The Distribution of Inorganic Phosphate and Malate between Intra- and Extramitochondrial Spaces

RELATIONSHIP WITH THE TRANSMEMBRANE pH DIFFERENCE*

Nancy L. Greenbaum‡ and David F. Wilson
From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The steady-state distribution of inorganic phosphate and malate between the intra- and extramitochondrial spaces was measured in suspensions of nonrespiring and respiring rat liver mitochondria in which the transmembrane pH difference was incrementally varied. In respiration-inhibited mitochondria, the slope of log $[P_i]_{\text{int}}/[P_i]_{\text{ext}}$ (ordinate) versus $\Delta p \text{H}$ approached 2 by either chemical or isotopic determination of $[P_i]$, and the slope of log [malate]$_{\text{int}}$/[malate]$_{\text{ext}}$, versus $\Delta p \text{H}$ was 2.0 with an extrapolated log [P]$_{\text{int}}$/[P]$_{\text{ext}}$ value of 0.3 at $\Delta p \text{H} = 0$. We conclude that the distribution of $P_i$ and malate for nonrespiring mitochondria was quantitatively consistent with those predicted by exchange of $P_i^-$ for $OH^-$ (or cotransport with $H^+$) and of malate$^2-$ for $P_i^2-$. In respiring mitochondria using glutamate + malate as substrate, there was very little pH dependence of $P_i$ or malate accumulation (the slopes were <0.5) unless $n$-butylmalonate (inhibitor of $P_i$-dicarboxylate exchange) was added before the glutamate and malate, in which case the distribution patterns at $\Delta p \text{H} < 0.4$ were similar to those in nonrespiring mitochondria. In either case, however, after reaching a maximal value of 1.1, log [P]$_{\text{int}}$/[P]$_{\text{ext}}$ did not further increase with increasing $\Delta p \text{H}$. Thus, in normally metabolizing mitochondria, the distributions of $P_i$ and malate are not directly correlated with the difference in pH across the membrane.

Phosphorylation of extramitochondrial ADP within the mitochondrial matrix requires the inward transport of the reactants, ADP and $P_i$, and outward transport of the product, ATP. While ATP is stoichiometrically exchanged for ADP by the adenine nucleotide translocase, the majority of $P_i$ uptake occurs via a separate transport protein of approximately 34 kDa (1). Most investigators agree that $P_i$ transport, considered to be the most rapid of all mitochondrial transport processes, is an electroneutral movement of $P_i^-$ accompanied by $H^+$ or exchanged for $OH^-$ (2-7). An independent one-to-one exchange of $P_i$ for dicarboxylate ions has also been demonstrated, although a separate protein carrier has not yet been isolated. Phosphate-hydroxyl exchange is inhibited by low concentrations of SH reagents such as $N$-ethylmaleimide and mercurials such as mersalyl (although in high concentration, mersalyl inhibits both transport processes), but only the phosphate-dicarboxylate exchange is inhibited by substrate analogs such as $n$-butylmalonate and phenylsuccinate. This difference in sensitivity to inhibitors, combined with different rates of uptake for $P_i$ when exchanging for $OH^-$ or dicarboxylate, implicates independent transport proteins for the two mechanisms (8-10).

The factors affecting the uptake of $P_i$ and its steady-state distribution ratio, as well as the relationship of $P_i$ transport to that of other metabolites, is not well understood. Elucidation of these relationships under different conditions is important in evaluating a number of cellular and metabolic functions, including a postulated role for $P_i$ in the transport of citric acid cycle intermediates (11) and a regulatory role in oxidative phosphorylation (12-15).

A correlation between the transmembrane pH difference and $P_i$ distribution has been noted previously in respiration-inhibited mitochondria (4-7, 11, 16), as well as in vesicles into which the $P_i$ transport protein had been reconstituted (17). Where quantitative models were proposed, however, the observed distributions did not agree with quantitative predictions (4, 5, 7). Furthermore, the relationship between $P_i$ and dicarboxylate distribution ratios and their dependence on the pH difference remained to be established for respiring mitochondria.

In this study, we have undertaken a quantitative evaluation of $P_i$ and dicarboxylate distribution in the absence of other metabolic processes. Experiments were carried out in which these relationships were examined in respiration-inhibited and respiring mitochondria. From these studies, we have been able to conclude that while in the absence of other transport and metabolic processes, phosphate and dicarboxylate distributions follow a predictable (approximately second power) dependence on the pH difference, this behavior is not observed under more physiological conditions.

