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STEADY-STATE DISTRIBUTION OF PHOSPHATE ACROSS THE MEMBRANE OF THE EHRLICH ASCITES TUMOR CELL

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SUMMARY

1. The steady-state concentrations of chloride, mono- and divalent phosphate ions in the cellular and extracellular phases of Ehrlich ascites tumor cells were determined.

2. The distribution ratios of these ions were: $[Cl^-]_{cell}/[Cl^-]_{ex.} = 0.365$, $[HPO_4^{2-}]^{1/2}_{cell}/[HPO_4^{2-}]^{1/2}_{ex.} = 0.385$ and $[H_2PO_4^-]_{cell}/[H_2PO_4^-]_{ex.} = 0.627$.

3. These results indicate that the divalent ion is passively distributed across the tumor cell membrane, while the monovalent ion is not.

INTRODUCTION

The importance of inorganic phosphate as a substrate in cellular metabolism is well documented. Recent evidence indicates that in the Ehrlich ascites tumor cell, phosphate also plays a major role in the regulation of glycolysis[1,2], respiration[2,3] and adenine nucleotide balance[4]. If this ion acts as a metabolic regulator, control of its intracellular concentration is necessary. Therefore, passive distribution across the cell membrane would reduce its effectiveness in metabolic regulation.

In a previous publication[5] it was shown that the cell membrane mediates the transport of phosphate into and out of the cell. In order to define the role of the cell membrane in phosphate transport and metabolism, a knowledge of the steady-state concentrations of phosphate in both the intracellular and extracellular phases is essential. Therefore, the measurement of the steady-state distribution ratio of phosphate and chloride at various extracellular concentrations was undertaken.

METHODS

Experiments were performed with Ehrlich–Lettré mouse ascites tumor cells ($n = 41$) that were maintained in Swiss white mice (HA/ICR) by weekly transplantation. Tumor-bearing animals with growths between 9 and 11 days were used.

Cell suspensions were removed from unanesthetized animals by peritoneal aspi-

Abbreviation: DMO, 5,5-dimethyl-2,4-oxazolidinedione.

ration and transferred to a large volume of sodium and potassium Ringer solution[6] (9 g NaCl, 40 ml 0.154 M KCl, 15 ml 0.11 M NaH_2PO_4 and 85 ml 0.11 M Na_2HPO_4 solutions to 1 l with glass-distilled water) at room temperature.

The cell suspension was divided into two or more equal aliquots and washed in Ringer solution of varying phosphate concentration. $[P_i]$ (1.44–135.1 mM) was varied by isosmotic replacement of NaCl in the environment. The final solutions were pH 7.37–7.44 and 305–315 mosM.

10–15 ml of cell suspension ($6 \cdot 10^7$–$8 \cdot 10^7$ cells/ml) were incubated in 1-l flasks under 100% O_2 at 37°. To insure that steady-state conditions with respect to cell water, chloride and phosphate content were obtained, cell suspension from each flask was sampled at 35 min and again at 50 min, and transferred to preweighed Wintrobe hematocrit tubes (5 mm × 60 mm) in triplicate, and centrifuged for 23 min at 2300 × *g*. After removal of the supernatant fluid, the tubes were reweighed, and the packed cells dried at 90° for 16 h. Wet weight, dry weight and cell water content, corrected for extracellular fluid volume (see below), was then calculated. The ratio of wet to dry weight in 36 determinations was 4.141 ± 0.0407 (S.E.), and the cell water content was 75.85 ± 0.75% (S.E.) of the wet weight.

The volume of the extracellular fluid phase was determined by mixing cell suspensions with tracer amounts of $Na_2{}^{35}SO_4$ and transferring cell suspension at various times to preweighed Wintrobe hematocrit tubes. After centrifugation at 2300 × *g* for 23 min the tubes were reweighed and the packed cell surface rinsed three times with Ringers solution followed by the addition of 0.3 ml of 6% (v/v) perchloric acid. Aliquots of the perchloric acid extract and supernatant solution were then counted in 10 ml of BRAY's cocktail[7] in the Packard Model 3375 liquid scintillation spectrometer. This procedure made it possible to correct for any uptake of $^{35}SO_4{}^{2-}$ by the cells by extrapolating to zero time. Fig. 1 shows the results of a representative experiment. The trapped volume in three separate experiments was 0.20 ± 0.01 (S.E.) ml

