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ACCUMULATION OF SUBSTRATES BY MITOCHONDRIA

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SUMMARY

1. Substrates are accumulated in the matrix space of rat-liver mitochondria under conditions where no energy can be generated.

2. The accumulation shows saturation characteristics: a plot of the inverse of the external *versus* the inverse of the intramitochondrial concentration gives a straight line.

3. The maximal uptake (at infinite external substrate concentration) is finite and approximately equivalent to the intramitochondrial K^+ concentration.

4. Substrates inhibit competitively the uptake of other substrates.

5. The uptake of all substrates is inhibited competitively by 2,4-dinitrophenol.

INTRODUCTION

It has been shown with different techniques that mitochondria are able to concentrate substrates from the suspending medium[1-8]. This paper describes an investigation of the concentration dependence of this accumulation process, using the technique of centrifugation through a silicone layer to collect the mitochondria[9,6,10].

In earlier publications the question as to where the substrates are accumulated was left open. However, the demonstration by HARRIS AND VAN DAM[10] that it is the sucrose-impermeable space that is osmotically active leaves little doubt that the concentration gradient is developed over the inner mitochondrial membrane. Furthermore, it will be shown in this paper that swelling of mitochondria in solutions of ammonium salts of substrates[3] results in a change in the sucrose-impermeable space only, again indicating that the membrane excluding sucrose is the same as that constituting a barrier for substrates.

Intramitochondrial concentrations of substrates in this paper refer to values calculated for the sucrose-inaccessible or matrix space of the mitochondria. Fundamentally, these calculations are the same as those used by others to correct for extraparticulate or adhering water in the mitochondrial precipitate.

The experiments reported here were all performed in the absence of an added source of energy, *i.e.* no oxidation of substrate or hydrolysis of ATP took place.

METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[11] as described by MYERS AND SLATER[12]. Protein was determined with the biuret method as modified by CLELAND AND SLATER[13].

The mitochondria were incubated at a concentration of approx. 5 mg/ml in a medium containing ^{14}C-labelled substrate and $^{3}H_2O$. Antimycin or rotenone was always present to prevent oxidation of endogenous substrates. In some experiments oligomycin was added to block the utilization of endogenous ATP of the mitochondria. At 30-sec intervals after starting the reaction by addition of the mitochondria, 0.2-ml samples were taken and layered on top of a 3-mm layer of silicone oil (Wacker Siliconöl AR 100), itself layered on top of 25 μl $HClO_4$ (1.5 M) in a small plastic centrifuge tube. Each sample was centrifuged immediately in a microcentrifuge (Coleman Model 6-811)[10].

Radioactivity was measured by dissolving 20-μl samples in 15 ml fluid consisting of toluene–ethanol (3:1, v/v) with 2 g 2,5-diphenyloxazole *plus* 30 mg 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene per l. To correct for any quenching induced by the $HClO_4$, 20 μl $HClO_4$ (1.5 M) were added to the samples taken from the medium remaining after centrifugation, while 20 μl water was added to the samples taken from the acid layer. The radioactivity in the mitochondrial extract did not change with the time of incubation, indicating that equilibrium had been established already at 30 sec. Therefore, usually the average of four successive samples was used to calculate the intramitochondrial concentrations.

In each experiment the sucrose-permeable space of the mitochondria was determined in a parallel experiment under identical conditions, using [^{14}C]sucrose instead of ^{14}C-labelled substrate.

The K^+ content of the mitochondria was determined by comparing the atomic absorption of the acid extract of the mitochondrial suspension (after suitable dilution) with those of standards in a flame photometer (Evans Electroselenium Ltd., Great Britain).

^{14}C-Labelled substrates and $^{3}H_2O$ were obtained from the Radiochemical Centre, Amersham, Great Britain.

Valinomycin was a gift of Dr. B. C. PRESSMAN. Oligomycin was kindly supplied by the Upjohn Chemical Co.

For the reasons mentioned in the INTRODUCTION it was assumed in the calculations that the accumulation of substrates took place in the sucrose-impermeable space of the mitochondria. Therefore, the amount of labelled substrate carried by the mitochondria was taken to consist of one fraction in the sucrose-permeable space with the same concentration as that in the suspending medium, and a second fraction in the sucrose-impermeable space.

RESULTS

CHAPPELL AND CROFTS[3] showed that, under certain defined conditions, mitochondria swell when suspended in a medium containing the ammonium salt of a substrate. For the swelling to occur with dicarboxylic acid anions like malate the presence of some phosphate is necessary[3]. Since ammonium phosphate can induce

swelling by itself and ammonium malate does not, it is safe to assume that it is the malate anion that cannot freely penetrate into some mitochondrial compartment. The experiment described in Fig. 1 shows that during swelling in ammonium malate solution (initiated by the addition of phosphate) it is the sucrose-impermeable space that increases while the sucrose-permeable space remains constant. These results are in line with the findings of HARRIS AND VAN DAM[10] who showed that also under other swelling conditions the matrix space is the main variable. The fact that the swelling proceeds to a volume corresponding to an osmolarity smaller than that of 50 mM ammonium malate alone indicates that the phosphate renders the membrane permeable to the malate anion, and that it is not only phosphate permeation that causes the swelling.

