THE RELATION BETWEEN MITOCHONDRIAL ANION ACCUMULATION AND THE pH GRADIENT WITH SPECIAL REFERENCE TO PHOSPHATE DISTRIBUTION

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1. Introduction

It has been demonstrated that penetrating organic anions are accumulated in mitochondria in a charge-dependent way, as they would be in a Gibbs–Donnan system [1]. The purpose of this note is to extend the treatment to cover the phosphate ion and consequently the pH gradient.

2. Theory

Consider a two compartment system interconnected by a membrane permeable to both HPO_4^{2-} and $H_2PO_4^-$

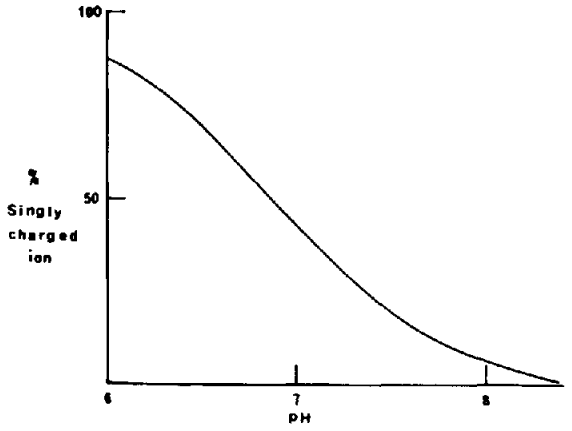

Fig. 1. Relation between pH of a phosphate buffer mixture and proportion of singly charged ion in the mixture, from reference tables. Ionic strenght 0.1, 25°.

and to organic anions; the two compartments will be referred to as 'inside' and 'outside'. With the anions present, the minimum potential energy condition corresponds to no work being obtainable by interchanges between the different anions. As in the Gibbs–Donnan system, this condition requires that the ratios between inside and outside concentrations of anions A^-, B^{2-}, C^{3-} having charges 1, 2 and 3 e^- per ion are related by

$$\frac{A_i^-}{A_o^-} = \left(\frac{B_i^{2-}}{B_o^{2-}}\right)^{1/2} = \left(\frac{C_i^{3-}}{C_o^{3-}}\right)^{1/3} = n$$

Between the limits pH 6 and pH 8 the phosphate ion is essentially a mixture of singly and doubly charged forms; the proportions of the forms can be found in reference tables. For convenience, the fraction of singly charged form in a mixture to provide an ionic strength of 0.1 is plotted in fig. 1. For a pH selected for the outside solution the proportions of the two forms can be read off. Now when, for some reason such as an electrical asymmetry or presence of non-penetrant ions, the value of n exceeds unity, the singly charged form is concentrated n times and the doubly charged form is concentrated n^2 times. The inside will then be more alkaline than the outside.

Provided two or more forms of the phosphate ion are permeant the pH difference across the membrane must adjust to conform to the ratio n. This can be shown by substituting n and n^2 respectively for the ratios of singly and doubly charged forms in the dissociation equations for inside and outside. The measurable quantity is the ratio between the total inorganic phosphate concentrations on the two sides.

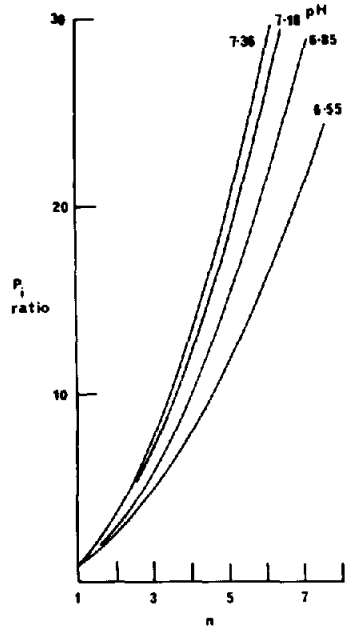

Fig. 2. The relation between the factor by which inorganic phosphate is accumulated and n the factor applying to the singly charged form. The curves are calculated for different outside pH values.

From the ionisation curve (fig. 1) one can calculate the proportions of the two forms and hence obtain the ratio for total phosphate at different external pH (fig. 2). Since n can be found using other anions a comparison with the figure deduced from the phosphate ratio is obtainable.

3. Methods

Rat liver mitochondria were prepared as before [1,2]. Ratios between inside content of malate or citrate and the external concentration were measured using the ^{14}C-labelled compounds with centrifugal separation of the mitochondria through silicone exactly as before [3]. Parallel measurements were always made with ^{14}C-labelled sucrose to find the amount of external solution and internal sucrose-accessible solution carried down with the protein. The inorganic phosphate distribution was obtained by estimations of ^{32}P added to carrier phosphate at the desired outside concentration. The inorganic phosphate in the supernatant over the silicone centrifugation, and in the acid extract of the protein recovered from beneath the silicone, was extracted as the phosphomolybdate complex by means of isopropyl acetate, an aliquot of which was used for radioactivity measurement. To convert to the phosphomolybdate, the phosphate solutions were mixed with 2 ml of 3.3% by volume sulphuric acid having 2% ammonium molybdate dissolved in it, then isopropyl acetate (2 ml) was shaken with the acid mixture for 1 min.

