Intramitochondrial Substrate Concentration as a Factor Controlling Metabolism

THE ROLE OF INTERANION COMPETITION

By E. J. HARRIS AND J. R. MANGER

*Departments of Biophysics and Biochemistry, University College London,
Gower Street, London, W.C. 1*

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1. The concentration-dependence of the intramitochondrial accumulation of L-malate and succinate was measured and expressed in the form of adsorption isotherms. The accumulation, however, may arise because of an internal positive potential. 2. The competition for accumulation offered by some other anions, including phosphate, was measured and is expressed conventionally by additional terms in the adsorption equation. 3. The interactions between anions were also studied when one was acting as oxidized substrate. 4. In some examples there is a parallel between the effects of an added anion on both accumulation and oxidation; in other cases chemical participation of the added substance in metabolism is presumed to remove the correlation. 5. It is suggested that by combining kinetic data on penetration with stoichiometric data on accumulation and specific reaction rates it may be possible to account for the rates of respiration obtained with intact mitochondria. 6. It is possible to show that there is a certain phosphate/substrate ratio for maximum phosphorylation rate with some substrates. This is to be expected when phosphate and substrate compete for accumulation.

By application of the technique of centrifugation through silicone (Werkheiser & Bartley, 1957) as modified to a micro scale by Harris & van Dam (1968) it is possible to measure the quantities of substrate and water carried by mitochondria. Proceeding from the observations of Chappell and his colleagues (Chappell, 1964; Chappell & Crofts, 1966; Chappell & Haarhoff, 1967) and Ferguson & Williams (1966) that malate and other dicarboxylic acids facilitate the entry of tricarboxylic acids into mitochondria depleted of endogenous substrate, we found (Harris & Manger, 1968) that citrate uptake is accompanied by a displacement of malate. This observation supported the concept that there is a general competition between anions for occupation of the mitochondrial sucrose-inaccessible space. Haslam & Krebs (1963) discussed inter-substrate competitions and Haslam (1966) described competitions between pyruvate and oxoglutarate for oxidation in homogenates and mitochondria. One factor influencing the relative rates of oxidation is the relative accumulations in the mitochondria, and these depend in turn on what competitors are presented, as well as the concentrations of the substances themselves.

We have measured the mitochondrial accumula-

tion of L-malate and succinate and some mutual interactions between these anions as well as the competitive effects of phosphate and malonate on them. The results are expressed in terms of ' K_m ' and ' K_i ' values as if the mitochondrion was an anion-exchanger. The values of the parameters are compared with those deduced from metabolic rates: in many examples there is a close correspondence, suggesting that passage through the membrane and ability to be accumulated determine the supply of substrate to the enzymic system in intact mitochondria. A particularly important competition can arise between phosphate and substrate, so setting an optimum ratio for the concentrations necessary to conduct phosphorylation. The control exercised by the membrane accumulating system can be modified by disruption, so comparisons of anion interactions have been extended from intact to disrupted mitochondria.

A further important feature of the ability of the mitochondria to accumulate anions is that it can lead to disparities between the ratios relating total cellular contents of two or more given substances (e.g. glutamate, oxoglutarate and ammonia) and the separate ratios pertaining respectively in the cytoplasm and in the mitochondria. In that event

the analyses for total cytoplasmic contents may not lead to correct calculation of the redox state in one compartment of the system.

MATERIALS AND METHODS

Mitochondria were isolated from rat liver by the method of Schneider (1952), with homogenization in 0.25 M-sucrose containing 1 mM-ethylene glycol bis-(2-aminoethyl)-tetra-acetate. Three washes were given by resuspension in 0.25 M-sucrose-1 mM-ethylene glycol bis-(2-aminoethyl)-tetra-acetate, special care being taken to discard all loosely packed fluffy material from the mitochondrial pellet. The mitochondrial protein content of the final suspension was determined by the biuret method and was about 40–50 mg./ml. The medium used for accumulation and competition experiments was either 120 mM-KCl-20 mM-tris chloride, pH 7.4, or 0.25 M-sucrose-10 mM-KCl-20 mM-tris chloride, pH 7.4. The competition experiments were carried out in the low-K⁺ medium generally, but in certain cases the high-K⁺ medium was used. Disruption of the mitochondria was effected by three consecutive freezings and thawings.