MATERIALS AND METHODS

Mitochondria were isolated from livers of male Sprague-Dawley rats in a medium containing 0.225 M mannitol, 0.075 M sucrose, and 0.4 mM EGTA, as described by Schneider (18), and suspended to a concentration of approximately 50 mg of protein/ml in the same medium. When the distribution of $P_i$ was to be measured in nonrespiring, nonphosphorylating mitochondria, this stock suspension was diluted 10-fold into a medium containing 90 mM choline chloride, 10 mM KCl, 15 mM MOPS, and 0.4 mM EGTA at pH 6.5.

Suspensions of mitochondria were incubated aerobically at 25 °C and supplemented with hexokinase (0.6 IU/ml), 0.2 mM MgCl₂, 0.05 mM ADP, and 2 mM glucose for 90 s in order to deplete the mitochondria of endogenous $P_i$ and dicarboxylic acid substrates. Phos-
phate and/or substrate depletion in well-coupled mitochondria was considered to be complete when oxygen consumption reached minimal rates. Electron transport and ATPase activity were then inhibited by rotenone (2 mmol/mg) and oligomycin (1 mg/ml/mg protein), respectively. Complete inhibition by rotenone was verified in separate experiments by its effect on oxygen consumption with glutamate + malate as substrate; complete inhibition of mitochondrial ATPase by oligomycin was independently verified by its ability to completely block stimulation of respiration by ADP plus P_i. Varying concentrations of P_i (0.2-2 mmol/mg) + [32P]Pi were then added, with or without malate (0.3-3 mmol/mg), and incubation was allowed to proceed for 90 s. Although P_i has been shown to equilibrate across the mitochondrial membrane within several seconds (10), this longer time of 90 s was found to be necessary for full equilibration of the malate-P_i exchange. All P_i transport processes were then stopped by rapidly mixing in an excess of mersalyl (0.2 mg/ml) before samples were removed for centrifugation.

In experiments with phosphorylating mitochondria, stock suspensions were diluted 10-fold in 120 mM choline chloride, 15 mM MOPS, 0.4 mM EGTA, pH 7.0, and were supplemented with substrate (3-OH-butyrate or glutamate + malate) with or without prior addition of 0.35-1.0 mg/ml n-butylmalonate, after which 0.5 mg/ml ADP was added, concurrently. At the indicated times, either during state 3 or state 4 respiration, aliquots of the suspensions were removed and rapidly mixed into a solution containing rotenone and oligomycin. After ATPase was fully inhibited (15 s), mersalyl (0.2 mM) was added to inhibit subsequent P_i transport.

Samples (400 µl) to be used for determination of inorganic phosphate and malate distribution were layered over 50 µl of silicone oil, which in turn were layered above 60 µl of cold 4% HClO_4, 1.5% NaCl solution in a 400-µl Eppendorf microfuge tube (actual capacity, 520 µl). By this technique, mitochondria were rapidly separated from their suspending medium, and enzymatic activity was quenched upon centrifugation. Aliquots from each resulting supernatant fraction were separately quenched by addition to equal volumes of cold 4% HCIO_4, 1.5% NaCl solution in a 400-µl Eppendorf microfuge tube (actual capacity, 520 µl). By this technique, mitochondria were rapidly separated from their suspending medium, and enzymatic activity was quenched upon centrifugation. Aliquots from each resulting supernatant fraction were separately quenched by addition to equal volumes of cold 4% HClO_4; the remaining supernatant and oil were carefully suctioned from the tube, and the sides of the microcentrifuge tube were blotted with a pipe cleaner. The pellet fraction was then resuspended in the quenching solution and allowed to sit on ice for 10 min. Both pellet and supernatant samples were then recentrifuged. The final supernatants were divided; an aliquot for [32P]Pi determination was assayed immediately, and the remainder was neutralized with 2 M K_2CO_3 and frozen for later analysis of malate.

