THE DISTRIBUTION OF INORGANIC PHOSPHATE IN AMPHIBIAN MUSCLE

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ABSTRACT

The Na^+, K^+, and inorganic phosphate levels of the plasma and sartorius muscle of the toad *Bufo marinus* were determined.

Soaking in normal Ringer brought about the usual cation shifts, but did not alter the level of inorganic phosphate in the cell.

Increases in the external phosphate level brought about an increase in the internal phosphate, but the apparent phosphate space of muscle is somewhat smaller than the apparent Cl^- space.

Phosphate spaces were compared with inulin spaces and were found to be significantly greater.

Alteration of the H^+ concentration of the high phosphate Ringer did not alter the partition of phosphate across the cell membrane.

These results have been found to be consistent with the theory of a three compartment system for muscle, wherein the tissue is assumed to consist of an extracellular phase, and two intracellular phases. The inorganic phosphate of the cell is assumed to be adsorbed onto the "ordered phase," and increments in organic phosphate found on raising the external level are assumed to take place in the "free intracellular phase."

The nature of the distribution of inorganic phosphate between a muscle cell and its environment has been the subject of extensive investigation. Eggleton (4) noted that the inorganic phosphate of the medium appeared to penetrate approximately 25 per cent of the muscle, and it was assumed that this represented the extracellular phase, and phosphate was not able to penetrate the cell. This result was compared with muscles in heat rigor, when it was found that all the cell water was available for phosphate diffusion.

The apparent impermeability of the muscle membrane to phosphate, as was found with the apparent impermeability of the membrane to Na^+, was disproved with radioactive isotope experiments, and many workers (1, 5, 13) have demonstrated a rapid exchange of phosphate across the membrane. It is known (4, 9) that there is a considerable concentration gradient for phosphate across the membrane, there being some 20 mM P per kg. in whole muscle, and approximately 1.5 m.eq. per liter in plasma. On an intracellular basis, assuming 15 per cent extracellular space, this yields a ratio $\text{phosphate}_{in}/\text{phosphate}_{out}$ approximately equal to 16.

The nature of the accumulation of phosphate has been attributed to various causes. Kamen and Spiegelman (11), and Causey and Harris (1) assume all intracellular inorganic phosphate to be an artifact, due to the breakdown of labile phosphate esters during the extraction process. A somewhat similar theory is proposed by Roberts *et al.* (15) in the form of an exchange adsorption hypothesis. Mitchell (13) has invoked an active transport mechanism, coupled with exchange diffusion, such as has been put forward by Ussing (22) for Na^+ exchange. Rothstein (7) has also proposed that phosphate is actively transported into the yeast cell.

We have previously (20) proposed a three phase system to account for ionic partitions in the sartorius muscle, and it was decided to investigate whether the data about phosphate accumulation could be fitted into such a system. The cell is assumed to consist of an extracellular phase and two intracellular phases. The greater part of the intracellular compartment will be occupied by the "ordered phase" onto whose semirigid lattice structure will be adsorbed K^+, inorganic phosphate, and all other cellular constituents which are selectively accumulated by the tissue. Other inorganic constituents of the extracellular phase will be excluded from this region of the cell, and will be confined to the "free intracellular phase." This will result in an apparent concentration gradient for these substances across the membrane, although as a first approximation they will be in a diffusion equilibrium with the external phase. Movements of Na^+ and Cl^- have previously been shown (20) to be closely correlated in the muscle cell, and are largely independent of K^+ movements.

It will be determined in this paper whether alteration of the external phosphate level leads to a partition of this ion across the membrane in a manner consistent with the above theory.

Methods

The treatment of sartorius muscles which were taken from the toad *Bufo marinus*, collection of blood, etc., was essentially similar to that reported previously (20). At the conclusion of each experiment the muscles were frozen on solid carbon dioxide, and rapidly cut in two with a sharp scalpel. One-half was analysed for Na^+, K^+, and Cl^- by the usual methods (20) the other half was ground in a chilled mortar with 3 per cent trichloracetic acid, and assayed for phosphate by the method of Lowry and Lopez (12). It was shown by analysing two ends of a cut muscle that there was no difference in ionic composition between them.