Accumulation and competition experiments. A reaction volume of 3.0 ml., containing 20–30 mg. of mitochondrial protein, ¹⁴C-labelled substrates (0.1 mM) and usually an inhibitor, to prevent the oxidation of the substrate to be measured, was used. The labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., and the non-labelled compounds from Sigma (London) Chemical Co. Ltd., London, S.W. 6. During the course of an experiment 15 samples (0.2 ml.) were taken from the reaction mixture and added to Coleman plastic centrifuge tubes containing 40 μ l. of 1.5 M-HClO₄ beneath a layer (2–3 mm. thick) of silicone oil. The density of the oil was varied according to the medium used, e.g. that used for the high-K⁺ medium had a density of 1.038, whereas that used for the sucrose-low-K⁺ medium had a density of 1.058. The tubes were centrifuged at 12000 rev./min. in a Coleman 811-K5 microcentrifuge for 30–60 sec. The mitochondria passed through the silicone oil into the acid and the resulting denaturation liberated the contents of the mitochondria into an acid-soluble extract, which was divided for K⁺ and ¹⁴C and ³H analyses. Metal ions were analysed by using an atomic absorption spectrophotometer (model AA-100; Techtron Pty. Ltd., Melbourne, Vic., Australia). The scintillation counting was done on a Packard Tri-Carb spectrometer with toluene-ethanol (4:1, v/v) containing 0.2% of 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen as scintillator. The use of ³H₂O gave a measure of the total mitochondrial water carried through the silicone oil (Harris & van Dam, 1968).

Respiratory rates. A polarograph with a Hagihara (1961) rotating platinum cathode maintained at 20° was used for the oxidation studies. The reaction volume was 3.0 ml. and included 4–5 mg. of mitochondrial protein, 30 μ M-2,4-dinitrophenol and 1 mM-substrate, and additions of 0–10 mM-competitor were made during each experiment. Measurements were made on a Kent recorder with a full-scale deflexion of 2 mv. Unless otherwise stated, the high-K⁺ medium was used for the oxidation studies.

Calculation of results. Radioactivity in each sample was measured and the resulting counts were processed by using a computer programme written in FORTRAN IV and used on

the University College London IBM system 360/65. Samples taken under constant conditions usually gave values for ¹⁴C and ³H within a range of 10% about the mean. Counting accuracy was better than 1.5%. Control of the centrifugation was obtained from the ³H content of the protein extract and, when this was carried out, from the K⁺ analysis. Samples for which either of these quantities differed from prevailing values were rejected.

RESULTS

Anion uptake without induced K⁺ uptake. The mitochondria were incubated with labelled substrate at 22–25° and three or four samples for centrifugation were taken at intervals of 30 sec. There was a considerable uptake of substrate anion from an applied concentration of 1 mM. The values found at 30 sec.–2 min. for the contents of labelled substrate were usually constant within an experimental error of $\pm 10\%$, so the entry process must have been completed within the time required for separating the first sample. Hence they are values for a completed process and are not related to kinetics.

With untreated mitochondria, all anions tested at 1 mM concentration reached concentrations of two- to five-fold the quantity that, at 1 mM concentration, would be dissolved in the total mitochondrial water (3.5–5 ml./g. of protein). Since the values were obtained from radioactivity measurements, it is important to note that the compounds were accumulated in conditions in which they were not oxidized. Citrate, malate and glutamate were measured in the presence of rotenone, succinate in the presence of antimycin A, and α -oxoglutarate and pyruvate in the presence of 0.5 mM-sodium arsenite. In the citrate experiments, conversion of citrate into aconitate and isocitrate accounted for about 10% of the total mitochondrial radioactivity no longer existing as citrate, but this conversion was small compared with observed concentrations of citrate. After allowance for the anion in the sucrose-accessible water assumed to be present at the same concentration as in the medium, the concentrations of the anions in part of the water inaccessible to sucrose were between 5 and 18 mM. Table 1 shows that citrate, even after allowance for 10% isomerization, was the anion most accumulated from the 1 mM external concentration.

