Biochimica et Biophysica Acta 849 (1986) 236–243
Elsevier

BBA 41979

A theoretical analysis of the effect of phosphate on apparent H^+/O stoichiometries in oxygen-pulse experiments with rat liver mitochondria

Ian C. West *

Glynn Research Institute, Bodmin, Cornwall, PL30 4AU (U.K.)

(Received November 25th, 1985)

Key words: H^+/O stoichiometry; Phosphate porter; (Rat liver mitochondria)

It is now generally accepted that, in oxygen-pulse experiments on rat-liver mitochondria suspended in KCl-based media, the rapid import of H^+ with phosphate leads to an approx. 33% lowering of apparent H^+/O stoichiometry. However, in low-K^+ media, *N*-ethylmaleimide has no effect on stoichiometry, and there appears to be no import of H^+ with phosphate. In this paper the quantitative effect of extramitochondrial phosphate on apparent H^+/O stoichiometry is calculated theoretically, on the basis of internal and external buffering powers. The lack of appreciable phosphate uptake in low-K^+ media is quantitatively explained in terms of several factors, including the initial pH gradient and initial phosphate distribution.

Introduction

In rat liver mitochondria the most rapid of the H^+-coupled porters is the H^+-phosphate symporter (or OH^--phosphate antiporter). In is now recognized that a serious lowering of the apparent $\leftarrow H^+/O$ stoichiometry in O_2-pulse experiments may be caused by the rapid entry of H^+ with phosphate unless phosphate entry is prevented, for example by inhibiting the phosphate porter with *N*-ethylmaleimide [1,2]. It appears that the lost protons cannot be corrected for by extrapolation of the traces [3]; phosphate entry is largely complete within the response time of the pH-sensitive glass-electrode. Until recently one reason for doubting this role of the phosphate porter was the observation that, while *N*-ethylmaleimide raised the apparent $\leftarrow H^+/O$ stoichiometry by 30–40% in KCl medium, it had no effect in 250 mM sucrose or 150 mM choline chloride media, where the apparent $\leftarrow H^+/O$ stoichiometry was already 30–40% higher than in KCl medium [4].

Mitchell considered the effect of a generalized H^+-anion symport on the collapse of ΔpH, and presented [5] an equation that took into account the small volume changes that accompany anion import. The distribution of phosphate at equilibrium has been discussed by many authors, most recently by Greenbaum and Wilson [6], who pointed out the different predictions of $OH^-/H_2PO_4^-$ and $2OH^-/HPO_4^{2-}$ antiport models when the internal and external dissociation con-

* On leave of absence from the University of Newcastle-upon-Tyne, NE1 7RU, U.K.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; subscripts O and I indicate outer and inner aqueous phases; P, quantity of orthophosphate per ml of suspension including all states of protonation; $K'(K'')$, first (second) dissociation constant of phosphoric acid; $V_O(V_I)$, volume of outer (inner) aqueous phase per ml of suspension; $\leftarrow H^+/O$, number of hydrogen ions translocated out of the mitochondrion per O atom reduced; $\rightarrow H^+/P$, number of hydrogen ions withdrawn from the outer medium per phosphate-phosphorus imported; ΔpH, $pH_I - pH_O$; B_I, buffering power of inner aqueous phase, i.e., nmol H^+ required per ml of suspension to lower pH_I by 1 pH unit.

0005-2728/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

stants of phosphate are not equal. The present calculations combine these two approaches to predict the effect of extramitochondrial phosphate on apparent $\leftarrow H^+/O$ stoichiometry in both KCl-based on sucrose-based suspension media.

Method of calculation

First, one can calculate the pH_O and pH_I that result from the translocation by the respiratory burst of a given amount of H^+ out of the mitochondria. Then, allowing x nmol phosphate to move into the mitochondria, taking with it y nmol H^+ (y/x is between 1 and 2, when pH_O lies in the range 6–8; see below), one can calculate both the rising $[P]_I/[P]_O$ ratio and the falling $[H^+]_O/[H^+]_I$ ratio. After the translocation of a certain amount of phosphate, the phosphate concentration ratio will come into thermodynamic equilibrium with ΔpH, and no more inward movement of H^+ or phosphate will occur on the porter. The net number of hydrogen ions taken in with each phosphate will depend on pH_O and pH_I, while the exact relationship between $[P]_I/[P]_O$ and $[H^+]_O/[H^+]_I$ at equilibrium will depend on pH_O, pH_I and the reaction mechanism of the porter. These relationships will now be derived.