4. Results

The distributions of phosphate, citrate, and other different organic anions, were determined in mitochondria drawn from chemically identical mixtures separated into a number of portions each with a different anion marked with radio isotope. An additional portion was set up with labelled sucrose to find the amount of medium and sucrose accessible space in the pellet. The comparisons had to be made in parallel sets with each mitochondrial preparation because the value of n holding varies between preparations, and probably with time. The contents of the pellet were corrected for the amount of anion which would have been accommodated in the measured sucrose-accessible space. These corrected contents have been regarded as a measure of the concentration of the anion in the matrix water, and are divided by the applied concentrations to obtain the ratios which are thus contents μmole/g protein/external concentration (mM). The matrix was as deduced from the difference between tritium and sucrose space was 0.5–1.0 ml/g. Departure of the value from unity will after the concentration ratios equally but introduces errors when the $(1/n)^{th}$ roots are taken.

The results of the measurements of content/concentration ratios are summarised in table 1. The rows refer to individual parallel incubations.

In order to test the use of the phosphate relations in fig. 2, the outside pH was set at different values in certain of the experiments. The data show that the values of n deduced from the total phosphate ratios are in reasonable accordance with values derived from the ratios holding for the organic acids.

Valinomycin will induce uptake of K^+ along with phosphate. The consequent increase in concentration

Table 1
Use of total inorganic phosphate ratio inside/outside to find n (from fig. 2) and comparison with values of n found from organic anion ratios.

Extl pH	Extl phosphate (mM)	Ratio of inorganic phosphate inside/outside	Ratio of citrate	Ratio of dicarboxylic acid	n from P_i	n from cit	n from dca	n from pyr
7.4	0.5	18.0	57	15.4 (malate)	4.8	3.8	3.9	–
7.4	0.4	5.7	20	5.3 (succinate)	2.5	2.7	2.3	–
7.4	0.26	12.1	46	13.7 (malate)	3.8	3.5	3.7	–
7.4	0.22	30.4	129	–	6.3	5.05	–	–
6.5	0.16	10.2	73	–	4.7	4.2	–	–
7.0	0.076	20.6	82.5	15.9 (malate)	5.6	4.3	4.0	5.7

cit: citrate; dca: dicarboxylic acid; pyr: pyruvate

of phosphate (with allowance for water entry) will, according to the inverse relation between protons and anions, give rise to an alkalinisation. In an experiment made with 5 mM K^+ salt and 2 mM phosphate in the medium at pH 7.4 the internal phosphate before valinomycin was 20 μmoles/g protein after correction for the phosphate in the sucrose space.

This total phosphate ratio corresponds to $n = 3.4$ (from fig. 2) and Δ pH 0.53 units. With valinomycin at 30 μg/g protein the inorganic phosphate rose to 44 μmole/g and the concentration in the medium fell to 1.7 mM. There was swelling of the matrix, as revealed by the difference between the total water in the pellet exchangeable with tritiated water and the water accessible to sucrose: the difference rose from 1.02 to 1.38 ml/g protein. Taking account of the additional water the new total phosphate ratio comes to 19, the corresponding n is 4.85 and the Δ pH is 0.69. In this experiment the valinomycin induced K^+ uptake (which amounted to 100 μmole/g) along with phosphate causes an alkalinisation of 0.16 units.

5. Disussion

The evidence presented supports the proposition that both singly and doubly charged forms of phosphate accumulate across the mitochondrial membrane as they would in a passive system. This would give rise to a proton gradient inversely related to the gradient of the singly charged anions. The origin of the asymmetry giving rise to anion accumulation is presumably the energy-linked accumulation of the cations Ca^{2+} and K^+. It has been shown that cations emerge when the energy supply is inhibited [4,5] and the cation content is diminished when anion accumulation is impaired [6,7]. The redistribution of phosphate allows an explanation to be given for the proton ejection accompanying uptake of Ca^{2+} or K^+ (in presence of an ionophore). A gain of cation is accompanied by an increased capacity for anions [8] and the consequent increased anion ratio requires that the internal proton concentration should fall to maintain the inverse relation. Either protons emerge or the doubly charged phosphate inside is protonated and is replaced by more from outside.

One may proceed to ask how many protons can so be shifted. With $n = 10$ the interior will be 1 pH unit alkaline to the outside. With the latter at pH 7.4 the internal pH will approach the condition of inhibition of certain enzymes. The buffering power of the mitochondrial content is about 50 nequiv/mg protein [9] so the maximum proton output is likely to be about the same before inhibition occurs. It is presumably no mere coincidence that the maximum uptake of Ca^{2+} in absence of added penetrating anion is about 50 μmoles/g [10] with which is associated a numerically equal proton output. The pH differential associated with the Ca^{2+} uptake as deduced from the distribution of dimethyloxazolidinedione is about 1 unit [11] while that associated with valinomycin-induced K^+ uptake is less, about 0.2 unit [12]. The valinomycin-induced K^+ uptake is in general associated with less than equivalent proton output which, in fact, seems to reach a maximum of little more than 50 μequiv/g protein [13]. The limitation is likely to

arise from the water entry associated with the K^+ uptake since this will restrict the ratio between inside and outside anion concentrations.

The relations between the accumulation of certain anions, such as citrate, malate, pyruvate and phosphate may perhaps be explained by an electrostatic absorption on neighbouring internal negative sites. This mechanism offers a possibility of explaining why certain anions behave differently to those just mentioned [1].

Acknowledgements

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