Inspection of the values shows that the quantities, expressed as μ moles/g. of protein, are directly proportional to the net number of negative groups in the substrate molecule. Acetate, pyruvate and glutamate, with one net negative charge, are taken up to an internal concentration of about 5.0 μ moles/g. of protein, corresponding to a concentration of about 7 mM in the sucrose-inaccessible water. Malate, malonate, succinate and α -oxoglutarate,

Table 1. *Quantities of labelled anion found in the space inaccessible to sucrose of rat liver mitochondria after exposure to 1 mM concentrations at 20°.*

Values are means from between two and four readings taken in the first 2 min. of incubation. Exposure up to 10 min. did not lead to significant alterations. Separate values are from different preparations. The sucrose-accessible space was either measured in a special experiment or assumed to be 0.7 ml./g. of protein less than the total water (see the text). At the time of mixing the metabolism was blocked by an appropriate inhibitor when measuring oxidizable substances (see the text). The medium contained either sucrose (250 mM), KCl (10 mM) and tris chloride, pH 7.4 (20 mM), or KCl (120 mM) and tris chloride, pH 7.4 (20 mM). Since the observed uptakes did not appear to differ systematically between the two media no distinction has been made in the Table.

Anion measured	Uptake in sucrose-inaccessible space (μ moles/g. of protein)
Acetate	4.1
L-Glutamate	5.5, 5.8, 3.4
Pyruvate	4.1, 5.5
DL- β -Hydroxybutyrate	2.9, 4.0
L-Malate	10.9, 9.7
Malonate	13.3, 11.1, 10.6
α -Oxoglutarate	9.8
Succinate	9.9, 8.2
Citrate*	15.8, 12.3, 18.9
DL-Isocitrate*	16.4

* Citrate and isocitrate are presumably accumulated as the equilibrium mixture.

with two non-neutralized carboxyl groups, are taken up to about 10 μ moles/g. of protein, whereas citrate, with three carboxyl groups, is accumulated to a content of about 17 μ moles/g. of protein. This relationship suggests that electrostatic force leads to anion accumulation because of an internal positive potential.

Whether the mitochondrion is regarded as a container with a charged interior or it is supposed that the membrane enforces certain anion exchanges, as suggested by Chappell & Haahrhoff (1967), the consequences will be similar. There will be a limited capacity for the anions according to the requirements of electroneutrality. The steady-state distribution will depend on the anion's charge and the potential difference across the membrane bounding the relevant compartment, which may not be common to all anions. Formally, the distribution can be described by using the Langmuir adsorption isotherm relating quantities, not rates, to concentrations, which is similar to the Michaelis-Menten expression for the kinetics of an enzyme reaction involving a reversible combination between sites, X^+ , and substrate, S^- :

Since the adsorption of anions shows this behaviour, the use of eqns. (1) and (2) relating the quantities of substrate taken up, Q_i to the applied concentrations of substrate, S , and inhibitor, i (Dixon & Webb, 1964), permits certain analogies

$$Q_i = \frac{Q_{\max} \cdot [S]/K_m}{1 + [S]/K_m + [i]/K_i} \quad (1)$$

for 'competitive' inhibition of accumulation

$$Q_i = \frac{Q_{\max} \cdot [S]/K_m}{1 + [S]/K_m} \left(\frac{1}{1 + [i]/K_i} \right) \quad (2)$$

for 'non-competitive' inhibition of accumulation

between $Q_{\max}$ and the $V_{\max}$ for oxidation to be drawn, and the values of K_m and K_i are related to the affinity of the anion exchanger for different ions. The fact that there is competitive adsorption of substrate ions does not necessarily imply that the ions are transported across the membrane by an enzyme or a specific carrier system, only that anion exchangers are involved. The value Q_i is a measure of the new steady-state concentration reached inside the mitochondrion when a certain concentration of substrate is applied. Other methods have to be used to follow the kinetic course of the entry. The accumulation and oxidation data were obtained over a range of concentrations, but most weight is placed on results for concentrations less than 1 mM. Not only are these more nearly physiological, but also the correction necessary for the material in the sucrose-accessible space is a smaller fraction of the whole than when saturation of the accumulating system is approached.

Accumulation of succinate and malate: phosphate competition. Measurements of the total malate or succinate associated with the protein were corrected for the amounts in the sucrose-accessible space. In some sets of experiments separate determinations of sucrose and $^3\text{H}_2\text{O}$ spaces were made, but in others it was assumed that the sucrose space was 0.7 ml./g. of protein less than the $^3\text{H}_2\text{O}$ space, which was always measured. The value of 0.7 ml./g. of protein is taken from Harris & van Dam (1968), and is similar to values given by other workers for the space inaccessible to sucrose.