For each set of conditions, the intra- and extramitochondrial water volumes were measured in incubations carried out concurrently in separate, continuously stirred glass vials under identical conditions. The water volumes were calculated from the measured content of [3H]_2O, [14C]sucrose, [3H]- and [14C]acetate, [14C]dimethoxyazolidine-2,4-dione, and [14C]triphenylmethylphosphonium, by substituting a range of values for ApH in these equations. In Fig. 2, the relationships are as follows.

\[
\log \left( \frac{[\text{Pi}]_{\text{in}}}{[\text{Pi}]_{\text{out}}} \right) = \log \left( \frac{([\text{Pi}]_{\text{in}} + K_{\text{d}}} {([\text{Pi}]_{\text{out}} + K_{\text{d}})} + 2 \Delta pHe \right) (2)
\]

\[
\log \left( \frac{[\text{malate}^+]_{\text{in}}}{[\text{malate}^+]_{\text{out}}} \right) = \log \left( \frac{([\text{Pi}]_{\text{in}} + K_{\text{d}}} {([\text{Pi}]_{\text{out}} + K_{\text{d}})} + 2 \Delta pHe \right) (3)
\]

Generation of theoretical curves (shaded areas in Fig. 2) was achieved by substituting a range of values for ΔpH in these equations. In considering the distribution of phosphate, the relative concentrations of mono- and divalent forms are significant at all times; the pH_out for malate, however, is approximately 5, and therefore only the divalent species is present in any appreciable percentage of the total.

**RESULTS**

**Distribution of P_i in Suspensions of Nonrespiring Mitochondria at Various ΔpH Values**—Mitochondrial suspensions were depleted of endogenous P_i by addition of ADP, glucose, and hexokinase. After the respiration returned to minimal values, the inhibitors rotenone and oligomycin were added as described under “Materials and Methods.” Although the respiratory activity had returned to minimal values, suggesting that all available P_i had reacted, samples taken at this point still had a measurable amount of endogenous P_i, approximately 2 nmol/mg of mitochondrial protein.

The initial value of ΔpH was approximately 1.0 unit, and this was decreased in some incubations by addition of Na-propionate (up to 3 mM) and/or varying the amount of P_i (0.2-2 mM) in the assay medium. The measured steady-state intra- and extramitochondrial concentrations of P_i and malate from these representative experiments are shown in Table I, and the average data from eight experiments are plotted as the log [P_i]_in/[P_i]_out (ordinate) versus the measured ΔpH in Fig. 2. The slope of best-fit straight lines, including experiments with P_i alone, P_i + malate, or P_i + glutamate and malate, with or without n-butylmalonate, approached 2 (there

![Fig. 1. Schematic diagram of P_i−OH and P_i−malate exchange across the mitochondrial inner membrane.](image-url)
plotted as the log of the metabolite distribution ratio versus ΔpH. The shaded regions predicting the theoretical behavior were generated by substituting a range of ApH values into Equations 2 and 3. The following constants were used in the calculations: $K_{ApH} = 2.0 - 2.6 \times 10^{-6} \text{ (pK} = 6.6 - 6.7$), $K_{mal} = 1.3 - 1.6 \times 10^{-7} \text{ (pK} = 6.8 - 6.9$), and $pH_{out} = 6.5$.

**TABLE I**

Representative data of $P_i$ and malate distributions and their relationship with ΔpH from experiments with nonrespiring mitochondria

Isolated mitochondria (approximately 5 mg of protein/ml) were suspended in 90 mM choline chloride, 10 mM KCl, 15 mM MOPS, 0.4 mM EGTA, pH 6.5, with varying amounts of Na propionate. Suspensions were aerobically depleted of endogenous $P_i$ and citric acid cycle intermediates, and then inhibited as described under “Materials and Methods.” $P_i \pm$ malate were then added, and then after 90 s, $P_i$ transport was inhibited with 0.2 mM mersalyl. The data were taken from three independent experiments.

<table>
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<th>Starting concentrations</th>
<th>Final concentrations</th>
<th>ΔpH</th>
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<td>mM</td>
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<tr>
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<td>-0.92</td>
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was a minimum slope of 1.5 at low ΔpH). The fit of the data to the behavior predicted in Equations 2 and 3 is indicated in Fig. 2 by superimposing the experimental data on the predicted behavior. Similar data were obtained if a low ionic medium (125 mM mannitol, 40 mM sucrose, 15 mM MOPS, 0.4 mM EGTA) was used. The distribution of $P_i$ was similar whether it was determined by chemical or isotopic techniques (separate data for $[^{32}P]P_i$, distribution not shown). This indicates that, although there was a small but consistent amount of $P_i$ which was not removed in the depletion procedure, the internal $P_i$ was fully exchangeable with extramitochondrial $P_i$ in the time course of these experiments. Full exchange was observed even when only 10 s was allowed before inhibition by mersalyl. Exchangeability of this pool was also assessed in separate experiments in which mitochondria were first allowed to equilibrate with $[^{32}P]P_i$, FCCP, which has previously been demonstrated to cause an immediate efflux of $P_i$ from mitochondria (23-25), was then rapidly mixed into the suspension, and an excess of mersalyl was added 1 s later. Samples were then processed as in other experiments, and relative specific activity of $[^{32}P]P_i$ in the intra- and extramitochondrial space was measured. There was no measurable difference between the specific activity of the compartments, indicating full equilibration of the intra- and extramitochondrial $P_i$ pools.