The overall process catalysed by the phosphate porter is the electroneutral uptake of phosphate either with H^+ or in exchange for OH^-, but the detailed mechanism of the porter is not known. Several models have been proposed, but it is not yet possible to decide conclusively between them. The original Chappell and Crofts [7] scheme described $OH^-/H_2PO_4^-$ antiport (Fig. 1c). Mitchell pointed out [5,8] that it was conceptually simpler to regard the process as H^+-$H_2PO_4^-$ symport (Fig. 1b), and equivalent to the entry of H_3PO_4 by acid uniport (Fig. 1a), though the faster operation of the carrier at alkaline pH [9] would seem to argue against both acid uniport and H^+-$H_2PO_4^-$ symport. Freitag and Kadenbach [10] obtained evidence in favour of an exchange of HPO_4^{2-} for 2 OH^- (Fig. 1d). Recently, Greenbaum and Wilson [6] have pointed out that these schemes are thermodynamically equivalent only if the acid dissociation constants of phosphate (K', K'') are the same in the inner and outer media. Phosphate ionization is quite sensitive to ionic strength [11], and, as the ionic composition of the matrix is likely to differ from that of the suspending medium, small differences in pK_a values are to be expected. In their calculations, Greenbaum and Wilson [6] adopted values for the second dissociation constant of phosphate in the range $(2.0–2.6) \cdot 10^{-7}$ M in the matrix and $(1.3–1.6) \cdot 10^{-7}$ M in the outer medium, and concluded that $OH^-/H_2PO_4^-$ antiport best fits the equilibrium distribution of phosphate and malate. For the present calculations the inner and outer dissociation constants will initially be taken as equal; subsequently the effect of a small increase in K_I'' will be examined in terms of the alternative models.

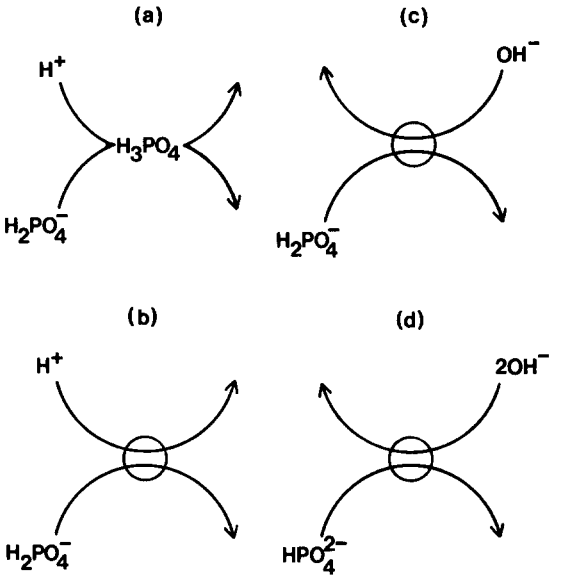

Fig. 1. Possible reaction mechanisms for an electroneutral phosphate porter. (a) Phosphoric acid uniport; (b) H^+-$H_2PO_4^-$ symport (which differs from (a) in that here it is the carrier, not the phosphate, that is protonated); (c) monovalent antiport; (d) divalent antiport.

When inner and outer dissociation constants are equal, it is permissible to consider the phosphate porter to be thermodynamically equivalent to one catalysing the equilibration of the electroneutral complex H_3PO_4, independently of the physical mechanism actually involved (equation 1).

$$HPO_4^{2-} \underset{K_O''}{\overset{H^+}{\rightleftharpoons}} H_2PO_4^- \underset{K_O'}{\overset{H^+}{\rightleftharpoons}} H_3PO_4 \left| \xrightarrow{\text{membrane}} \right| H_3PO_4 \underset{K_I'}{\overset{H^+}{\rightleftharpoons}} H_2PO_4^- \underset{K_I''}{\overset{H^+}{\rightleftharpoons}} HPO_4^{2-} \quad (1)$$

The constants K' and K'', representing the first and second dissociation constants of phosphate, are here being assumed to have the same value inside as outside the mitochondrion. The third ionization is ignored; it occurs at highly alkaline pH, and under our conditions the PO_4^{3-} ion will make an insignificant contribution to the total.

