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A Physico-chemical Basis for Anion, Cation and Proton Distributions between Rat-liver Mitochondria and the Suspending Medium

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The distribution of ions, including protons, between the mitochondrial interior and the medium can be treated, most simply, as a physico-chemical problem akin to that met with in cell suspensions, particularly when sufficient is known about the system. More is now known about mitochondrial properties, and the aim of the present review is to summarize the evidence, some new and much derived from earlier reports, that the distributions of permeant anions and of protons between the mitochondrial interior and the exterior provide an example of the Gibbs–Donnan law applied to a situation with an ionized internal buffer. This can only hold of course when the metabolic fluxes are low. Some anions behave as non-penetrants and so can take on any distribution ratio. The cations Ca^{2+} and K^+ are subject to energy-linked inward transport.

The Basis of the Physico-chemical Interpretation

The classical Gibbs–Donnan system has 2 compartments ("in" and "out"), and one at least carries a complement of charged particles which cannot pass to the other. The requirement for equalities of electrochemical potential of the salts of permeable species lead to the equations:

$$r = H^+_{out}/H^+_{in} = Anion^{1-}_{in}/Anion^{1-}_{out} = (Anion^{2-}_{in}/Anion^{2-}_{out})^{1/2} \qquad (1)$$

for protons and permeant anions bearing the number of charges shown as superscript. Across the membrane there is potential difference $E = RT/F \ln r$.

When, as in the present case, cations are moved by energy-linked process, the charge balance leads to:

$$H^+ + K^+ + 2Ca^{2+} = [X^-] + rA^-_{out} + r^2 A^{2-}_{out} + r^3 A^{3-}_{out} + \sum nB^{n-}_{in} \qquad (2)$$

In this A^- denotes the sum of singly charged penetrant anions and so on.

and B_{in}^{n-} is an internally generated n-charged anion which remains trapped inside.

The values of r and the potential difference will rise or fall as the energy-linked cation accumulation process provides more, or fewer internal cations. In addition the parameters will respond to the content of non-buffer anions denoted by A^- and B^- in equation (2). A further factor related to r is the internal pH (pH_i) because it determines the degree of ionization of the buffer (X^- in equation (2)). As pH_i falls the buffer becomes protonated and to maintain internal electroneutrality more anions A^- and/or B^- can be accommodated.

Between pH 7 and 8, in presence of uncoupler to preclude energized ion movements, Harris *et al.* (1966) found that mitochondria have a buffer power of about 50 nequiv per mg dry weight. The value corresponds to about 55 nequiv per mg protein because the dry weight is about 1·1 × the biuret protein. The result means that when internal pH falls from 8 to 7 electroneutrality requires entry of 55 nequiv anions, provided no cations are lost. This explanation of why mitochondrial anion capacity increases with acidity, a phenomenon described by Palmieri *et al.* (1970) does not involve assumptions about the mechanism of proton movement.

To relate the ionization of the internal buffer to the anion content the transmembrane pH difference (ΔpH) can be calculated from the concentration ratio holding for a selected permeant anion using the relation:

$$\Delta pH = \frac{1}{n} \log \frac{A_{in}^{n-}}{A_{out}^{n-}}$$

TABLE I. Comparison between transmembrane pH differences deduced from distributions either of dimethyloxazolidinedione (DMO) or phosphate using the data of Hoek *et al.* (1971) and the curve relating phosphate concentration ratio to Donnan ratio given by Harris (1970)

Phosphate concn. in medium μM	200	560	700
ΔpH deduced from DMO ratio	1·10	0·93	0·87
ΔpH deduced from phosphate ratio	1·17	0·99	0·96

Hoek *et al.* (1971) have shown that the ΔpH deduced from the distribution of the weak acid dimethyloxazolidinedione agrees fairly well with that deduced from the phosphate distribution. Calculated values derived from their data are given in Table I. A slight excess phosphate concentration (giving an apparently higher pH) can be ascribed to binding, probably to Ca^{2+}. Palmieri *et al.* (1970) demonstrated an inverse relation between the ΔpH (deduced from the dmo distribution) and the concentration ratio for acetate and the square root of the ratio

for the doubly charged malate anion. Although these authors do not accept the applicability of the Donnan concept with its associated internal positivity their data are in precise accord with the behaviour predicted by the theory.