**Malate Distributions in Nonrespiring Mitochondria**—Malate concentrations were measured in the same samples as $[P_i]$. Results of these determinations are presented with the corresponding $P_i$ data in Table I and Fig. 2. It can be seen that the plot of log $[malate]_{in}/[malate]_{out}$ versus ΔpH forms a straight line with a slope of 2, as predicted by the model; this line has an intercept of approximately 0.3, equivalent to the difference between the intramitochondrial and extramitochondrial $P_i$ values (see Equation 3).

**Distributions of $P_i$ in Respiring Mitochondria**—Suspensions of isolated mitochondria were incubated in media supplemented with respiratory substrate (either 10 mM DL-3-OH-butyrate or 1.0-3 mM glutamate + malate with or without prior addition of 3 mM n-butylmalonate) and varying concentrations of $P_i$ (1.5-4 mM). The intramitochondrial pH was increasingly decreased by addition of varying amounts of Na propionate (up to 5 mM) or K acetate (up to 20 mM). ADP (0.5 mM) was then added, stimulating maximal respiratory rates (state 3). Samples were taken (as detailed under “Materials and Methods”) when approximately 25% of the ADP had been phosphorylated, corresponding to the point at which 20% of available $P_i$ had been consumed (and ADP-stimulated respiration would have continued for at least 20 s). A second set of samples was inhibited and quenched 30 s after maximal phosphorylation was achieved (hence, when respiratory rates had returned to minimum values, but the suspension was still aerobic).

Samples from maximally respiring mitochondria (state 3) consistently had slightly lower ΔpH and lower log $[P_i]_{in}/[P_i]_{out}$ than their state 4 counterparts (for each set of conditions, ΔpH was 0.66 ± 0.02 higher and log $[P_i]_{in}/[P_i]_{out}$ 0.14 ± 0.04 higher in state 4 for five experiments, accompanied by an increase in the transmembrane electrical potential of 13 ± 2 mV in state 4), but the inter-relationship between the $[H^+]_{in}$ and $[P_i]$ differences remained essentially constant. Because state 4 incubations have maximal flux rates and unchanging levels of $P_i$, all further reported data are from these samples.

Plots of log $[P_i]_{in}/[P_i]_{out}$ versus ΔpH from experiments in which glutamate + malate were used as substrate are shown in Fig. 3A (data from five experiments are superimposed upon the predicted behavior from Equation 2). The data had a slope of approximately 3.5 at ΔpH values of less than approximately 0.5 units. Once the value of log $[P_i]_{in}/[P_i]_{out}$ reached 1.1 (corresponding to ΔpH of approximately 0.5), there was no further increase with increasing ΔpH. Data from experiments in which 3-OH-butyrate + malate were used as substrate (not shown) were indicative of similar behavior.

Plots of log $[P_i]_{in}/[P_i]_{out}$ versus ΔpH from experiments in which glutamate + malate were used as substrate following preincubation with n-butylmalonate are shown in Fig. 3B.
Fig. 3. Dependence of distribution of Pi and malate in respiring mitochondria on ΔpH using glutamate + malate as substrate. A, stock suspensions of mitochondria were diluted in 120 mM choline chloride, 15 mM MOPS, 0.4 mM EGTA, pH 7.0, and supplemented with varying amounts of Pi (1.5-4 mM) and acetate (0-20 mM) or propionate (0-5 mM). Maximal respiratory rates were stimulated by addition of 0.5 mM ADP. 30 seconds after return to minimal respiratory rates, further activity was inhibited by addition of oligomycin and rotenone, followed 15 s later by mersalyl. B, the mitochondrial suspensions were preincubated with 3 mM n-butyrolmalonate before addition of the glutamate and malate. For both A and B, data ± S.E. from five experiments are shown with the range of predicted behavior calculated for these conditions, using Equations 2 and 3. Lines are drawn through the data for better visualization of the pH-dependent patterns.