According to Eqn. 1, the condition for thermodynamic equilibrium will be that $[H_3PO_4]_O = [H_3PO_4]_I$. The relationship between total phosphate (i.e., all states of protonation) in the outer medium ($[P]_O$) and the concentration of H_3PO_4 in the outer medium will be governed by pH_O, as described by the Henderson–Hasselbach equation; similarly on the inside, thus:

$$[P]_O = [H_3PO_4]_O\left(1 + \frac{K'}{[H^+]_O} + \frac{K'K''}{[H^+]_O^2}\right) \quad (2)$$

$$[P]_I = [H_3PO_4]_I\left(1 + \frac{K'}{[H^+]_I} + \frac{K'K''}{[H^+]_I^2}\right) \quad (3)$$

Therefore, at equilibrium:

$$\frac{[P]_I}{[P]_O} = \frac{1 + \frac{K'}{[H^+]_I} + \frac{K'K''}{[H^+]_I^2}}{1 + \frac{K'}{[H^+]_O} + \frac{K'K''}{[H^+]_O^2}} \quad (4)$$

Because of the equilibria shown in Eqn. 1, the phosphate that crosses the membrane will be drawn from the external pools of H_3PO_4, $H_2PO_4^{1-}$ and HPO_4^{2-} in the ratios in which they exist at the pertaining pH_O, i.e., in the ratios given by the three terms on the right hand side of Eqn. 2. The free acid will take up no protons, the monovalent ion will take up 1 H^+, the divalent ion will take up 2 H^+. The total number of moles of H^+ taken up from the outer medium per mole of phosphate taken up can therefore be expressed as:

$$\frac{\rightarrow H^+}{P} = 0 + \frac{1}{\frac{[H^+]_O}{K'_O} + 1 + \frac{K''_O}{[H^+]_O}} + \frac{2}{\frac{[H^+]_O^2}{K'_O K''_O} + \frac{[H^+]_O}{K''_O} + 1} \quad (5)$$

At pH_O close to 7.0 the ratio $\rightarrow H^+/P$ will be close to 1.6. The amount of H^+ released on the inside is given by a similar equation written in terms of $[H^+]_I$, K'_I and K''_I.

The amount of phosphate that moves into the mitochondria to establish equilibrium can be determined with a simple iterative calculation, an example of which is indicated here in the form of a short computer program written in BASIC.

ITERATIVE CALCULATION OF H^+ AND PHOSPHATE MOVEMENTS DURING THE COLLAPSE OF ΔpH:

```
 10 LET HO = 10↑ − 7: LET HI = 10↑ − 7: REM INITIAL H + CONCENTRATIONS
 20 LET K1 = 0.01: LET K2 = 1.4E − 7: REM 1ST AND 2ND pKA FOR PHOSPHATE
 30 INPUT "PULSE SIZE"; PULSE: REM NMOL H + EJECTED/ML
 40 INPUT "INITIAL PHOSPHATE (uM)"; IP
 50 INPUT "NMOL P MOVED"; X: REM MOVED BACK INTO MATRIX
 60 FOR N = 1 TO 9: REM ITERATE, AS H + MOVEMENTS CHANGE HO, HI
 70 LET  PHO = 7 − (PULSE − X*(2/(1 + HO/K2 + HO*HO/(K1*K2)) + 1/(HO/K1 + 1 +
    K2/HO)))/712
 80 LET  PHI = 7 + (PULSE − X*(2/(1 + HI/K2 + HI*HI/(K1*K2)) + 1/(HI/K1 + 1 +
    K2/HI)))/73
 90 LET HO = 10↑ − PHO: LET HI = 10↑ − PHI
100 LET PI = (IP/166.7 + X)*166.7: REM VOLUME RATIO (VO/VI) = 166.7
110 LET PO = (IP − X)
120 LET HR = (1 + K1/HI + K1*K2/(HI*HI))/(1 + K1/HO + K1*K2/(HO*HO))
130 PRINT PULSE, IP, X, PI/PO, HR
140 NEXT N
150 GOTO 50
```

