ex}} \tag{6}$$

where γ_{Cl} is the ratio of the activity coefficients of the chloride ions in the extra- and intracellular phases, respectively ($f Cl_{ex}/f Cl_c$).

The distribution ratio, R_{Cl}, determined in each individual experiment by means of eqn. 6, is used as an expression of the Donnan distribution in the given cell suspension. Consequently, this is the magnitude with which the distribution ratio calculated for the monovalent phosphate ions is to be compared.

In the course of the derivation of the equation for the distribution ratio $R_{P^-} = {}^aP\text{-}_c/{}^aP\text{-}_{ex}$ (the subscript P^- referring to the monovalent phosphate ion $H_2PO_4^-$), we first arrive at an equation analogous to (6), which gives us, however, the concentration ratio of total inorganic intracellular to extracellular phosphate. The concentrations of the monovalent phosphate ions are calculated from the concentrations of total inorganic phosphate by means of eqn. 9, which is obtained by combination of eqns. 7 and 8:

$$K'' = \frac{[HPO_4^{--}]\ {}^aH^+}{[H_2PO_4^-]} \tag{7}$$

$$[H_3PO_4] + [H_2PO_4^-] + [HPO_4^{--}] + [PO_4^{---}] = [P_{total}] \tag{8}$$

At pH values between 7.0 and 7.2, the concentrations of unionized and completely ionized phosphoric acid are negligible. When they are left out, combination of eqns. 7 and 8 gives 9:

$$[H_2PO_4^-] = \frac{{}^aH^+}{({}^aH^+ + K'')}[P_{total}] \tag{9}$$

This equation is valid for calculation of the concentration of the monovalent phosphate ions in the extracellular as well as the intracellular phase. In eqn. 9 the hydrogen ion activity in the extracellular phase is the measured value in the cell suspension corrected to 37°. The corresponding value in the cellular phase, ${}^aH^+_c$, is calculated by means of eqn. 10 and the resulting 11:

$$R_{Cl} = \frac{{}^aCl^-_c}{{}^aCl^-_{ex}} = \frac{{}^aOH^-_c}{{}^aOH^-_{ex}} = \frac{{}^aH^+_{ex}}{{}^aH^+_c} \tag{10}$$

$${}^aH^+_c = \frac{{}^aH^+_{ex}}{R_{Cl}} \tag{11}$$

This presupposes that the hydroxyl ions (partly by a rapid equilibration of the HCO^-_3-ions) are distributed among the intra- and extracellular phases in the same concentration ratio as the chloride ions. As for R_{Cl} we are unable to calculate an accurate value of it. Experimentally we determine the concentration ratio $[Cl_c]/[Cl_{ex}] = R_{Cl}\gamma_{Cl}$. Considering the uncertainty of the above estimation of intracellular ionic strength, we have not tried to calculate γ_{Cl}, but have simply taken it as unity.

Let P^t_c and P^t_{ex} denote the analytically determined concentrations of inorganic phosphate in the intra- and extracellular phases, respectively. The corresponding concentrations of monovalent phosphate ions may then be calculated, partly from a combination of eqns. 9 and 11, and partly from eqn. 9:

$$[P^-_c] = [P^t_c]\frac{{}^aH^+_{ex}}{(K''_cR_{Cl} + {}^aH^+_{ex})} \tag{12}$$

$$[P^-_{ex}] = [P^t_{ex}]\frac{{}^aH^+_{ex}}{(K''_{ex} + {}^aH^+_{ex})} \tag{9a}$$

From eqns. 9a and 12 we obtain eqn. 13:

$$R_{P^-} = \frac{{}^aP^-_c}{{}^aP^-_{ex}} = \frac{1}{\gamma_{P^-}}\frac{[P^t_c]}{[P^t_{ex}]}\frac{(K''_{ex} + {}^aH^+_{ex})}{(K''_cR_{Cl} + {}^aH^+_{ex})} \tag{13}$$

This shows that the equation giving the distribution ratio $R_{P^-}\gamma_{P^-}$ may be obtained from an equation analogous to 6 by multiplying its right-hand member by the last fraction occurring in 13:

$$R_{P^-}\gamma_{P^-} = \frac{\left(\frac{100}{1.75\,t_c}P_c - 0.098\,P_{ex}\right)50\,(K''_{ex} + {}^aH^+_{ex})}{(96.5 - 1.01\,t_c)\,P_{ex}\quad(K''_cR_{Cl} + {}^aH^+_{ex})} \tag{14}$$