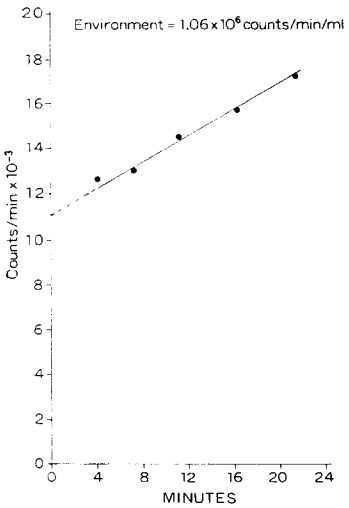

Fig. 1. The measurement of extracellular fluid volume with $Na_2{}^{35}SO_4$. Consult METHODS for details of the procedure. Data from a representative experiment.

per g wet cell weight, which agrees with the values reported by HEINZ AND MARIANI[8]. This correction factor was applied to all determinations of cell water content.

Concomitant with the determination of wet weight, dry weight, and water content, samples of cell suspension were removed to centrifuge tubes, centrifuged for 35 sec and stopped by mechanical braking. The cellular environment (ex.) was saved for analysis of pH, chloride, and P_i and the packed cells washed twice with unbuffered ice-cold isosmotic $NaNO_3$ solution (7.25 g $NaNO_3$ to 500 ml distilled water). Less than 3 min elapsed from the initial to final centrifugation. The packed washed cells were immediately placed in an ice bath and 3 ml of cold water were added followed by 0.50 ml of 35 % (v/v) cold perchloric acid. 2 ml of the perchloric acid extract were used for the analysis of P_i (ref. 9). To correct for possible spontaneous hydrolysis of labile phosphate esters, 0.80 μmole of ATP contained in a final volume of 3.5 ml perchloric acid–water was run parallel to the experimental samples. In 25 experiments ATP breakdown was 2.84 $\pm$ 0.46 % (S.E.). Since the ATP content of the tumor cell is 0.02–0.05 μmole per 10^7 cells[10,11] and in these experiments each cell sample contained $6 \cdot 10^7$–$8 \cdot 10^7$ cells, the total ATP content of the perchloric acid extract never exceeded 0.40 μmole. Consequently, 2.84 % breakdown of ATP represents a maximum value.

Chloride analysis of the perchloric acid extract and cellular environment was done with the Buchler-Cotlove automatic titrator at low titration rate. The chloride and P_i concentration of each sample was calculated from the ion content of the perchloric acid extract of the packed, washed cells (μmoles per g wet weight) divided by its corresponding cell water content (mg water per g wet weight). No attempt was made to measure the activity coefficient of phosphate or chloride ions. Therefore, in this paper it is assumed that the ratio of intracellular to extracellular activity coefficients was unity.

At the end of the experiment, samples of cell suspension were transferred to centrifuge tubes and centrifuged for 35 sec at 2300 $\times$ g. The cellular environment was saved, and the packed cells washed twice with unbuffered, cold, isosmotic $NaNO_3$ solution. The washed, packed cells were then frozen and thawed three times in a dry ice–acetone mixture. The cellular environments and cytolyzed cells were then incubated at 37° for 15–25 min. After temperature equilibration, the pH of the samples was measured with a Corning Model 10 expanded pH meter equipped with a Corning No. H-4332-1 combination pH electrode. The pH meter was standardized at 37° with 0.01 M potassium phosphate buffer (pH 7.05).

RESULTS

Table I summarizes the results of 20 experiments performed in exactly the same manner on 10 different tumor cell populations. In each experiment the values for cellular and environment chloride and P_i represent the mean of samples taken at 35 min and 50 min. Preliminary experiments indicated that cells when incubated in increasing concentrations of P_i (decreasing chloride) reached a steady state with respect to pH, chloride and P_i within 30 min at 37°. Consequently, the 50-min sample was in most cases a duplicate of the 35-min sample. However, at high phosphate concentrations (50–135 mM) a difference between cell samples taken at 35 min and 50 min was noted. This difference was usually 5–10 % greater P_i concentration or

TABLE I

RESULTS OF 20 EXPERIMENTS WITH 10 DIFFERENT POPULATIONS OF TUMOR CELLS

In each experiment the values listed for environment (ex.) and cellular chloride and phosphate represent the mean of samples taken after 35 min and 50 min of incubation at 37° under 100% O_2. pH measurements were made after 50 min incubation.