In the light of equation (2), let us consider how the situation is changed when a penetrant anion is added. As more anion enters the mitochondria there is a trend to saturation because the maximum capacity is limited to equality with the cation equivalents. Hence, the concentration ratio between inside and out falls and ΔpH falls,

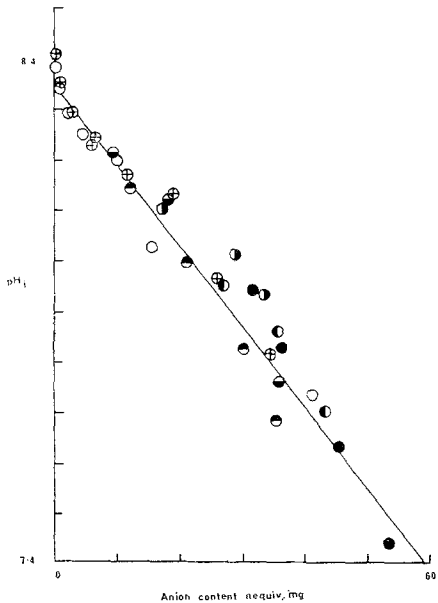

Figure 1. The relation between the content of internal anions in equivalents and the internal pH. The latter was deduced by adding:

$$\Delta\text{pH} = \frac{1}{n}\log\frac{A_{\text{in}}^{n-}}{A_{\text{out}}^{n-}}$$

to the external pH. The differently marked points refer to different experiments in which various anions were used. Experimental details of the experiments are given elsewhere (Harris and Bangham, 1972).

with the interior becoming less alkaline. The internal buffer is protonated ($X^- \rightarrow HX$) equivalent to the anion gain so, in effect, the internal buffer is titrated by the anions entering. This fact is used by plotting the ΔpH deduced from the ratio of a permeant anion against the total content of anions other than the buffer. In this way a curve is obtained corresponding to a buffering power of about 60 nequiv per pH per mg protein (Fig. 1).

This explanation of the nature of the response of internal pH to anions requires a means by which protons can be supplied to combine

with X^-. As originally suggested by Chappell and Crofts (1966) a cycling of the two forms of the phosphate ion present at physiological pH can act as proton transporter. That phosphate distributes according to the ratio holding for other permeant anions has been shown by Harris (1970). Most mitochondrial preparations carry sufficient phosphate to obscure the requirement when relatively small anion movements are studied. However, Chappell and Crofts (1966) did find a phosphate requirement for penetration in quantity of dicarboxylates.

A demonstration of the applicability of another feature of equation 1; namely, the dependence of anion content on cation content is perhaps most directly obtained by comparing anion and cation contents before and after induction of K^+ uptake by valinomycin. Permeant anions are found to enter in amount almost equivalent to the K^+ gain (Harris, 1969). Less extensive movements of anions with Ca^{2+} have also been described (Rasmussen *et al.*, 1965; Rossi *et al.*, 1967; Kimmich and Rasmussen, 1968; Harris and Berent, 1970). Uptake of phosphate along with Ca, even when the prevalent concentration of phosphate is only that provided by leakage from the mitochondria, will provide a quantitative explanation of the acidification of the medium accompanying Ca^{2+} uptake. In absence of penetrant anions, about 1 H^+ appears in the medium per Ca^{2+} removed from it. If one calculates the acidity left in the medium when trebly charged phosphate is removed to form either tricalcium phosphate or hydroxyapatite, the results (Table II) depend on external pH as do the observed values. Best agreement is obtained on the assumption of formation of a compound having the composition of hydroxyapatite, though evidently this is not formed in the crystalline state (see review by Lehninger, 1970).

Another consequence of Ca^{2+} uptake which has attracted considerable interest is the transient internal alkalinization which accompanies it (Chance and Mela, 1966; Reynafarge *et al.*, 1967; Rossi *et al.*, 1966;

TABLE II. Calculation of ratio of protons in medium to Ca^{2+} uptake for different pH media. It is assumed that the Ca^{2+} is accompanied by phosphate to form internal hydroxyapatite: $3.Ca_3(PO_4)_2.Ca(OH)_2$

pH of medium	7·62	7·15	6·48
Net charge on P_i	−1·85	−1·60	−1·30
Protons freed when 6 P_i converted to P_i^{3-}	6·9	8·4	10·2
Protons freed by dissocn. of water to provide $2OH^-$	2	2	2
Protons per hydroxyapatite (10 Ca)	8·9	10·2	12·2
Protons/Ca calc.	0·89	1·02	1·22
Protons/Ca obs.	0·89	1·00	1·13

The experimental determination (last line) was made in a medium having 150 mM KCl, 20 mM tris-chloride, 10 mM tris-hydroxybutyrate, 60 mM mannitol.