Inulin spaces were determined using the method of Wilde (23). Muscles were first soaked for 2 hours in Ringer containing 1 per cent inulin, then the inulin was leached out of the muscle into an inulin-free Ringer, and the amount of inulin left in the Ringer estimated (see Tasker *et al.* (21)).

Solutions.—Ringer solutions were modifications of those used previously (17), and will be described in the text.

RESULTS

Na^+, K^+, and Phosphate in Blood.—These ions were determined in 9 samples of toad plasma, with the following results; Na^+ 108 ± 1.9 m.eq. per liter (± S.E. K^+ 3.1 ± 0.4 m.eq. per liter, phosphate 1.76 ± 0.07 mM per liter. There was no correlation between the variations of any of the three ions. It should be noted that the Na^+ level of the plasma was particularly low in this series, presumably owing to seasonal variation.

Na^+, K^+, and Phosphate in Muscles in Vivo.—Toads were killed by pithing, and the muscles (32 in number) were rapidly excised and frozen on dry ice. They were then cut in two and analysed as described in the methods section. The Na^+ content ± S.E. was 31.4 ± 1.03 m.eq. per kg., the K^+ content was 75.7 ± 1.3 m.eq. per kg., and the phosphate 22.3 ± 0.8 mM per kg. There was no correlation between variation in the values of the three ions. Intracellular levels were not calculated as plasma samples were not taken from each toad.

The Effect of Soaking in Normal Ringer on the Ionic Content.—The ionic content of muscles *in vivo* was compared with that of the companion muscle soaked for 4 hours in normal Ringer. The usual gain in Na^+ and loss of K^+ was found, the mean Na^+ increase for five pairs of muscles was 39.8 m.eq. per kg., and the mean K^+ loss 24.7 m.eq. per kg. The phosphate content did not change significantly, there being a loss of only 0.3 mM per kg. Concomitant changes in the organic phosphate fractions are being investigated and will be reported at a later date.

The mean phosphate level *in vivo* (22.3 ± 0.8 mM per kg.) was compared with the mean phosphate content in normal Ringer, which was 23.8 ± 0.6 mM per kg. (96 observations). It is clear that soaking in normal Ringer does not alter the phosphate content of muscle, although it markedly alters the Na^+ and K^+ levels.

The Effect of High Phosphate Ringer on Ionic Content.—The ionic content of muscles soaked in normal Ringer was compared with that of muscles soaked in Ringer, part of the Cl of which had been substituted with phosphate. A suitable mixture of the disodium and monosodium salts was used to maintain the pH at 7.2. It was found that the phosphate solutions produced swelling of the muscles, and in one experiment the Na^+ content was raised somewhat to maintain the volume of the muscle. (See Table I.) Groups of ten pairs of muscles were used in each experiment, and the muscles were in all cases soaked for 4 hours. In these experiments all figures refer to intracellular levels, assuming 15 per cent extracellular volume. The results are shown in Table I, and may be summarised as follows:—

1. High phosphate Ringer does not alter either the Na^+ content of the cell, or the ratio Na^+_{out}/Na^+_{in}, when the Na^+ content of the Ringer is kept constant. There is an increase in internal Na^+ when the external Na^+ is raised, and this is accompanied by a slight, but not significant drop in Na^+ ratio out/in.

2. The chloride content of all phosphate Ringers was less than the 110 m.eq. per liter of the control, and there was a drop in the internal Cl^- of the cell also. This drop was, however not proportional to the drop in external Cl^- and there is a consequent decrease in the ratio Cl^-_{out}/Cl^-_{in}.

3. There is a slight, but significant, drop in the K^+ content of the cell at 60 mM per liter external phosphate. In those experiments in which swelling oc-

TABLE I

The Effect of High Phosphate Ringer on Ionic Content of Muscle

Phosphate in Ringer, mM per liter	40	Ratio	60	Ratio	60	Ratio
Cl in Ringer, *m.eq. per liter*	47		13		52	
Na in Ringer, *m.Eq. per liter*	130		130		171	
Volume change, *per cent*	+ 7.7		+ 11.6		+ 0.8	
Na, *m.eq./kg.*						
Control	41.4	3.2	52.2	2.5	44.6	2.9
Treated	42.8	3.1	50.9	2.6	68.6*	2.6
Cl, *m.eq./kg.*						
Control	27.9	3.9	28.4	3.9	33.3	3.3
Treated	22.7*	2.1*	3.9*	3.3*	20.3*	2.6*
Phosphate, *mM/kg.*						
Control	30.7	5.5	29.9	4.4	28.8	3.6
Treated	37.9* (7.2)		43.5* (13.6)		45.4* (16.6)	
K, *m.eq./kg.*						
Control	73.8		91.2		75.5	
Treated	62.0		62.0*		60.4*	