In addition to the experimentally determined phosphate quantities, this equation contains the values K''_{ex}, K''_c, R_{Cl} and $\gamma_{P^-} = {}^fP_{-ex}/{}^fP_{-c}$. The second ionization constant of phosphoric acid (eqn. 7) was computed from the equation given by BJERRUM[22] for the ionic strength range up to 0.1:

$$pK'' = 7.165 - 1.545\sqrt{\mu} + 1.12\mu \qquad (15)$$

where μ is the ionic strength of the solution. The ionic strength of the extracellular phase, μ_{ex}, can easily be calculated with sufficient accuracy, and we have found it to vary between 0.160 and 0.185. In expts. 1 through 5 we have chosen to use the value 0.18 and in expts. 6 through 13 the value 0.17, which give pK''_{ex} values of 6.71 and 6.72, respectively. The ionic strength in the intracellular phase cannot be computed, but considering the content of organic phosphates in this phase we have chosen to use the value 0.20, giving a pK''_c value of 6.70.

We have chosen to express the final result of an experiment by the ratio $Q = R_{P-}\gamma_{P^-}/R_{Cl}\gamma_{Cl}$, and have not attempted to calculate the activity coefficients of the monovalent phosphate and chloride ions in the extra- and intracellular phases. If, however, the ratios of the activity coefficients in the extra- to the intracellular phases are identical for primary phosphate and for chloride ions, Q should be close to unity provided that the distribution of the primary phosphate ions is determined solely by the Donnan potential, and provided, of course, that all the constants used have been chosen correctly.

As a mean of twelve determinations (not including donor No. 10, the one with hypochromic anemia), $R_{Cl}\gamma_{Cl}$ was found to be 0.78 ± 0.01. The average value of Q found from all thirteen experiments was 0.98 ± 0.01, the relative standard deviation of a single determination of Q being 4.2 %.

The standard deviation to be expected for a single Q value was calculated in the following manner: The partial derivatives of Q with respect to the various parameters involved were calculated by means of the equation for Q obtained by division of eqn. 14 by eqn. 6. In the calculation were used the average values pH = 7.1, $R_{Cl}\gamma_{Cl} = 0.78$, $R_{Pi} = 0.68$, and $t_c = 35$, and for pK''_{ex} and pK''_c the value 6.70. For the standard deviations of the values for chloride, phosphate, dry matter, and pH, the figures quoted under METHODS were used. For pK''_{ex} and pK''_c the standard deviations were taken as 0.005 and 0.01, respectively (see below). In this way the relative standard deviation for a single Q determination was calculated as 2.5 %, a somewhat lower value than was found experimentally.

Column 14 of the table shows that Q seems to rise slightly when the equilibrium concentration of phosphate decreases. This apparent rise, which amounts to 2 % within our concentration interval, might be due to a too small correction for organic phosphate hydrolyzed, or the $R_{P-}\gamma_{P^-}$ value might be subject to a systematic error due to possible adsorption of phosphate ions to the membrane or stroma, since the expression for $R_{P-}\gamma_{P^-}$ was derived on the assumption that the concentration of free phosphate ions was equal to the analytically determined phosphate concentration. It is difficult to make an estimate of the magnitude of an error of this kind, but the suggested correlation between Q and the found phosphate concentration may be explained by the occurrence of such an adsorption. However, experiments with phosphate labelled with ^{32}P have given results indicating only a very slight effect of adsorption.

Among the systematic sources of error the choises of the values of R_F and pK''_c are particularly important. In the section on methods, R_F has been given as $(1.75 \pm 0.02)\ t_c$. The standard deviation of 0.02 stems partly from the uncertainty of the analytical determination, partly from a possible variation in R_F from one precipitation to another, which is, however, involved in the quadruplicate determinations of the phosphate and chloride concentrations. A relative change of 1 % in R_F gives rise to a relative change in Q of 0.8 %. The pK'' depends on the ionic strength, which was easily calculated for the extracellular phase with reasonable accuracy. In the numerical treatment of the experimental data we even used two different values of pK''_{ex}, so we feel justified in ascribing a small standard deviation to this magnitude. As already mentioned, however, it is very difficult to motivate the choice of any value of ionic strength of the intracellular phase. According to eqn. 15, a change of μ_c from for example 0.18 to 0.20 changes the value of pK''_c from 6.71 to 6.70, corresponding to a change of −1.6 % in Q.

Finally it should be mentioned that in expt. 4, Q was determined not only at $t = 150$ min, but also after 20 and 40 min. The results were: at $t = 20$ min, $Q = 0.55$, at $t = 40$ min, $Q = 0.85$; and at $t = 150$ min, $Q = 1.06$.

DISCUSSION

The results and considerations reported in the foregoing sections suggest that, under the given experimental conditions, the distribution of inorganic phosphate ions between the intra- and the extracellular phases is governed by a simple principle. In this connection the absolute value of Q is less significant than the rather pronounced constancy of the Q value found within the rather large concentration interval studied. In our opinion, such a regular distribution can only be found if the phosphate ions pass the erythrocyte membrane chiefly by a passive penetration process.