There was a slope of approximately 2 at low ΔpH values, extrapolating to a value of log [Pi]i/[Pi]o, at 0.4 at a ΔpH of 0. This was equivalent to 2.5 mm [Pi]o, at A [Pi]o, of 1 mM, not correcting for endogenous or bound Pi. As in the experiments without n-butyrolmalonate, log [Pi]i/[Pi]o, increased to 1.1 at ΔpH = 0.4 (i.e., an intramitochondrial pH of 7.4), after which there was no further increase with increasing ΔpH. Experiments using 3-OH-butrate as substrate (not shown) yielded similar data.

Malate Distribution in Respiring Mitochondria—Final malate concentrations were measured in all experiments with respiring mitochondria in which this substrate had been included. When glutamate + malate or 3-OH-butrate + malate were used as substrates, intramitochondrial [malate] ranged from 8.3 mM (with 3-OH-butrate) to 17.0 mM (with glutamate) when extramitochondrial [malate] was 1.1-1.5 mM. There was very little dependence of log [malate]i/[malate]o, on ΔpH, in marked contrast with the behavior for malate distribution in nonrespiring mitochondria. Data from three experiments with glutamate + malate are shown in Fig. 3A. The distribution of malate predicted by exchange of malate− for P2− (Equation 3) is also shown for comparison. The log [malate]i/[malate]o, remained almost constant at 1.2 for experiments with glutamate + malate, although ΔpH ranged from 0.2 to 1.2 units. For experiments with 3-OH-butrate plus malate, the log [malate]i/[malate]o, was nearly constant at 0.9, while ΔpH ranged from 0.6 to 0.2 units.

When n-butyrolmalonate was included in the incubation medium, there was approximately half as much intramitochondrial malate as in its absence. There was an approximately first-power dependence of [malate]i/[malate]o, on the H+ distribution ratio (Fig. 3B).

Concentrations of intra- and extramitochondrial K+ and Na+, as well as the transmembrane electrical potential, were measured for each of the experimental conditions. There was no measurable amount of intramitochondrial Na+ in any experiment, and no change in the electrical potential was observed, with the exception of the state 3-state 4 transition mentioned previously. Intramitochondrial [K+] remained constant with or without added propionate (116 ± 14 mM at high ΔpH, 118 ± 5 mM at low ΔpH, for five experiments, when extramitochondrial [K+] was 11 mM) for all experiments using glutamate + malate (with no n-butyrolmalonate present). The apparent small increase in [K+]o, with decreasing ΔpH (from 131 ± 9 to 139 ± 13 mM, +3 mM Na propionate, respectively, for a [K+]o, of 16 mM) observed when n-butyrolmalonate was included in the incubation is not statistically significant.

DISCUSSION

Phosphate transport is essential to a number of mitochondrial processes, including ADP phosphorylation and transport of various citric acid intermediates. It has also been postulated to maintain a role in the transport of divalent cations (26-30). Because of the importance of inorganic phosphate in cellular energy metabolism (see, for example, Ref. 12), the kinetics and energetics of phosphate accumulation by mitochondria and their relationship with other metabolite distributions are of great interest.

Evidence for two independent P; transport mechanisms, one in exchange for hydroxyl ions and one in exchange for dicarboxylate ions, was first provided by Chappell and Crofts (2) and Chappell and Haarhoff (3). Since that time, most investigators have supported this concept (Refs. 1-4, 5, and 7-11, for example), although it has been argued that a single carrier develops altered transport properties in response to incubation with selective inhibitors such as N-ethylmaleimide or n-butyrolmalonate (31-33). Strong evidence supporting the original proposal of two independent carriers has been supplied by Wohlrab (1), with the demonstration that the isolated and reconstituted P; transport is inhibited by mersalyl but is insensitive to n-butyrolmalonate.

Exchange of one P; for one OH−, the most commonly accepted mechanism for P; transport, is supported by several lines of investigation (Refs. 4, 5, and 10, for example). Freitag and Kadenbach (34), conversely, have provided evidence that divalent P; is exchanged for two hydroxyl ions by showing that monofluorophosphate and thiophosphate, which have either mono- or divalent forms, were accumulated by mitochondria, while difluorophosphate, which is exclusively monovalent, was not. Contrary to the measured electroneutrality of exchange observed by other investigators. McGivan and co-workers (11) have suggested that a fraction of P; uptake is electrogenic, as charge compensation for ATP−−ADP3− exchange.