Cell preparation	$[P_i, total]_{ex.}$ *(mM)*	$[Cl^-]_{ex.}$ *(mM)*	*pH (ex.)*	$[P_i, total]_{cell}$ *(mmoles/kg cell water)*	$[Cl^-]_{cell}$ *(mmoles/kg cell water)*	*pH (cell)*
6	1.44	165.38	7.18	13.94	62.88	6.590
3	1.70	160.16	7.23	16.28	63.66	6.625
7	2.49	168.68	7.19	13.31	68.11	6.595
9	2.76	162.89	7.24	16.67	64.58	6.620
1	11.00	161.70	7.31	12.95	69.41	6.655
2	11.50	163.50	7.25	15.79	56.67	6.625
4	11.53	167.55	7.32	15.69	70.11	6.670
5	11.65	171.98	7.27	13.65	64.49	6.350
3	12.28	167.00	7.33	16.88	71.55	6.665
8	18.11	155.21	7.31	17.12	61.50	6.685
10	23.06	135.92	7.37	17.66	67.23	6.685
7	31.00	137.96	7.30	20.05	58.21	6.675
1	44.12	132.00	7.40	17.06	57.34	6.705
2	50.21	125.61	7.38	23.50	51.00	6.695
10	57.23	118.70	7.40	22.97	46.65	6.705
8	57.67	104.02	7.41	23.41	53.97	6.720
9	65.11	90.11	7.38	26.50	38.01	6.695
5	102.13	71.00	7.42	33.74	30.93	6.720
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4	135.10	52.53	7.41	46.64	21.13	6.745

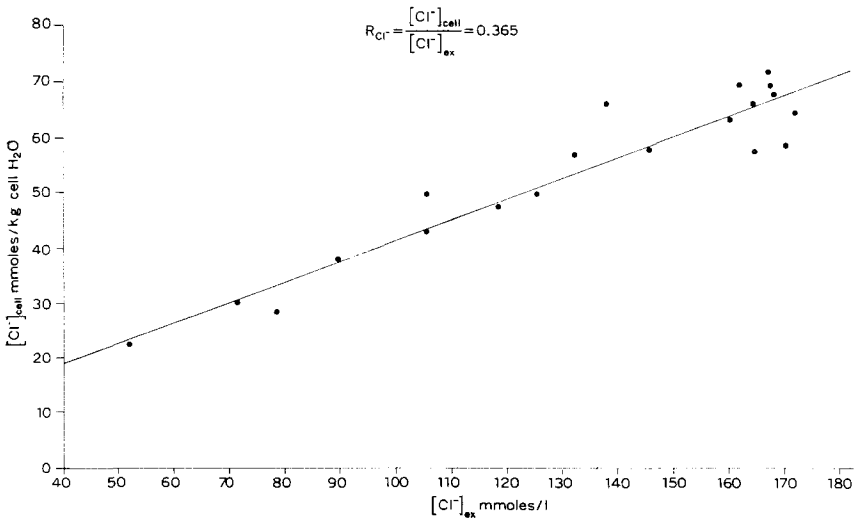

Fig. 2. The effect of extracellular chloride concentration on steady-state intracellular chloride concentration. Each point represents the mean value for samples taken after 35 min and 50 min incubation at 37°. $y = 5.528 + 0.3648\,x$.