In this and subsequent tables figures in parenthesis in phosphate colum are differences between control and treated, and "ratio" in this column means phosphate out/phosphate treated–phosphate control. All figures refer to intracellular levels, assuming an extracellular space of 15 per cent.

* Only figures significantly different from controls.

curred this would be partly explained by the increase in volume, assuming the dry weight K^+ level to remain nearly constant. In the experiment in which there was no volume change this must be considered a genuine loss of K^+.

4. The phosphate level of the cell has in all cases increased significantly. If it is assumed that the increase in internal phosphate is due to the diffusion of these ions into the free intracellular phase (20), then the ratio of external phosphate to the *increase* in internal phosphate should be of the same order of magnitude as the Cl^- ratio. It will be seen that this is only approximately true, and this difference will be dealt with in the discussion.

The Effect of High Potassium Phosphate Ringer on Ionic Content.—The ionic content of muscles soaked in normal Ringer was compared with the ionic content of paired muscles soaked in Ringer to which had been added potassium phosphate. The high potassium phosphate Ringer contained the usual quantities of all other constituents. The techniques were identical with those described in the previous paragraph. The results are presented in Table II, and may be summarised as follows:—

1. The addition of K^+ and phosphate ions to normal Ringer, which consequently rendered it hyperosmotic, did not produce any volume change in the muscle.

TABLE II

The Effect of High Potassium Phosphate Ringer on Ionic Content of Muscle

Phosphate in Ringer, *mM per liter*	30	Ratio	60	Ratio
K^+ in Ringer, *m.eq. per liter*	50		100	
Na, *m.eq./kg.*				
Control	34.8	3.7	35.0	3.7
Treated	24.5*	5.3*	31.6*	4.1*
Cl, *m.eq./kg.*				
Control	25.4	4.3	30.6	3.5
Treated	33.4*	3.3*	45.1*	2.4*
Phosphate, *mM/kg.*				
Control	23.1		27.3	
Treated	26.1* (3.0)	10.0	34.3* (7.0)	8.6
K, *m.eq./kg.*				
Control	95.2		90.8	
Treated	120* (25.2)	2.0	125* (34)	2.9

Figures in parentheses in K columns are differences between control and treated, and "ratio" in this column means $K_{treated} - K_{control}/K_{out}$.

2. There was a significant decrease in the Na^+ content of the cell in high potassium phosphate Ringer, and since the external Na^+ was not altered there was a consequent increase in Na^+ gradient.

3. There was a significant increase in Cl^- content of the muscles in high phosphate Ringer, and a decrease in Cl^- gradient. These three results are similar to those found with Ringer containing high KCl (see below).

4. There was a small but significant increase in phosphate content of the test muscles. If we use the calculation given in paragraph 4 of the previous section it is apparent that a smaller amount of phosphate has entered than was found in high sodium phosphate Ringers, and consequently the ratio of external phosphate/increase in internal phosphate is much higher. This may be due to

variation in extracellular volume between different groups of muscles, and may have no theoretical significance.

5. There was a significant increase in the K^+ content of cells in high potassium phosphate Ringer. When the ratio of the external K^+/increase in K^+ in the cell is calculated, it is seen to be roughly similar in magnitude to the ratio Cl^-_{out}/Cl^-_{in}.