A number of investigators have reported correlations between transmembrane pH differences and steady-state distributions of P; and/or dicarboxylic acids in nonrespiring mitochondria (1-11). Quantitative models have been proposed by some of these authors to describe the distribution of monovalent P; and divalent malate in a nonrespiring system, but there was a large discrepancy between the predicted and observed behavior or large empirical corrections were required to fit the data to the model.

In this work, we have derived quantitative expressions (Equations 2 and 3) for the steady-state distributions of total P; and malate at different values of ΔpH in the absence of other metabolic and transport activities, assuming the transport scheme depicted in Fig. 1. 1) For the major transport system, only one species of phosphate can be transported in stoichiometric exchange for OH− (or cotransport with H+); the extent of accumulation is determined by activities of transported species and [OH−] on both sides of the mitochondrial inner membrane; and 2) the divalent form of malate (or other transported dicarboxylic acid) is exchanged for divalent P;.
The measured distributions of $P_i$ and malate for nonrespiring mitochondria are consistent with the model, with distributions of $P_i$, displaying a nearly second-power dependence on the distribution of the hydroxyl ion (although there was a slight deviation from the theoretical values for $P_i$ distribution at lower $\Delta pH$ values), and a second-power dependence of the malate distribution on that of the hydroxyl ion distribution. Measurement of intramitochondrial phosphate subject is several possible sources of inaccuracy, any of which would contribute to the observed deviation at low values of log $[P_i]_{in}/[P_i]_{out}$: (a) the approximately 2 nmol/mg of protein of $P_i$, which remained in the depleted mitochondria, which we have not subtracted from the total intramitochondrial $[P_i]$ values, may be tightly bound and therefore cause overestimation of the intramitochondrial $[P_i]_{int}$ by up to 1.5 mm; (b) hydrolysis of ATP or ADP in the acid-quenched sample, resulting in added $[P_i]_{in}$ (such hydrolysis has been reported to cause underestimation of the intramitochondrial ATP/ADP in respiring mitochondria; see Ref. 36); and (c) residual transport or metabolic activities not completely inhibited by our treatment. On the other hand, malate is not rapidly metabolized under the conditions utilized in these experiments, does not bind significantly to intramitochondrial proteins and phospholipids, and is neither hydrolized nor a product of hydrolysis in acid-quenching procedures. It is for this reason that the malate measurements are particularly important to the data analysis. When combined with the $P_i$ distribution data, the data for malate distribution lend significant support to the conclusion that the mechanisms of $P_i$ and malate transport described here represent the major transport mechanisms for these metabolites in nonrespiring mitochondria.

Several investigators have suggested alternative transport schemes to account for the observed dependence of $P_i$ distribution on the pH difference. Examination of the equations for the following mechanisms (see “Appendix”) shows us that in each case the alternative mechanisms fail to explain either the $P_i$ or malate distributions. Other proposed mechanisms include the following.

1) For exchange of $P_i^-$ for $2OH^-$ (34), the predicted behavior of the $P_i$ distribution does not fit the data as well as the curve predicted by $P_i^-$:OH- exchange, but cannot be clearly excluded. On the other hand, although this mechanism predicts that a plot of log $[malate]_{in}/[malate]_{out}$ versus $\Delta pH$ would have a slope of 2.0, log $[malate]_{in}/[malate]_{out}$ would be equal to 0.6 at $\Delta pH = 0$ (i.e. twice the difference between the intramitochondrial and extramitochondrial $pK$ values for $P_i$; see Equation 19 in the “Appendix”). As the experimentally measured value for log $[malate]_{in}/[malate]_{out}$ is 0.3 at $\Delta pH = 0$, the data are inconsistent with this mechanism.

2) Concerning malate$^{2-}$ exchanging for $2P_i^-$, this proposal also calls for log $[malate]_{in}/[malate]_{out}$ to have a second-power dependence on [OH$^-$], but for the ratio to be equal to 0 when $\Delta pH$ equals 0 (Equation 20 in the “Appendix”). This transport system would also result in log $[P_i]_{in}/[P_i]_{out}$ being larger than log $[malate]_{in}/[malate]_{out}$, in contrast with the experimental results.