With untreated mitochondria the reciprocals of the corrected uptakes were linearly related to the reciprocals of the applied concentrations. The lower curves in Figs. 1(a) and 1(b) show operational K_m values (eqns. 1 and 2) of 3.1 mM for malate and 1.3 mM for succinate accumulation. The upper curves show the effects of phosphate competition and the capacity of the mitochondrion for malate is decreased. The competitive constant of phosphate (K_i in eqn. 2) can be evaluated by two slightly different procedures. Either parallel sets of

Fig. 1. Double-reciprocal plots of mitochondrial contents of malate (a) and succinate (b) against applied concentrations with (●) and without (○) phosphate (2 mM in a, 1.8 mM in b). The contents have been corrected for the quantities present in the space accessible to sucrose. The medium used contained sucrose (250 mM), KCl (10 mM) and tris chloride, pH 7.4 (20 mM), and rotenone for (a) and antimycin for (b) at $1 \mu\text{g./ml.}$ The protein concentration was 4 mg./ml.

measurements are made at a series of different concentrations of substrate with a constant amount of competitor present in one set (as in Fig. 1), or the competitor can be titrated into an incubation having a constant substrate concentration. The former lends itself to the double-reciprocal plot and the latter to one of the other treatments described by Dixon & Webb (1964), in which the reciprocal of the amount accumulated is plotted against the concentration of competitor. The second method

Fig. 2. Competitive displacement of mitochondrial malate (a: ○, with 0.20 mM-malate; △, with 0.64 mM-malate) or succinate (b: ○, with 0.08 mM-succinate; ●, with 0.18 mM-succinate; △, with 0.67 mM-succinate) by phosphate. The contents were corrected and the media were as described in Fig. 1.

was used to confirm the inhibitory effect of phosphate on the accumulation of malate (Fig. 2a) and succinate (Fig. 2b). Estimates of the operational K_i values are given in Table 2. There is a considerable latitude in fitting the points and different preparations lead to other values, but there is a fair concordance between the results from the two methods.

Interaction between malate and succinate. Succinate accumulation is decreased by malate (Fig. 3a) and vice versa (Fig. 3b). However, if accumulation is the consequence of an internal positivity and the capacity depends on the cation content, it would follow that any penetrating anion applied would occupy some of the capacity and so act as a competitor.

Fig. 3. Competitive displacements of mitochondrial malate by succinate (a: O, with 0.07 mM-malate; ●, with 0.16 mM-malate) and of mitochondrial succinate by malate (b: O, with 0.17 mM-succinate; ●, with 0.53 mM-succinate). The contents were corrected and medium was as described in Fig. 1.

Oxidation of succinate and malate: effects of competitors. The rates of respiration were measured in the presence of optimum concentrations of 2,4-dinitrophenol, with a series of concentrations of succinate and malate. These gave the K_m values in Table 2 (column 1), which can be compared with those found from the accumulation studies (column 3). When phosphate or malate (Fig. 4) (in the presence of rotenone to block its oxidation) is titrated into an incubation mixture with succinate as substrate, there is in each case a progressive inhibition of respiration. The changes can be described by the conventional enzyme-inhibition formula analogous to eqn. (2) and the values of K_i (in Table 2) may be compared with those found for accumulation. The effect seen includes the consequences of displacement of succinate, which has been described, and a possible displacement of 2,4-dinitrophenol. Evidently specific competitors at the enzyme site, e.g. malonate on succinate dehydrogenase, are often more effective as inhibitors than those that, perhaps like malate, act solely by keeping the substrate out. The effect of phosphate

on malate oxidation does not conform to the concept of its acting only as a competitor for accumulation; in fact it stimulates the rate of oxidation. This may be due perhaps to removal of the inhibitory product (oxaloacetate) by the operation of phosphoenolpyruvate carboxykinase (Scholte & Tager, 1965).

The results summarized in Table 2 include a considerable scatter between different preparations of mitochondria. This is probably related to the differing K^+ contents of the preparations. A trial was made to find the effect of overnight storage at 0° on the substrate uptake. It was approximately halved by this procedure, as also was the K^+ content. The observation that the saturation capacity ($Q_{\max.}$) is about $50 \mu\text{moles}$ of the dibasic acids malate and succinate/g. of protein suggests that the acid can reach equivalence with the exchangeable part of the total K^+ , i.e. about $100 \mu\text{moles/g.}$ in fresh preparations (Harris & van Dam, 1968). Some K^+ is apparently not free to exchange, and this part may be less likely to provide an equivalent anion-exchange capacity.