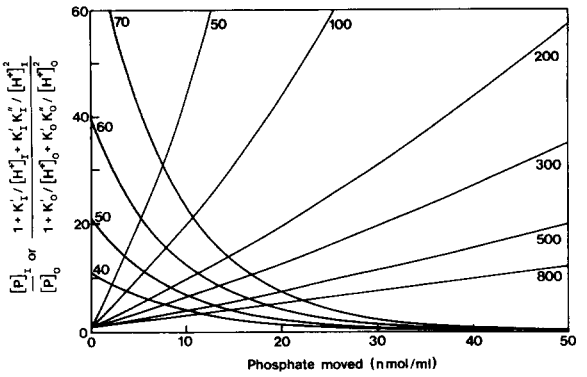

Fig. 2. The effect of the total orthophosphate concentration and the size of a respiratory pulse on the relationships between amount of phosphate imported and the ratios $[P]_I/[P]_O$ and

$$\frac{1+\frac{K'_I}{[H^+]_I}+\frac{K'_I K''_I}{[H^+]_4^2}}{1+\frac{K'_O}{[H^+]_O}+\frac{K'_O K''_O}{[H^+]_O^2}}$$

For the calculations the following were assumed: 6 mg mitochondrial protein/ml, $V_I = 1$ µl/mg protein, $pH_O = pH_I = 7$, $K'_O = K'_I = 0.01$ M, $K''_O = K''_I = 1.4 \cdot 10^{-7}$ M. Rising curves are drawn for total inorganic orthophosphate concentrations in the range 50–800 nmol/ml suspension. Falling curves are drawn for respiratory pulses translocating 40–70 nmol H^+/ml suspension.

For small O_2-pulses (less than 100 nmol H^+/ml), it is permissible to ignore the small volume changes (less than 18%) that result from cation and anion inflow. The results of such calculations, for a range of phosphate concentrations and for different amounts of H^+ translocated in the respiratory burst, are shown in Fig. 2. As more phosphate is considered to have moved into the mitochondrial matrix, the rising curves indicate the $[P]_I/[P]_O$ ratio, and the falling curves indicate the function on the right-hand side of Eqn. 4. Equilibrium occurs where these lines cross.

Results

The precise amount of phosphate moved into the mitochondria by the time equilibrium is established will depend on the internal volume of the mitochondria, the amount of phosphate present and the initial distribution of that phosphate, as well as on the H^+-buffering power of the inner and outer aqueous phases. These quantities, in turn, will depend upon the media in which the mitochondria are suspended.

KCl media

Consider a suspension of rat liver mitochondria (6 mg protein/ml) in 150 mM KCl, 25 mM sucrose, 3.3 mM glycylglycine at a pH_O of 7.0. It was found that the inner buffering power of 1 ml of such a suspension is 73 nmol H^+/pH unit and is virtually independent of pH in the range 7.0–8.5 [12], while the combined outer buffering power (mitochondria + glycylglycine) is 712 nmol H^+/pH unit [12]. After 20 min anaerobic incubation in this medium the internal volume (V_I) appears to be about 1 µl/mg protein, and pH_I