High potassium phosphate Ringer was compared with high KCl Ringer, using paired muscles in each Ringer. The Ringer solutions contained 100 m.eq./liter K, 60 mM/liter phosphate, and 100 m.eq./liter KCl respectively. All other ionic constituents of the Ringer solutions were normal. The following details are apparent from Table III:—

1. Neither solution produced a significant volume change in the muscles, although both were hyperosmotic.

TABLE III

A Comparison of the Effect of High KCl Ringer and High Potassium Phosphate Ringer on Ionic Content

	KCl Ringer	Ratio	Potassium phosphate Ringer	Ratio
Volume Change, *per cent*	+4.8		+1.0	
Na, *m.eq./kg.*	45.4	2.9	42.2	3.1
Cl, *m.eq./kg.*	91.5	2.3	75.5*	1.5*
Phosphate, *mM/kg.*	25.4		39.5*	4.3
K, *m.eq./kg.*	138.0		118*	

* Only figures significantly different from companion muscles.

2. There was no significant difference between the Na^+ content of the muscles in high phosphate or high Cl^- Ringer, and the increase in Na^+ gradient seen in Table II must be regarded as due to the K^+ level of the Ringer, rather than the nature of the anion.

3. The Cl^- gradient in high phosphate Ringer is significantly lower than in high Cl^- Ringer, that is, a disproportionate amount of Cl^- has entered the cell in the phosphate-treated muscles.

4. In high phosphate Ringer a significant amount of phosphate has entered the cell, and the increase in phosphate content compared with the muscles in high KCl Ringer yields a ratio $phosphate_{out}/phosphate_{in}$ of 4.3. This gradient is considerably greater than the Cl^- ratio of 1.5.

5. Muscles in high KCl Ringer contain a significantly greater amount of K^+ than do those in high phosphate Ringer.

The Effect of Variation in Hydrogen Ion Concentration on the Penetration of Phosphate into the Cell.—It has been reported (7, 14) that the H_2PO_4 ion penetrates the membrane of *Staphylococcus aureus* and of yeast, but that $HPO_4^=$ is almost impermeant. By a suitable variation in the proportion of mono- and disodium phosphate, Ringers were made up at pH 6.5 and 8.5. Both solutions contained 60 mM/liter phosphate. Sixteen pairs of muscles were soaked for 4 hours in these solutions, and analysed in the usual manner. The results may be seen in Table IV, and the following points emerge:—

1. There has been a slight but significant decrease in volume in the alkaline solution, which may be attributed to the higher Na^+ concentration in this Ringer.

TABLE IV

The Effect of Variation in pH on Phosphate Content of Muscle

	pH 6.5	Ratio	pH 8.5	Ratio
Na in Ringer, *m.eq./liter*	140		190	
Cl in Ringer, *m.eq./liter*	52		52	
Volume changes, *per cent*	−0.4		−9.0	
Na, *m.eq./kg.*	44.2	3.2	60.6	3.1
Cl, *m.eq./kg.*	15.4	3.4	16.0	3.2
Phosphate, *mM/kg.*	53.5		52.5	
K, *m.eq./kg.*	70.8		71.7	

2. There has been an increase in the absolute amount of Na^+ at the higher pH, but the ratio Na^+_{out}/Na^+_{in} has remained constant.

3. There has been no change in the absolute amount of Cl^- in the cell, nor in the Cl^- gradient.

4. The amount of phosphate in the cell is the same at both levels of hydrogen ion concentration, and as the external concentration of phosphate was the same in both series, the gradient is also unchanged.

5. The K^+ level of the cells is also unaffected by the alteration in pH. This last finding is in distinction to that reported previously (18) by the authors, who found in Ringer of normal phosphate content that increase in pH led to an increase in K^+ content.

Thus it would appear that the partition of phosphate across the cell membrane is unaffected by the hydrogen ion concentration, and consequently by the charge of the phosphate ion.

A Comparison of the Inulin Space of Muscle with the Apparent Phosphate Space.—In this paper all intracellular concentrations have been based on the assumption of an extracellular volume of 15 per cent. This would appear to be close to the average value, but it is known (21) that the extracellular volume may vary from 8 to 30 per cent. Since the apparent phosphate space is of the order of 25 to 40 per cent it would be possible that the population of toads used

TABLE V

A Comparison of Inulin, Phosphate and Chloride Spaces in Muscle

	Total concentration	Intracellular concentration	Ratio
Volume Change, *per cent*	−3.1		
Na, *m.eq. per kg.*			
Control	45.1	23.6	5.5
Treated	75.0*	46.3*	3.7*
Cl, *m.eq. per kg*			
Control	39.3	21.4	5.1
Treated	31.0*	24.4	2.1*
Phosphate, *mM per kg.*			
Control	19.3	23.8	
Treated	45.1*	40.4* (16.6)	3.6
K, *m.eq. per kg*			
Control	65.0	80.6	
Treated	58.4*	74.3*	
Inulin space			
Control	20.2		
Treated	22.8		