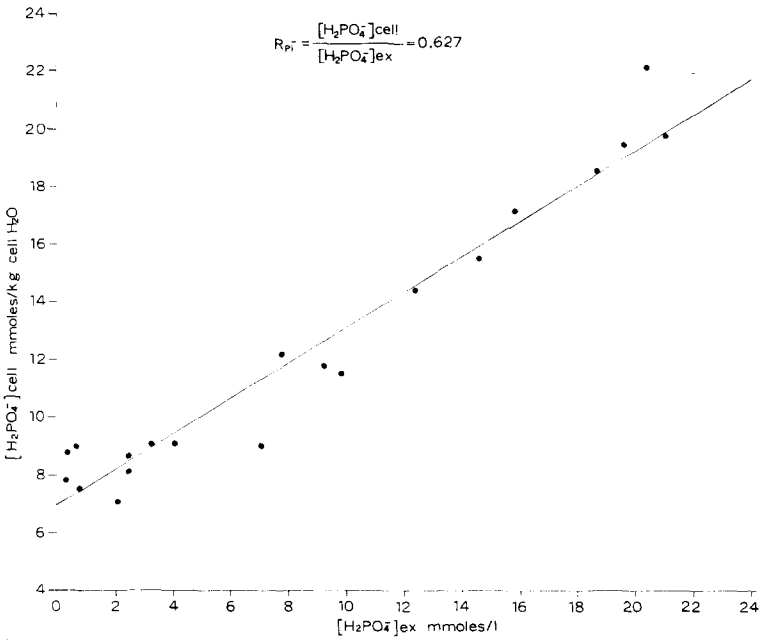

Fig. 3. The effect of extracellular monovalent phosphate concentration on steady-state intracellular monovalent phosphate concentration. Each point represents the mean value for samples taken after 35 min and 50 min incubation at 37°. $y = 6.922 + 0.6274\,x$.

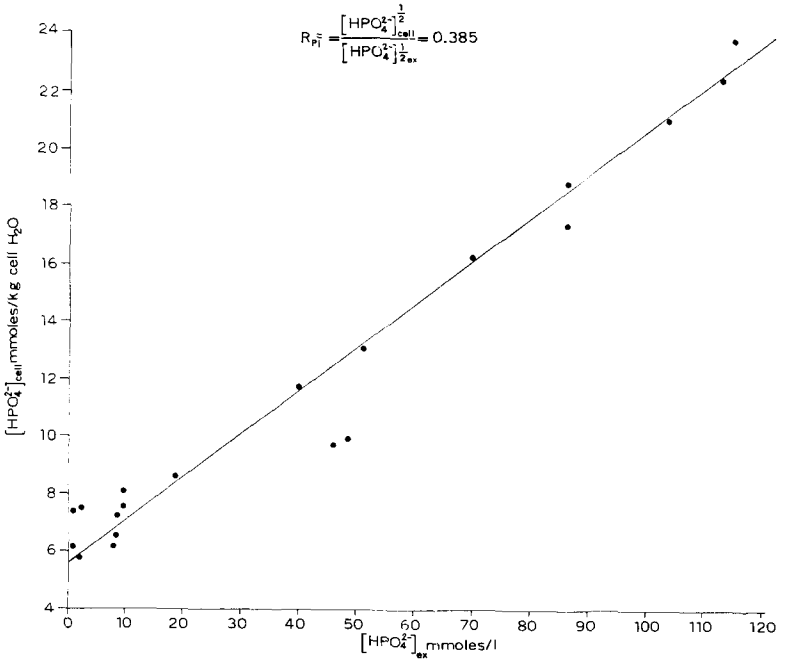

Fig. 4. The effect of extracellular divalent phosphate concentration on steady-state intracellular divalent phosphate concentration. Each point represents the mean value for samples taken after 35 min and 50 min incubation at 37°. $y = 5.515 + 0.148\,x$; $(0.148)^{1/2} = 0.384$.

lowered chloride concentration in the 50-min samples. Since incubation of tumor cells at 37° for more than 1 h often results in cell damage (unpublished observation), the difference between the 35-min and 50-min samples was considered small when compared to the adverse effects of prolonged incubation.

In order to determine whether P_i distribution across the tumor cell membrane is determined solely by the membrane potential, the analytical data presented in Table I were plotted as shown in Figs. 2–4. Fig. 2 shows the distribution of chloride between the intracellular and extracellular phases. Since the available information suggests that chloride distribution across the tumor cell membrane is passive[12,13], the intracellular chloride concentration should vary in a predictable manner with changing environmental chloride concentration. This prediction was realized and the slope of the linear regression line which describes $[Cl^-]_{cell}/[Cl^-]_{ex.}$ was 0.365. Consequently, if P_i is similarily distributed, a plot of intracellular to extracellular phosphate concentration should produce the same slope. However, P_i can exist as four distinct species, and the relative proportion of each form in solution is determined by the pH. Therefore, when calculating the P_i distribution ratio, it is imperative to specify the ionic species under consideration.