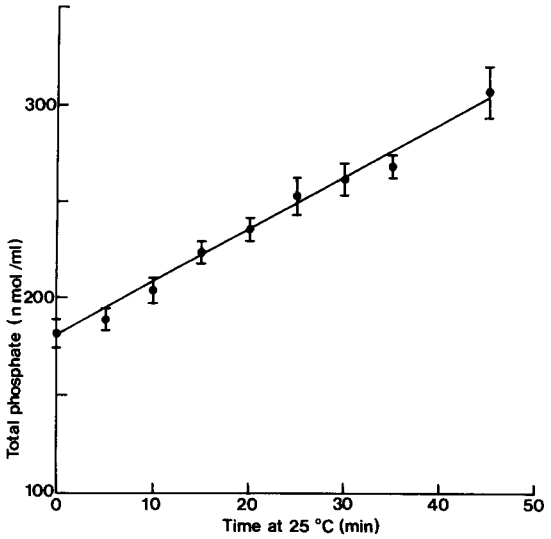

Fig. 3. Total orthophosphate in a suspension of rat-liver mitochondria during anaerobic incubation at 25°C. Mitochondria, prepared as previously described [2], were suspended at 6 mg protein/ml in a medium containing 150 mM KCl, 25 mM sucrose, 3.3 mM glycylglycine, 1 mM EGTA, and were incubated anaerobically at 25°C. At intervals a 1.0 ml sample of the suspension was withdrawn anaerobically via a glass needle into a glass syringe that had been freed of O_2 by previous flushing with an O_2-free solution of 1 mM $Na_2S_2O_4$. The sample was immediately injected into an equal volume of 10% trichloroacetic acid. After centrifugation, the neutralized supernatant was assayed for orthophosphate by a modification of the method of Fiske and Subbarow (described previously [2]). The points indicate the means of five experiments on five different mitochondrial preparations and the bars indicate the standard errors of the means.

appears to be the same as pH_O. The total orthophosphate concentration under these conditions varies considerably from preparation to preparation, but lies in the range 200–300 μM (Fig. 3; see also Refs. 1 and 2). The inner and outer phosphate concentrations will initially be equal, in accordance with Eqn. 4.

The fraction of the pulse that is collapsed when the H^+-phosphate symporter reaches equilibrium turns out to be a function both of pulse size and of initial phosphate concentration. This is illustrated in Fig. 4. If we assume for the present that the correct $\leftarrow H^+/O$ stoichiometry, without phosphate movement, is 9 (as has been reported for respiratory pulses with β-hydroxybutyrate as substrate [1]), a pulse of 1.25 ngatom O/mg protein represents the translocation of 68 nmol H^+/ml suspension. Fig. 4 shows that such an O_2-pulse would be expected to give an apparent $\leftarrow H^+/O$ stoichiometry of 6.0 in the presence of 250 μM phosphate. An O_2 pulse of half that size would give an apparent stoichiometry value near 7 (Fig. 4). The addition of a further 100 nmol phosphate/ml suspension would be expected to lower the apparent $\leftarrow H^+/O$ stoichiometry of the larger pulse to 5.5. These stoichiometries correspond quite closely with the results obtained under similar conditions by Mitchell and Moyle [13] (see Table I).

If the $\leftarrow H^+/O$ stoichiometry in the absence of phosphate movements were 6 (as was assumed for β-hydroxybuturate respiration by Mitchell and Moyle [13], but for succinate respiration by Brand et al. [1]), a pulse of 1.25 ngatom O/mg protein would translocate only 45 nmol H^+ per ml of our suspension, containing 6 mg protein/ml. The appropriate line in Fig. 4 shows that as little as 100 μM total phosphate in the suspension should

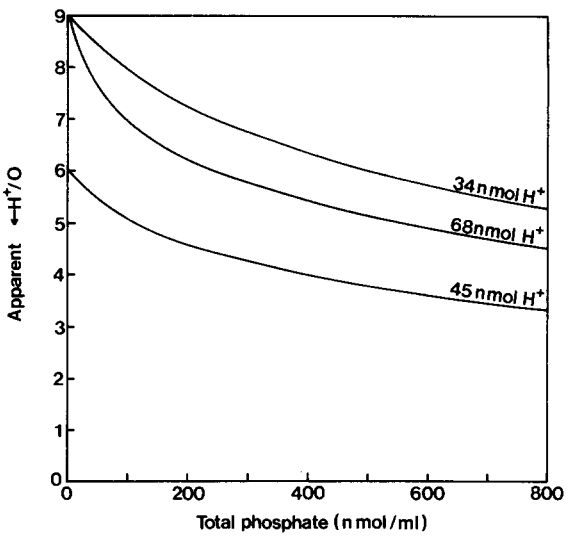

Fig. 4. The effect of total orthophosphate concentration and respiratory pulse size on the apparent $\leftarrow H^+/O$ stoichiometry. See Fig. 2 for assumptions involved in the calculations, and the text for further details.