This passive process might consist in a simple diffusion of free phosphate ions through the membrane or pores in it. Another possibility would be that a complex of phosphate ion with a carrier molecule was formed at the surface of the membrane, diffused through, and decomposed at the other side of the membrane. Such a passive carrier transport mechanism of penetration of the human erythrocyte membrane by glucose has been demonstrated by ROSENBERG AND WILBRANDT[23,24].

Our finding is at variance with the conclusions from studies carried out by HALPERN[2], by EISENMAN, OTT, SMITH AND WINKLER[19], and with the results of MUELLER AND HASTINGS[3]. HALPERN[2] and EISENMAN *et al.*[19] find the distribution of phosphate ions in human whole blood (freshly drawn, respectively incubated) so irregular that an active uptake of phosphate ions seems to them the only reasonable assumption. HALPERN[2] even claims to have observed active transport of phosphate ions in both directions through the membrane. Also MUELLER AND HASTINGS[3] find a definitely irregular distribution, but most often with considerably higher intracellular phosphate ion concentrations than the values calculated, according to the Donnan distribution, from the concentrations in the corresponding sera. Nevertheless, these authors conclude, partly on other grounds, that the membrane is penetrated by slow diffusion.

These earlier investigations were done on freshly drawn or incubated whole blood. In contrast, all of our studies were performed with washed cells that had been

incubated for shorter or longer times in salt solutions containing glucose. Furthermore, it is undoubtedly an important fact that our experiments were carried out with total inorganic phosphate concentrations considerably higher than in the experiments of the above authors. In this connection, the significance of the large pool of organic phosphate in the human erythrocyte should be strongly emphasized. The size of this pool, of which about 40 % is 2,3-DPGA[25], may fluctuate considerably because of phosphorylation and dephosphorylation, *in vitro* as well as *in vivo*[25]. In work with phosphate ions a suspension of human erythrocytes will thus behave like a polycompartment system containing extracellular inorganic phosphate, intracellular inorganic phosphate, and intracellular organic phosphate. Since the phosphate ion penetrates the erythrocyte membrane rather slowly, decomposition of a relatively small amount of organic phosphate will be able to give rise to a considerable concentration gradient across the membrane if the total inorganic phosphate concentration is as low as 1 or 2 mmoles/l suspension or whole blood. In such a case considerable fluctuations around the concentration ratio corresponding to passive distribution might be expected. In incubation of whole blood a certain dephosphorylation often takes place, and consequently increased amounts of intracellular inorganic phosphate are often found in such incubation experiments. If, however, the total inorganic phosphate concentration is somewhat higher, say 5 to 6 mmoles/l, this effect of the organic phosphate pool hardly makes itself felt.

As mentioned before, most of the available analytical methods for determination of inorganic phosphate in the cell phase lead to some hydrolysis of organic phosphate. In none of the works mentioned has this hydrolysis been adequately controlled, and so greater or smaller quantities of the organic phosphate present have presumably been determined as inorganic phosphate. Therefore, the most favourable conditions for a correct determination of the inorganic phosphate in the cell phase are those of comparatively high concentrations of inorganic phosphate, relative to which the percentage of organic phosphate erroneously included will be at a minimum.

The results given here do not, however, exclude the occurrence *in vivo* of some active transport of the phosphate ions, a transport which might make itself felt at the low phosphate concentrations prevailing in the plasma.

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THE HAEMOLYSIS OF HUMAN ERYTHROCYTES IN RELATION TO THE LATTICE STRUCTURE OF WATER*

I. DELAYED HAEMOLYSIS IN HYPOTONIC MALONAMIDE SOLUTIONS

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SUMMARY

The haemolysis of human erythrocytes in hypotonic aqueous malonamide has been examined in relation to the quasi-crystalline properties of liquid water. It is suggested that the controlling factor in the mechanism of this process is the order promoting effect of malonamide on the water lattice; this suggestion is supported by a study of the kinetics of malonamide induced haemolysis.

INTRODUCTION

During an investigation of the permeability of human erythrocytes to organic nitrogenous substances, in which the haemolysis technique was employed, a delayed action effect was observed with hypotonic solutions of malonamide.

A similar effect occurs with hypotonic solutions of glucose and this has been described by HENDRY[1] as delayed haemolysis.

The results of an investigation of delayed haemolysis in hypotonic malonamide solutions are described.

MATERIALS AND METHODS

Preparation of the blood

The experiments reported here were carried out on fresh, normal human blood. Immediately after its withdrawal by venepuncture, a 20-ml sample of blood was

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