Addanki *et al.*, 1968; Ghosh and Chance, 1970). This effect is predictable from equations (1) and (2) because cation gain leads to gain of anions and so to a higher inside/outside concentration ratio with, in turn, fewer internal protons. The fact that the entering Ca^{2+} combines with phosphate means that there will be a tendency for any other available anions to accumulate inside, but in any event the system should adjust so that the ratio of ionized internal phosphate to external phosphate is higher than before the Ca^{2+} addition, because of removal of phosphate from the medium. One corollary of the proposal made is that one would expect that substances forming highly insoluble phosphates (such as the rare earths) should inhibit uptake of Ca^{2+}, as indeed they do (Mela and Chance, 1968; Ghosh and Chance, 1970).

Having discussed the consequences of changes in anion concentration, cation content and internal pH, one may turn to the effect of external pH. This factor has two opposing results. As noted by Harris and Berent (1970), acidification can lead to discharge from the mitochondria of Ca^{2+} with citrate. Loss of cation and anion corresponds to a diminished accumulation of anions. On the other hand, to the extent that the interior moves towards acidity in response to acidification of the medium there will be a protonation of the buffer and an increase of non-buffer anions. What actually happens experimentally depends on the content of Ca^{2+} at the time the acidification is carried out. When the mitochondrial Ca^{2+} is more than about 8 nmole/mg protein, acidification leads to loss of Ca^{2+} and hence of anions. In Table 3, Expts. 1 and 2, Ca^{2+} losses of 41 and 21 nequiv/mg occur to offset the protonation of the buffers so smaller anion changes are needed than in Expts. 3 and 4 where the Ca^{2+} contents are little changed. Examples of concomitant changes of citrate and Ca^{2+} are given by Harris and Bangham (1972).

TABLE III. Effect of acidification on Ca^{2+} content of rat liver mitochondria

Expt.	pH of medium	Ca^{2+} nmole/mg	Expt.	pH of medium	Ca^{2+} nmole/mg
1	7·4	26	3	7·2	6·9
	7·0	21·5		6·8	6·0
	6·75	13·2		6·2	5·2
	6·2	5·5			
2	7·2	16	4	7·4	1·8
	6·8	7·5		6·8	2·2
	6·2	5·5		6·2	2·7

The medium contained 150 mM KCl, 30 mM sucrose and 29 mM tris chloride.

Other Evidence for, and Consequences of Internal Positivity

The most direct evidence that the interior is positive and that the potential behaves like that of a Donnan system towards anions has been given by Tupper and Tedeschi (1969), who used microelectrodes. Protagonists of the view that the interior is negative have rejected the result on the grounds that it is an artifact. However, the behaviour of the cations also points to internal positivity. Ca^{2+} uptake is associated with energy consumption, as is also the case when K^+ uptake is induced by valinomycin (Harris *et al.*, 1966). The electrochemical gradients against which the cations move will be higher if the interior is positive than if it is negative. Indeed, with sufficient internal negativity K^+ uptake would not demand energy expenditure. It has been shown that discharge of the internal K^+ to an initially K-free medium can be used to phosphorylate ADP, either endogenous (Cockrell *et al.*, 1967) or exogenous (Rossi and Azzone, 1970). About 1 ATP is obtained per 4 K^+ ions discharged. If one assumes an internal K concentration of 100 mM and an average external concentration during the discharge of 1 mM and an internal positivity amounting to 45 mv (so that unibasic anions are accumulated by a factor of 6) then the energy obtainable per K lost is 3700 cal per g-ion. Loss of 4 K g-ions furnishes 14,800 cal which is close to the energy required to phosphorylate ADP under comparable conditions of concentration. This stoichiometry has been reported by Rossi and Azzone (1970). If the interior had been negative many more K ions would have had to be lost. Cl^- is normally an impermeant anion. Its distribution will however reach the same ratio as that applying to the permeant pyruvate after induction of Cl permeability by alkyl tin salts (Harris, Bangham and Zukovic, 1972).

Further Discussion

Mitchell (1968) has used as evidence for an energy consuming proton ejection process the observation that restoration of energy to previously stored particles leads to an acidification of the medium. Most reports concerning phosphate movements are couched in terms of necessary phosphate-OH^- exchanges. It has been shown that the acidification of the medium is linked to entry of leaked Ca^{2+} (Thomas *et al.*, 1969) which in the light of the calculations of Table 2 has its effect on pH because of co-entry of phosphate. It is not necessary to invoke an obligate OH^--for-phosphate exchange if it is accepted that both protons and phosphate ions are at electro-chemical equilibrium.

The interaction between Ca^{2+} and phosphate to form a non-crystalline compound having the composition of hydroxyapatite could provide a sink for OH^-. Such a process, associated with protonation of X^- to remove H^+ would allow removal of water from phosphate or ADP ions at an enclosed locus so as to promote formation of ATP.

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