Kinetics of respiration of frozen-and-thawed mitochondria. The kinetics of a process that involves the step of membrane penetration followed by reaction with an enzyme would be expected to differ from the kinetics in the absence of the membrane. Some experiments were made with mitochondria disrupted by repeated freezing and thawing to find the operational K_m values for malate and succinate, and to test for competitive effects. The results are included in the summary in Table 2 (right-hand columns). There is neither competition nor stimulation from phosphate, and malonate has its well-known effect on succinate oxidation but is without effect on malate oxidation. Inhibition of succinate oxidation by malate (in the presence of rotenone) is of the kind called by Dixon & Webb (1964) 'competitive' inhibition, i.e. the competitor can be displaced by excess of substrate, and in this respect differs from the 'non-competitive' inhibition seen in intact mitochondria.

Optimum concentrations for phosphorylation. If the progress of phosphorylation involved only two completely independent processes, namely reaction of the substrate with its dehydrogenase and reaction of phosphate with ADP, each with conventional Michaelis kinetics and values of K_m for substrate and phosphate of about 1 mM, it would follow that the overall process would have a constant rate over a wide range of concentrations of phosphate and substrate. So long as the respective external concentrations were each several times the K_m the internal substrate and phosphate concentrations would be saturated. As the substrate/phosphate ratio was varied the rate would rise to a constant saturation value, only to fall when the concentration of the second reactant was sufficiently decreased.

Table 2. *Parameters describing rates of respiration, V , of intact mitochondria in the presence of 2,4-dinitrophenol at optimum concentration, and of broken mitochondria, and accumulation of substrate as functions of applied concentrations, C_e*

The additional parameters describing the inhibitory effects of added substances follow. The rates are given by the following equations: eqn. (1) for 'competitive' inhibition (C) and eqn. (2) for 'non-competitive' inhibition (NC):

$$V = V_{\max} \left(\frac{C_e/K_m}{1 + C_e/K_m + [i]/K_i} \right) \quad (1)$$

$$V = V_{\max} \left(\frac{1}{1 + [i]/K_i} \right) \left(\frac{[S]/K_m}{1 + [S]/K_m} \right) \quad (2)$$

The accumulations, Q , are given by similar equations with Q and $Q_{\max}$ replacing the V values.

	Intact mitochondria	Broken mitochondria	Accumulation
Substrate: succinate			
K_m (mM)	2.6	0.5	1.3
$V_{\max}$ ($\mu\text{g. atom of O/g. min.}$)	90	90	$Q_{\max}$. ($\mu\text{moles of substrate/g. of protein}$) 40
K_i for phosphate	3.2 (C)	No inhibition	3.1 (C) (Fig. 2b) 4.4 (C) (Fig. 1b)
K_i for malate*	2.6 (NC) (Fig. 4a)	2.8 (C) (Fig. 4b)	4.5 (NC) (Fig. 3b)
K_i for malonate	0.15 (C)	0.8 (C)	1.7 (NC)
Substrate: malate			
K_m (mM)	2.0	0.3	3.1
$V_{\max}$ ($\mu\text{g. atom of O/g. min.}$)	60	40	$Q_{\max}$. ($\mu\text{moles of substrate/g. of protein}$) 50
K_i for phosphate	Stimulates	No effect	2.0 (NC) (Fig. 1a) 2.0 (NC) (Fig. 2a)
K_i for succinate†	8.5 (NC)	—	2.0 (NC) (Fig. 3a)
K_i for malonate	0.3 (NC)	No effect	2.0 (C)

* Measured in the presence of rotenone.

† Measured in the presence of thenoyltrifluoroacetone (Tyler, Gonze & Estabrook, 1966). The increment in respiration due to addition of malate was measured because succinate oxidation is incompletely blocked.

The behaviour of α -oxoglutarate approximates to this pattern (Fig. 5). However, with β -hydroxybutyrate or succinate the rate continues to rise up to 50mM-substrate. This can be formally accounted for if the phosphate tends to compete with substrate for access to the enzyme, as seen in Fig. 1. With a mixture of glutamate and malate as substrate there is a marked maximum rate at 50mM each of mixed substrate and phosphate: this could arise if there is competition both ways, so that excess of phosphate tends to displace substrate and excess of substrate tends to displace phosphate.