TABLE I

OBSERVED AND EXPECTED $\leftarrow H^+/O$ STOICHIOMETRIES FOLLOWING O_2-PULSES IN THE PRESENCE OF PHOSPHATE

Calculations were made as detailed in the computer program, assuming 250 nmol/ml endogenous orthophosphate and other conditions as in Fig. 2. Conditions in Ref. 13 differed as follows: medium contained 150 mM KCl/25 mM sucrose/3.3 mM glycylglycine ($pH_O = 7.2$)/2 mM β-hydroxybutyrate/6.5 mg protein per ml suspension; pulsed with 0.8 ngatom O/mg protein. Conditions in Ref. 1 differed as follows: medium contained 109 mM KCl/23 mM sucrose/2.7 mM Hepes (pH 7.1)/0.5 mM succinate/5 μM rotenone/5 mg protein per ml; pulsed with 1.13 ngatom O/mg protein.

Phosphate concentration (nmol/ml suspension)		Apparent $\leftarrow H^+/O$ stoichiometry			
		β-hydroxybutyrate		succinate + rotenone	
added	presumed total	observed Ref. 13	expected	observed Ref. 1	expected
0	250	6.0	6.0	4.0	4.4
50	300	–	–	4.2	4.2
100	350	5.5	5.5	3.8	4.0
500	750	5.0	4.6	2.6	3.3
2000	2250	3.6	3.4	2.0	2.2

depress the apparent $\leftarrow H^+/O$ to 5.1. With the phosphate concentrations that we now find in this medium (Fig. 3, and Refs. 1, 2) the expected apparent $\leftarrow H^+/O$ stoichiometry would be 4.2–4.4. With 2.0 μmol/ml added phosphate, the expected apparent stoichiometry would be 2.25. These stoichiometries are clearly incompatible with experimental results using β-hydroxybutyrate as substrate. On the other hand, they correspond quite well with stoichiometries found in this medium in the presence of 0.5 mM succinate, when rotenone was included to prevent electron flow through complex I (Table II).

Low K^+-media

When mitochondria are incubated anaerobically for 20 min at $pH_O = 7$ in media containing low K^+ concentrations, the pH_I falls by up to 1.0 pH unit, as the outwardly diffusing K^+ is replaced by H^+. This is particularly marked when valinomycin is present [2]. In addition, it is probable that there is a significant shrinking of the mitochondria as K^+ leaks out during the 20 min anaerobic preincubation. Woelders et al. [14] report a value of 1.0 μl/mg protein for the internal water space of coupled mitochondria in a mannitol medium, but 0.4 μl/mg protein for "fully de-energized mitochondria" that have lost their K^+. When the extent of phosphate and proton inflow is calculated for the conditions pertaining in low-K^+ media, with initial $pH_O = 7$ and $pH_I = 6.0$, the predictions are strikingly different from the previous predictions for high-K^+ media; equilibrium of phosphate on the porter is achieved when a very small amount of phosphate has been taken up. Consequently, very little of the H^+ ejected in a respiratory pulse would be expected to be lost back into the mitochondria under these circumstances, as is indeed found to be the case [3,2].

It is interesting to examine the contributions made by the various factors that minimize the loss of H^+ on the phosphate porter when mitochondria are suspended in low-K^+ media. First, and of least significance, the shrinkage of the mitochondria causes a proportional lowering of the amount of phosphate imported at equilibrium. Second, the internal buffering power rises steeply, from 73 to 193 nmol H^+ per pH unit per ml of our suspension, as the pH_I falls from 7 to 6 [12]. This means that a given respiratory pulse will cause a smaller change of pH_I in low-K^+ media than in 150 mM KCl. Third, if pH_i is one unit more acidic than pH_O during the preincubation, the phosphate will be displaced almost completely out of the mitochondria into the outer medium by the pH gradient, so that the ratio $[P]_I/[P]_O$ has the value 0.0475 ($[P]_I/[P]_O = 1.0$ when $pH_O = pH_I$). Because the internal phosphate concentration is 21-times smaller than in the case where $pH_I = 7.0$, the ratio $[P]_I/[P]_O$ initially responds 21-times more sensitively to the inflow of phosphate.

The combined effect of these three factors is such that, in a medium initially containing a total of 250 nmol phosphate per ml, a respiratory pulse that displaces 68 nequiv H^+/ml suspension will come into equilibrium with phosphate when only 0.21 nmol phosphate has moved into the matrix. This will carry 0.34 nmol H^+, which represents the loss of 0.5% of the pulse – an insignificant portion.