The equations derived in the following were used subsequently for calculating the concentrations of monovalent phosphate ($H_2PO_4^-$) and divalent phosphate (HPO_4^{2-}) in both the environment and cell phases.

$$H_3PO_4 \overset{K_1}{\rightleftarrows} H_2PO_4^- + H^+ \overset{K_2}{\rightleftarrows} HPO_4^{2-} + H^+ \overset{K_3}{\rightleftarrows} PO_4^{3-} + H^+ \tag{1}$$

The analytically measured phosphate concentration $[P_i, \text{total}]$ is given by:

$$[P_i, \text{total}] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^{2-}] + [PO_4^{3-}] \tag{2}$$

but at pH 7.1–7.4 only negligable amounts of H_3PO_4 and PO_4^{3-} exist. Therefore,

$$[P_i, \text{total}] = [H_2PO_4^-] + [HPO_4^{2-}] \tag{3}$$

and from (1),

$$K_2 = \frac{[H^+]\,[HPO_4^{2-}]}{[H_2PO_4^-]} \tag{4}$$

Substitution of (3) into (4):

$$[H_2PO_4^-] = \frac{[P_i, \text{total}]\,[H^+]}{[H^+] + K_2} = \frac{[P_i, \text{total}]\,10^{-pH}}{K_2 + 10^{-pH}} \tag{5}$$

Eqn. 5 shows that the monovalent phosphate ion concentration, $[H_2PO_4^-]$, can be obtained if the total phosphate concentration, the second ionization constant (K_2), and the pH are known. The divalent phosphate ion concentration, $[HPO_4^{2-}]$, can then be obtained from Eqn. 3.

In the present experiments, K_2 for the extracellular environments was calculated from the equation given by Bjerrum and cited by VESTERGAARD-BOGIND AND HESSELBO[14] for the ionic strength range up to 0.10: $pK_2 = 7.165 - 1.545\,I^{1/2} + 1.12\,I$, where I is the ionic strength of the solution. The ionic strength of the extracellular phase increased from 0.1914 to 0.331 as the extracellular phosphate concentration increased. This gave pK_2 (ex.) values of 6.701–6.649, or K_2 (ex.) values of $2.21 \cdot 10^{-7}$–$1.99 \cdot 10^{-7}$. Although K_2 (ex.) varied with the ionic strength of the extracellular phase,

the value $2 \cdot 10^{-7}$ was used for calculating $[H_2PO_4^-]_{ex.}$. This simplification introduced an error of less than 4 % in the final result. The ionic strength of the intracellular water is unknown, but based on the total cation and anion composition of the tumor cell[15] 0.197 would be a reasonable estimate. This gives pK_2 (cell) the value 6.70. The analytically determined [P_i, total] and pH values listed in Table I were also used in computing $[H_2PO_4^-]$.

Fig. 3 shows the results of plotting the calculated values of $[H_2PO_4^-]_{cell}$ as a function of $[H_2PO_4^-]_{ex.}$ for each experiment. In Fig. 4 the calculated values of $[HPO_4^{2-}]_{cell}$ as a function of $[HPO_4^{2-}]_{ex.}$ are presented in a similar manner.

The slopes of the resulting linear regression lines are: $[H_2PO_4^-]_{cell}/[H_2PO_4^-]_{ex.} = 0.627$, and $[HPO_4^{2-}]^{\frac{1}{2}}_{cell}/[HPO_4^{2-}]^{\frac{1}{2}}_{ex.} = 0.383$.

Among the systematic sources of error, the values selected for pK_2 and pH (cell) are particularly important. The pK_2 (ex.) which depends on the ionic strength was calculated for the extracellular phase. However, it is rather difficult to select a value of pK_2 (cell), since precise knowledge of the ionic strength of the intracellular phase is unknown. As mentioned, we have chosen 0.197 as a reasonable estimate for intracellular ionic strength. This is based on the total anion, cation and organic phosphorus content of these cells[5,15]. In these experiments intracellular pH was estimated on freeze–thawed, washed, packed cells with a conventional glass electrode. WADDELL AND BATES[16] have criticized results based on this technique. These authors suggest 5,5-dimethyl-2,4-oxazolidinedione (DMO) distribution as a more satisfactory indicator of hydrogen ion distribution. However, in their work with red cells, BROMBERG *et al.*[17] showed a closer correlation between R_{Cl^-} and R_{H^+} (measured on freeze–thawed hemolysates) than between R_{Cl^-} and R_{DMO^-}.

Our data show that changes in the extracellular pH resulted in only small changes in intracellular pH. This is in accord with the results of POOLE *et al.*[18] who calculated the intracellular pH of tumor cells based on DMO distribution. However, the absolute values for intracellular pH measured here were 0.10–0.30 pH unit lower than those measured by DMO distribution[18]. When the values of extracellular and intracellular pH (Table I) are converted to hydrogen ion concentrations, the mean ratio of $[H^+]_{ex.}/[H^+]_{cell}$ is 0.389 which agrees favorably with the ratio $[Cl^-]_{cell}/[Cl^-]_{ex.} = 0.365$.