3) Some exchange of $P_i^-$ for $OH^-$ and some exchange of $P_i^-$ for $2OH^-$ was proposed (5) in order to explain the observation that plots of log $[P_i]_{total} / [P_i]_{total}$ against $\Delta pH$ had slopes of between 1 and 2. Since the ionization $H_2PO_4^- \rightarrow H^+ + PO_4^{2-}$ occurs on both sides of the membrane, such a mixed transport system would result in a “ futile cycle” of transport which would dissipate the pH difference in a steady-state system. Thus, this model is inconsistent with the data.

Respiring Mitochondria—We have also extended our experiments to examine the relationships in respiring mitochondria. The results indicate that both the $P_i$ and malate distribution ratios follow a very different pattern in relation to the $H^+$ distribution ratio whether or not $P_i$-malate exchange is operative. There was significantly less dependence of $P_i$ and malate accumulation on $\Delta pH$ than predicted in the experiments in which malate could exchange for $P_i$ (Fig. 3A). Only when $P_i$-malate exchange was inhibited or when 3-OH-butyrate alone was used as substrate was the distribution of $P_i$ (or malate) dependent upon $\Delta pH$, but then over a very limited range of $\Delta pH$ (0.2 units) (Fig. 3B). With respiring mitochondria, however, is neither case did the $P_i$ and malate distribute as predicted by Equations 2 and 3, implying that other factors are more important than the transmembrane pH difference in determining the distribution of these metabolites.

Phosphate distribution in respiring mitochondria reached a maximal limit at a log $[P_i]_{in}/[P_i]_{out}$ of 1.1 at $pH_{0}$ of approximately 7.5. Because the same distribution ratios were measured for starting $[P_i]_{out}$ of 1.5 or 4 mm, this limitation in $[P_i]_{in}/[P_i]_{out}$ cannot be explained by a limited capacity of the mitochondria to accumulate $P_i$. Since a similar limit was not observed when ATP synthesis was inhibited, we propose that this limit in log $[P_i]_{in}/[P_i]_{out}$ may be the result of a mechanism of respiring mitochondria designed to maintain the rate of ADP phosphorylation at high $P_i$ concentrations. Regulation of respiration by [ATP]/[ADP]/[Pi] implies that, at constant [NAD$^+$]/[NADH] and [O$_2$], the [ATP]/[ADP] must increase proportionally with $[P_i]$ to maintain a constant respiratory rate. Thus, high $[P_i]_{in}$ as a result of increased $[P_i]_{out}$ would lead to decreased intramitochondrial [ADP]$_{out}$. If $[P_i]_{in}$ rose too high, [ADP]$_{in}$ could then kinetically limit the rate of phosphorylation and, consequently, the ability of the mitochondria to maintain intra- and extramitochondrial [ATP]/[ADP]/[Pi]. The existence of a mechanism to limit the $P_i$ distribution ratio in respiring mitochondria would therefore aid in maintaining cellular homeostasis in times of stress.

Accumulation of malate by mitochondria is believed to occur only by exchange with $P_i$, other dicarboxylates, or tricarboxylates. Therefore, in incubations to which $n$-butyramonlate was added to inhibit $P_i$-malate exchange, the measured net malate uptake may have been due to exchange with endogenous substrates (which, in the case of exchange with citrate, is pH-dependent; see Ref. 11) or to incomplete inhibition by $n$-butyramonlate.

Inclusion of $n$-butyramonlate may have effects in addition to inhibition of $P_i$-dicarboxylate exchange in order to increase the pH dependence of the $P_i$ distribution observed in respiring mitochondria (Fig. 3B). There was, however, no equivalent alteration in pH dependence when $n$-butyramonlate was added to the incubations with nonrespiring mitochondria. Also, $P_i$ distribution in mitochondria respiring with 3-OH-butyrate as substrate (i.e. with no n-butylmalonate present) and no malate present to exchange with $P_i$ demonstrated pH dependence similar to that in Fig. 3B, suggesting that the $n$-butyramonlate per se does not cause the observed change in $P_i$ and malate distributions.

The discrepancy between the behavior of the phosphate and malate distribution ratios in respiring mitochondria and that predicted by the proposed pH-dependent transport mechanisms, combined with the observed limit of phosphate accumulation, make it impossible to calculate the intramitochondrial pH from the distribution of phosphate or the dicarboxylate anions or the distributions of the latter two from $\Delta pH$. 