The different predictions for $OH^-/H_2PO_4^-$ and $2OH^-/HPO_4^{2-}$ antiports when $K_I'' > K_O''$

Eqn. 4 describes the equilibrium condition for an acid uniport mechanism (Fig. 1a). Analogous equations for $OH^-/H_2PO_4^-$ antiport (which, in this context, is equivalent to H^+-$H_2PO_2^-$ symport) and $2OH^-/HPO_4^{2-}$ antiport, may be derived as follows.

Equations corresponding to Eqn. 2 may be written expressing $[P]_O$ in terms of $[H_2PO_4^-]_O$ or $[HPO_4^{2-}]_O$, thus:

$$[P]_O = [H_2PO_4^-]_O\left(\frac{[H^+]_O}{K_O'} + 1 + \frac{K_O''}{[H^+]_O}\right) \quad (6)$$

$$[P]_O = [HPO_4^{2-}]_O\left(\frac{[H^+]_O^2}{K_O'K_O''} + \frac{[H^+]_O}{K_O''} + 1\right) \quad (7)$$

Remembering that the equilibrium condition for $OH^-/H_2PO_4^-$ antiport is:

$$\frac{[OH^-]_O}{[OH^-]_I} = \frac{[H_2PO_4^-]_O}{[H_2PO_4^-]_I} \quad (8)$$

and that for $2OH^-/HPO_4^{2-}$ antiport is:

$$\left(\frac{[OH^-]_O}{[OH^-]_I}\right)^2 = \frac{[HPO_4^{2-}]_O}{[HPO_4^{2-}]_I} \quad (9)$$

and that $[OH^-]$ varies reciprocally with $[H^+]$, it is possible to write down equations analogous with Eqn. 4 but describing the equilibrium conditions for $OH^-/H_2PO_4^-$ and $2OH^-/HPO_4^{2-}$ antiports, respectively:

$$\frac{[P]_I}{[P]_O} = \frac{\frac{[H^+]_I}{K_I'} + 1 + \frac{K_I''}{[H^+]_I}}{\frac{[H^+]_O}{K_O'} + 1 + \frac{K_O''}{[H^+]_O}} \frac{[H^+]_O}{[H^+]_I} \quad (10)$$

$$\frac{[P]_I}{[P]_O} = \frac{\frac{[H^+]_I^2}{K_I'K_I''} + \frac{[H^+]_I}{K_I''} + 1}{\frac{[H^+]_O^2}{K_O'K_O''} + \frac{[H^+]_O}{K_O''} + 1} \frac{[H^+]_O^2}{[H^+]_I^2} \quad (11)$$

Eqn. 5 applies to all the mechanisms shown in Fig. 1.

If the foregoing calculations are repeated for our standard conditions (150 mM KCl/3.3 mM glycylglycine medium, containing 6 mg protein/ml) but, following Greenbaum and Wilson [6], taking K_I'' as $2.3 \cdot 10^{-7}$ M and K_O'' as $1.4 \cdot 10^{-7}$ M, it is found that the two antiport models behave rather differently. The $2OH^-/HPO_4^{2-}$ antiport model now predicts very slightly less phosphate movement following an oxygen pulse (Fig. 5, dotted line), while the $OH^-/H_2PO_4^-$ model predicts appreciably more phosphate movement (Fig. 5, dashed line). This is explained quite simply in terms of the initial distribution of the phosphate; $[P]_I/[P]_O$ is initially 1 when the dissociation constants are equal, but becomes 0.84 for the $2OH^-/HPO_4^{2-}$ antiport and 1.37 for the $OH^-/H_2PO_4^-$ antiport, when K_I'' is raised. This is in accord with the equations of Greenbaum and Wilson [6].