The high phosphate Ringer contained 60 mM per liter phosphate, 171 m.eq. per liter Na^+, + 52 m.eq. per liter Cl^-.

had an unusually high extracellular volume, and consequently all increases in phosphate content in high phosphate Ringer were in the extracellular phase. To check this, estimations of extracellular volume using inulin as indicator were carried out on the same muscles as were used for the ionic estimation. Muscles were soaked for 2 hours in the normal and test Ringer, both of which contained inulin, and they were then transferred to similar solutions without inulin for a further 2 hours. The quantity of inulin released into the Ringer was estimated as described in the methods section, and the inorganic ions were determined in the muscle at the end of the soaking procedure.

It will be seen from Table V, in which total concentrations of the various ions, and intracellular levels calculated on the found inulin spaces are listed, that the phosphate space exceeds the inulin space. The general ionic pattern found conforms with that shown in Table I, and it is clear that the increment in intracellular inorganic phosphate is indeed genuine, and reflects the ability of the phosphate ion to penetrate a greater part of the cell water than does inulin.

DISCUSSION

It would appear from the results outlined above that there is a partition of phosphate across the membrane similar to, but not identical with that found for Cl^-. In other words the phosphate "space" is somewhat smaller than the Cl^- "space," but both are considerably larger than the extracellular space as measured with inulin. This is in agreement with the results of other workers. Desmedt (3) has found the extracellular space of frog muscle to be of the order of 12 per cent, and the authors have found a value of approximately 15 per cent in toad muscle. The phosphate space in muscles soaked in Ringer containing the normal amount of K^+ varies from approximately one-third to one-fifth, whereas the Cl^- space in the same muscles varies from one-half to one-third, (see Table I). Harris and Martins-Ferreira (10) also find a chloride space of 33 per cent, and a sulfate space of the same dimensions. These results will be discussed in a subsequent paper. Eggleton (4) found a phosphate space of approximately 25 per cent, which is in agreement with Causey and Harris, and also with our findings. The relationship between Na^+ and Cl^- in the muscle cell has been discussed previously, (20) and the relevant literature was also reviewed in this paper.

In any discussion of the apparent phosphate space one encounters the difficulty that part of the intracellular inorganic phosphate is already present in the control muscle, and must be subtracted from the amount found in the treated muscle in order to obtain the increment in phosphate content. This procedure assumes that there is an equal amount of phosphate in both paired muscles, which is known to be an approximation, and also that the treatment of the muscle with high phosphate Ringer does not alter this amount of "bound" phosphate in the treated muscle. There is no justification for this latter assumption, save that there appears to be no way of overcoming the difficulty. Consequently the absolute amount of phosphate entering the cell cannot be ascertained with any accuracy. Similar difficulties are discussed by Ennor and Rosenberg (5, 6) in their work using radioactive phosphorus to measure the turnover rates of organophosphates.

In common with other workers we find the muscle cell *in vivo* to contain approximately 22 mM per kg. intracellular phosphate. As was mentioned in the introduction this has been attributed to various forms of adsorption, in the

form of exchangeable labile phosphate esters, (1, 11, 15), or to active transport which is possibly linked with exchange diffusion (7, 13). It is known that isotopic P tends to accumulate in the membrane area (1), and Danielli (2) and Rothstein (16) have demonstrated phosphatases in the muscle and yeast cell membranes respectively. These authors have inferred that transport of phosphate across the membrane is an active process involving an enzymic reaction with the phosphatase. The process of transfer across the membrane appears to be rapid in mammalian muscle (6), and does not seem to be the rate-limiting step in the attainment of equilibriums. The nature of the transfer process has been reviewed by Greenberg (8).

We were unable to demonstrate a change in intracellular inorganic phosphate on soaking the muscle in normal Ringer for 4 hours, although there were marked cation shifts during this period. This is essentially in agreement with the finding of Harris (9) that very little inorganic phosphate is lost into P-free Ringer, and at variance with the finding of Ennor (6) that perfusion of mammalian muscle tends to lead to an increase in inorganic phosphate, although there is no concomitant breakdown of creatine phosphate.