DISCUSSION

There are two mechanisms by which anions can be accumulated in mitochondria; one is a process of anion exchange, which when endogenous reserves are depleted may become energy-dependent (Max & Purvis, 1965; Harris & Manger, 1968). The other process involves simultaneous gain of K^+ and anion; this is easiest to demonstrate when K^+ uptake is induced with an antibiotic such as valinomycin (e.g., see Fig. 5 of Harris & van Dam, 1968).

In the present paper we are concerned with a few of the numerous inter-anion effects possible in the exchange process. It has been shown that with intact mitochondria there is competition between phosphate and either malate or succinate for accumulation; the same holds for citrate (E. J. Harris & J. R. Manger, unpublished work; Harris, 1968). There is mutual interference between succinate and malate, and, though not measured in detail, malate has been found to displace glutamate. Displacement of phosphate by malate or citrate has been described (Gamble, 1965). The inter-anion effects have all been measured in the absence of appreciable metabolism, so they represent a process of readjustment of a system that is already charged with anions, to addition of a new anion. The question of how relevant these data are to metabolic control is to some extent answered by the comparisons made under conditions where only one of the two substrates is oxidized. With intact mitochondria phosphate inhibits oxidation of succinate, yet it is without effect on succinate oxidation by broken mitochondria, and so it is fair to conclude that the phosphate interference is related to internal accumulation. The same argument applies to the

Fig. 4. Inhibition by L-malate of succinate oxidation by intact mitochondria (a: Δ , with 1mM-succinate; $\bullet$, with 2mM-succinate; $\circ$, with 3mM-succinate) and by disrupted mitochondria (b: $\circ$, with 2mM-succinate; $\bullet$, with 4mM-succinate). The medium contained KCl (120mM), tris chloride, pH7.4 (20mM), rotenone ($1\mu\text{g./ml.}$) and (in a) 2,4-dinitrophenol ($50\mu\text{M}$). The protein concentration was 1.6mg./ml.

results obtained for malonate inhibition of malate oxidation. The other combinations are more complicated; malate inhibits succinate oxidation in both intact and broken mitochondria, so, like the malonate inhibition of succinate oxidation, the effect is partly on succinate dehydrogenase. It is important to underline the many other effects the malonate has: it promotes the reaction of oxoglutarate (Tager, 1963), and also competes with other acids for accumulation.

It has been shown (Harris, van Dam & Pressman, 1967) that succinate is accumulated less when mitochondrial K^+ is discharged with excess of uncoupling agent or with nigericin. The decrease in internal succinate concentration is one explanation

Fig. 5. Rates of phosphorylation of ADP by mitochondria as a function of the proportions of phosphate and substrate at 22° . Besides 10mM-KCl the media contained: $\circ$, tris phosphate and α -oxoglutarate as specified, tris malonate (3mM); Δ , tris phosphate and sodium DL- β -hydroxybutyrate as specified, sucrose (50mM); $\bullet$, tris phosphate and succinate as specified, rotenone ($1\mu\text{g./ml.}$), sucrose (25mM in the 90mM-phosphate mixture falling to zero with 10mM-phosphate); $\square$, tris phosphate and equimolar tris L-malate and glutamate as specified.

of decreased respiration and failure to respond to ADP; mere increase of the applied concentration of substrate restores the response to ADP (Pressman, Harris, Jagger & Johnson, 1967), doubtless because the internal concentration responds to the applied concentration. A similar finding based on the nigericin action on valinomycin-stimulated respiration has been made by Palmieri, Cisternino & Quagliariello (1967). Competition between phosphate and uncoupler (2,4-dinitrophenol) has been shown by Azzi & Azzzone (1965).

When kinetic data for uptakes are available and are combined with knowledge of the dependence of the rate of oxidation on local concentration, such as may be given by study of the pure enzymes, it should

be possible to relate the oxidation in intact mitochondria to permeability, accumulation and specific internal reaction rate.

Since formally the mitochondrion behaves like an anion-exchanger or a positively charged region and more-charged anions generally can replace less-charged anions, it is likely that the ratios holding between applied concentrations of substances will not be the same as the ratios holding in the mitochondrial interior. This in turn means that the overall ratios, as measured in intact cells, will be weighted means and will not necessarily tell us correctly either cytoplasmic or mitochondrial ratios.

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