Taking logarithms of both sides and making use of relationships pH and can be rearranged as:

\[ \text{pH} = \text{pH}_{\text{outside}} + \text{ApH} \]

where the subscripts i and e represent the intra- and extramitochondrial compartments. Because this is a purely physical (in space) separation, the equilibrium constant is equal to 1, and:

\[ K = \frac{[P_i^{-}][\text{H}^+]}{[P_i^{e}][\text{OH}^-]} = 1 \]  

or

\[ \frac{[P_i^{-}]}{[P_i^{e}]} = \frac{[\text{H}^+]}{[\text{OH}^-]} \]  

The second ionization of \( P_i^- \):

\[ P_i^- \rightarrow \text{H}^+ + P_i^{e-} \]  

for which the dissociation constant, \( K_d \), is:

\[ K_d = \frac{[P_i^{e-}][\text{H}^+]}{[P_i^{-}]} \]  

is important in this pH range (pH 6–8).

The total chemically measurable phosphate is the sum of \( P_i^- \) and \( P_i^{e-} \), i.e.:

\[ P_i \text{ total} = P_i^- + P_i^{e-} \]  

Substituting into Equation 5 we obtain:

\[ [P_i^-] = [P_i \text{ total} \left( \frac{[\text{H}^+]}{[\text{H}^+] + K_d} \right) \]  

Equation 7 may be written for \( [P_i^-] \) in both the inside and outside compartments and substituted into Equation 3 to give:

\[ [P_i \text{ total}]  = [P_i^-]  \frac{[\text{H}^+]}{[\text{H}^+] + K_d} + [K_d] \]  

and can be rearranged as:

\[ [P_i \text{ total}] = \left( \frac{[\text{H}^+]}{[\text{H}^+] + K_d} \right) [H^+] + [K_d] \]  

Taking logarithms of both sides and making use of the relationships pH = –log [H+] and [H+] = 10^{-14} [OH^-], we obtain

\[ \log \frac{[P_i \text{ total}]}{[P_i^e]} = \log \frac{([H^+]+K_d)}{[H^+]} + 2 \Delta pH \]  

where \( \Delta pH \) is defined as the pH inside minus the pH outside.

Exchange of \( P_i^{e-} \) for \( OH^- \)—In order to describe the equilibrium distributions of \( P_i^- \) and malate resulting from the stoichiometric exchange:

\[ P_i^{e-} + \text{malate}^- \rightarrow \text{Pi}^{e-} + \text{malate}^2^- \]  

the distribution of \( P_i^{e-} \) is calculated as in the previous derivation with the exception that in Equation 7 we solve only for \( [P_i^{e-}] \):

\[ [P_i^{e-}] = [P_i] \left( \frac{K_d}{[H^+] + K_d} \right) \]  

from which we can solve for the ratio of intra- to extramitochondrial \( P_i^{e-} \):

\[ [P_i^{e-}] = \frac{[P_i]}{K_d} \]  

Combining Equations 9 and 13, we obtain:

\[ \frac{[P_i^{e-}]}{[P_i]} = \frac{K_k}{K_d} \]  

Again, taking logarithms of both sides of the equation and using the relationship \( pK = -\log K_d \)

\[ \log \frac{[P_i^{e-}]}{[P_i]} = pK_d - pK_e + 2 \Delta pH \]  

From Equation 11 we make use of the fact that the equilibrium constant for the exchange is equal to 1:

\[ K = \frac{[P_i^{e-}][\text{malate}^2^-]}{[P_i^-][\text{malate}^-]} = 1 \]  

Therefore, at equilibrium:

\[ [\text{malate}^2^-] = \frac{[P_i^-]}{[P_i]} \]  

The expressions for the following alternate exchange mechanisms for \( P_i^- \) and malate can be similarly derived.

Exchange of \( P_i^{e-} \) for \( OH^- \)

\[ \log \frac{[P_i]}{[P_i]} = pK_d - pK_e + 2 \Delta pH \]  

Exchange of \( 2P_i^{e-} \) for \( \text{Malate}^2^- \) When \( P_i^- \) Exchanges for \( OH^- \)

\[ \log \frac{[\text{malate}^2^-]}{[P_i]} = \log \frac{[OH^-]^2}{[OH^-]} = 2 \Delta pH \]  

REFERENCES

Pi and Malate Distribution in Mitochondria