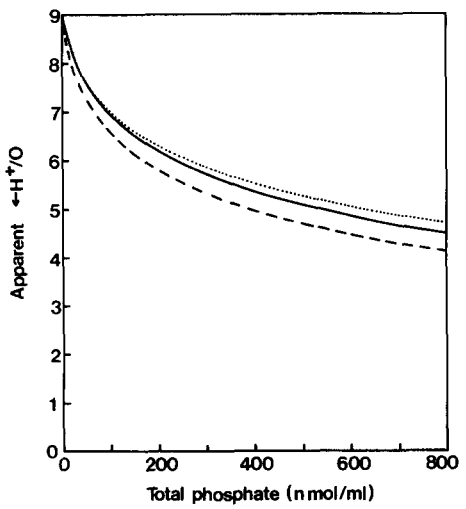

Fig. 5. The different predictions of $OH^-/H_2PO_4^-$ and $2OH^-/HPO_4^{2-}$ antiport models when $K_I'' \neq K_O''$. The solid line reproduces the conditions of Fig. 4 with $K_I'' = K_O'' = 1.4 \cdot 10^{-7}$ M and a respiratory pulse translocating 68 nmol H^+/ml suspension. The dotted line refers to $2OH^-/HPO_4^{2-}$ antiport, the dashed line to $OH^-/H_2PO_4^-$ antiport, when K_I'' is changed to $2.3 \cdot 10^{-7}$ M.

The substrate-analogue experiments of Freitag and Kadenbach [10] favour $2OH^-/HPO_4^{2-}$ antiport, but the data of Greenbaum and Wilson [6], on phosphate and malate distributions, favour $OH^-/H_2PO_4^-$ antiport. Because of the number and size of the uncertainties involved, the present calculations cannot be used to discriminate between the two alternative antiport models, though the experimental data do seem to fit the monovalent model slightly better than the divalent model.

Conclusions

It is not being suggested that the present calculations confirm that the actual $\leftarrow H^+/O$ stoichiometries are 9 in the absence and 6 in the presence of rotenone. Indeed, there are reasons for believing that the NADH dehydrogenase segment of the respiratory chain translocates 4 $H^+/2e^-$ [15,16]. Other factors than the phosphate porter may operate to lower the observed stoichiometries. What the calculations do show is (a) that the phosphate porter is expected to operate, quantitatively as well as qualitatively, in the manner proposed by Brand et al. [1]; (b) that when more protons are ejected in a respiratory burst a greater proportion will be lost via the phosphate porter; and (c) that H^+-phosphate symport (OH^-/phosphate antiport) is not expected to lower the apparent $\leftarrow H^+/O$ stoichiometry under the conditions pertaining in low-K^+ media.

Acknowledgements

This work was supported by the Medical Research Council and all benefactors of The Glynn Research Foundation Ltd. named in the brochure of that registered charity. I am grateful for much

discussion with R. Mitchell, A.J. Moody and P. Mitchell, and for technical help from Alan Jeal and Robert Harper.

References

1 Brand, M.D., Reynafarje, B. and Lehninger, A.L. (1976) J. Biol. Chem. 251, 5670–5679
2 Mitchell, R., West, I.C., Moody, A.J. and Mitchell, P. (1986) Biochim. Biophys. Acta 849, 229–235
3 Mitchell, P., Moyle, J. and Mitchell, R. (1979) Methods Enzymol. 55, 627–640
4 Mitchell, P. and Moyle, J. (1979) Biochem. Soc. Trans. 7, 887–894
5 Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research, Bodmin
6 Greenbaum, N.L. and Wilson, D.F. (1985) J. Biol. Chem. 260, 873–879
7 Chappell, J.B. and Crofts, A.R. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.) BBA Library Vol. 7, pp. 293–314, Elsevier, Amsterdam
8 Mitchell, P. (1970) in Membranes and Ion Transport (Bittar, E.E., ed.), Vol. 1, pp. 192–256, Wiley, Chichester
9 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 9, 149–155
10 Freitag, H. and Kadenbach, B. (1978) Eur. J. Biochem. 83, 53–57
11 Phillips, R.C., George, P. and Rutman, R.J. (1963) Biochemistry 2, 501–508
12 Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588–600
13 Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162
14 Woelders, H., Van der Zande, W.J., Colen, A.-M.A.F., Wanders, R.J.A. and Van Dam, K. (1985) FEBS Lett. 179, 278–282
15 Scholes, T.A. and Hinkle, P.C. (1984) Biochemistry 23, 3341–3345
16 Wikström, M. (1984) FEBS Lett. 169, 300–304