DISCUSSION

If the steady-state distribution of phosphate is determined solely by the Donnan potential, the ratio of intracellular to extracellular phosphate should be constant for all values of extracellular phosphate. In addition, this constant should be predictable from either the membrane potential (E_m) or equal to the distribution ratio of other ions distributed in accordance with E_m. Since in these experiments the membrane potential was not measured, the assumption is made that the chloride distribution ratio is a function of the Donnan potential or $[Cl^-]_{cell}/[Cl^-]_{ex.} = f(E_m)$.

There is ample precedence for this assumption. Many investigators[17,19] have shown in red blood cells that the distribution of chloride, bicarbonate, and hydrogen ions seems to accord with the Donnan potential, and there is no need to invoke a process involving the expenditure of metabolic energy to account for differences between intracellular and extracellular concentrations of these ions. A direct confir-

mation of this was recently made by JAY AND BURTON[20] who showed that the theoretical electromotive force corresponding to the ionic ratios of chloride was equal to the measured membrane potential.

Unfortunately, the situation in Ehrlich ascites tumor cells with regard to passively distributed ions is not as well defined. AULL[21] has recently measured the membrane potential of these cells using KCl-filled glass microelectrodes. Although the measured potentials were not stable for long periods of time, the mean potential difference was 11.2 mV inside negative, which was considerably less than that calculated from the chloride distribution ratio. However, it was concluded that the chloride ion was passively distributed and the discrepancy between the measured E_m and the potential predicted from the chloride distribution ratio (E_{Cl^-}) was due to high sodium permeability producing a shunt. Additional evidence supporting the view of passive chloride distribution comes from the work of GROBECKER *et al.*[22] who showed that the initial influx of chloride was directly proportional to the extracellular concentration. HEMPLING AND KROMPHARDT[23] also reported that the efflux rate coefficient remained constant when external chloride was varied. Consequently, the most reasonable criterion for assessing passive distribution of an ion across the tumor cell membrane is to compare its steady-state distribution ratio to that of the chloride ion.

The data shown in Fig. 2 demonstrate that when extracellular chloride was varied from 172 mM to 53 mM, intracellular chloride decreased by a constant amount. The slope of the linear regression line is 0.365. If phosphate were distributed in a similar fashion, the ratio of intracellular to extracellular phosphate should also be 0.365. Fig. 4 shows that $[HPO_4^{2-}]^{1/2}_{cell}/[HPO_4^{2-}]^{1/2}_{ex.}$ is 0.385, or 1.05 the chloride ratio. This result suggests that divalent phosphate ions are passively distributed. However, the distribution ratio of $[H_2PO_4^-]_{cell}/[H_2PO_4^-]_{ex.}$ as shown in Fig. 3 is 0.627, or 1.72 the chloride ratio. This indicates that monovalent phosphate ions are not passively distributed and some force other than the membrane potential must be operative.

Examination of Figs. 3 and 4 also indicate that when extracellular phosphate was reduced to near zero, cellular phosphate did not fall to zero. This fraction of phosphate may be weakly bound within the cell. Previous experiments have shown that this fraction of phosphate participates in glucose phosphorylation and exchanges with extracellular phosphate[5]. Therefore, while a constant fraction of cellular phosphate may be bound, this apparently does not influence the steady-state distribution of phosphate ions.

The results of these and previous experiments[5] support the view that membrane transport of phosphate is not an example of passive diffusion. Instead, some property of the membrane, mediating transport is suggested. In recent experiments made in this laboratory (to be reported), we have shown that the steady-state phosphate flux is sensitive to the pH of the extracellular phase. When the pH of the environment increased, intracellular phosphate and flux decreased. Since increasing the extracellular pH results in decreased $[H_2PO_4^-]_{ex.}$ (Eqn. 5), it is conceivable that the transport system is specific for the monovalent ion. A similar situation obtains with the phosphate transport system of yeast[24], while in fertilized sea urchin eggs the transport system is specific for the divalent ion[25]. The implication is that the transport system is capable of maintaining intracellular $[H_2PO_4^-]$ at a level above that predicted by passive forces only. Whether this represents active transport is currently under investigation.

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