An increase in the external phosphate level leads to an increase in the intracellular phosphate whether the phosphate is present as the Na^+ or the K^+ salt. (Tables I and II). The ratio external phosphate/increment in intracellular phosphate was in all cases greater than the ratio Cl^-_{out}/Cl^-_{in}. Thus it would appear that a smaller volume of the cell total is available for the entry of phosphate than for Cl^-. This is further emphasised by the fact that there is a decrease in Cl^- gradient in high phosphate Ringer, as if to maintain the anion balance of the cell. The increase in volume of muscles in high phosphate Ringer may no doubt be attributed to the lower osmotic activity of the divalent anion. The failure of muscles to shrink in Ringer rendered hyperosmotic by the addition of potassium phosphate is comparable to the failure of hyperosmotic KCl solutions to cause a decrease in volume, and must be related to the ability of the ions to penetrate the osmotic barrier of the cell. It is noteworthy that the potassium ion, when accompanied by Cl^-, is able to penetrate the cell to a greater extent than when accompanied by phosphate.

We have been unable to demonstrate a difference in phosphate penetration at pH 6.5 and 8.5, from which we infer that the charge of the ion does not affect its partition across the membrane. Causey and Harris (1) demonstrated a difference in the rate of incorporation of P^{32} in the muscle, at varying pH but this would seem to reflect an altered rate of turnover of organophosphate compounds, rather than a difference in phosphate partition. Mitchell *et al.* (14) have demonstrated that it is the monovalent phosphate ion which takes place in the phosphate exchange across the membrane in *Staphylococcus aureus* and assume the divalent ion to be impermeant. Rothstein (7) also finds the monovalent ion to be selectively absorbed in yeast.

The authors have previously proposed (20) a three compartment system to account for the partition of inorganic ions across the cell membrane in muscle. It remains now to discuss whether the data available for the distribution of phosphate in the cell can be fitted to such a scheme. Briefly, the cell is assumed to consist of an extracellular phase, containing the inorganic constituents in nearly the same concentration as is found in plasma, and an "ordered phase" and a "free intracellular phase" both contained within the cell membrane. The ordered phase may be considered to be a charged, semirigid lattice structure wherein the cellular constituents are arranged in a highly ordered fashion. It is assumed that the K^+ of the cell is adsorbed onto this structure, and would seem likely that the inorganic phosphate is similarly incorporated into the ultrastructure of the cell. This would account for the maintenance of a high phosphate gradient in the cell without the necessity of postulating a phosphate "pump." The phosphate adsorbed onto this structure would be free to exchange with external phosphate, and also free to exchange with the organic phosphate fractions of the cell.

The adsorption of K^+ onto the ordered phase has been inferred from the ability of the cell to maintain a constant level of intracellular K^+ in the face of large variations in external level. The increases in internal K^+ resulting from raising the external concentration can be accounted for by diffusion of the ion into the free intracellular phase, and are too small to be consistent with a Donnan distribution of the ion.

The free intracellular phase will be a region within the cell with a lesser degree of order than the ordered phase, and the inorganic constituents of the extracellular fluid are considered to diffuse freely into this compartment, but are excluded from the ordered phase. The isolation of these constituents in this phase, which will be considerably smaller than the ordered phase, will result in an apparent concentration gradient across the membrane when their concentration is expressed in terms of total cell volume. If this region were entirely randomised, then all the inorganic constituents of the plasma should have the same apparent ratio external concentration/internal concentration, except in so far as they may be incorporated in the ordered phase as well. If, however, one assumes the region to be intermediate in order between the extracellular fluid, and the ordered phase, then non-specific adsorption of ions may occur within it, which will alter the apparent partitions across the membrane. It is obvious from the results presented in this paper that phosphate enters the cell to a lesser extent than Cl^-, and consequently one must assume that less of the cell water is available to it than is available to Cl^-. In other words it cannot penetrate all the free intracellular phase, or alternatively, part of the Cl^- must be assumed to be in the ordered phase. At present the data are too meagre to reach any conclusion which must await an amplification of our knowledge of the properties of polyelectrolyte gels.

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