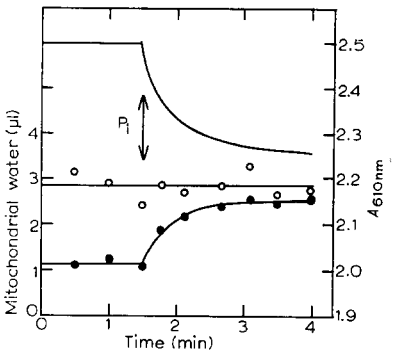

Fig. 1. Changes in sucrose-permeable and sucrose-impermeable space upon swelling of mitochondria induced by ammonium malate. Rat-liver mitochondria were incubated at 30° at a concentration of 5 mg protein per ml in a medium containing 50 mM ammonium malate, 5 mM Tris–HCl, 1 µg/ml rotenone, 0.33 mM ethyleneglycol-bis-(2-aminoethyl)-tetraacetate and 10 mM sucrose at pH 7.4. The medium further contained 0.2 µC/ml ^3H_2O and 0.5 µC/ml [^{14}C]sucrose. At the arrow 6.3 mM ammonium phosphate (pH 7.4) was added. 0.2-ml samples were withdrawn, centrifuged through silicone and the radioactivity measured as described under METHODS. ●—●, sucrose-impermeable space; ○—○, sucrose-permeable space; ———, $A_{610\ nm}$.

It may be noted that the concentration of ammonium phosphate used in this experiment is considerably higher than that in CHAPPELL AND CROFTS' original paper[3]. The reason for this is that in our hands the rate of swelling—as measured by the change in absorbance of the suspension—is very dependent on the phosphate concentration. Half-maximal rate of swelling is obtained only at a concentration of 15 mM ammonium phosphate.

Having thus established that the membrane enveloping the matrix is the substrate-anion barrier we may conclude that any concentration gradient of substrate anions will be across this barrier and, therefore, that the accumulated substrates are localized in the matrix space.

In Fig. 2 the results of an experiment measuring the uptake of succinate by rat-liver mitochondria are plotted. Fig. 2A shows that the internal equilibrium concentration of the succinate approaches a limiting value. In Fig. 2B the same data are plotted in a different way. There is clearly a linear relationship between the inverse of the extra- and intramitochondrial concentration of succinate. The intercept with the ordinate is finite and in this case the maximal internal concentration of succinate

(at infinite external concentration) is 50 mM. This value may be compared with the internal K^+ concentration which was 120 mM in this experiment.

Experiments in which oligomycin (1 μg/mg protein) was added to the reaction medium gave the same results as in the absence of this inhibitor (contrast ref. 6). Evidently, the endogenous ATP of the mitochondria plays no role in the accumulation studied here.

Similar results as described for succinate in Fig. 2 could be obtained with a number of other mitochondrial substrates. In all cases a straight line was found in the reciprocal plot of external *versus* internal concentration, with a finite intercept with the ordinate. These data are summarized in Table I, where K_m is defined as the graphically determined substrate concentration at which half-maximal accumulation occurs.

The intramitochondrial K^+ can be manipulated in two ways. First, by changing the tonicity of the medium the matrix space can be varied and since K^+ cannot move the intramitochondrial *concentration* changes. Secondly, by adding valinomycin *plus* potassium acetate in the presence of an energy source the intramitochondrial K^+

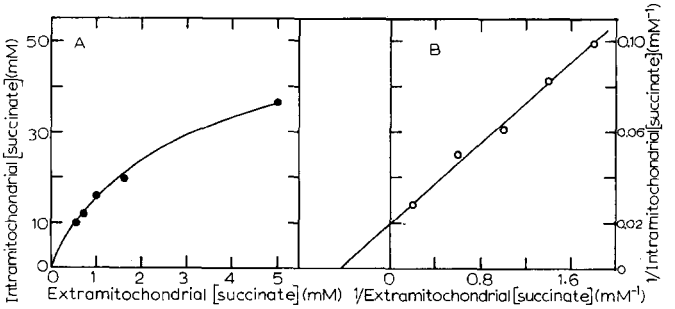

Fig. 2. Relation between intra- and extramitochondrial succinate concentration. Rat-liver mitochondria were incubated at room temperature at a concentration of 9.2 mg protein per ml in a medium containing 50 mM sucrose, 25 mM Tris–HCl, 25 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $NaAsO_2$, 1 μg/ml rotenone, 4 μg/ml antimycin and substrate at 0.55–5.0 mM; the final pH was 7.4. The medium contained 0.2 μC/ml ^3H_2O and 0.5 μC/ml [^{14}C]succinate. 0.2-ml samples were withdrawn and centrifuged as described under METHODS.

TABLE I

SUBSTRATE ACCUMULATION AND K^+ CONTENT OF RAT-LIVER MITOCHONDRIA

Incubation conditions as described under Fig. 2. Determinations of substrate and K^+ were made on the same acid extract of the mitochondria (see METHODS).

Substrate	K_m (*mM*)	*Maximal internal concn.* (*mM*)	*Internal* K^+ *concn.* (*mM*)
Succinate	1.5	45	121
Malate	1.7	36	84
Malonate	1.3	30	90
α-Oxoglutarate	2.5	50	156
Citrate	1.3	36	90
Pyruvate	6.3	100	106
Glutamate	4.5	50	124
β-Hydroxybutyrate	4.5	35	59

content can be increased. Since in this case the matrix space increases proportionally[10] the actual intramitochondrial K^+ concentration remains constant.

The substrate-accumulation pattern under the two conditions described is given in Fig. 3. It is clear that under conditions of changing K^+ concentration the intramitochondrial substrate concentration changes by a constant factor at all extramitochondrial concentrations (Fig. 3A). Upon changing the K^+ content, keeping its

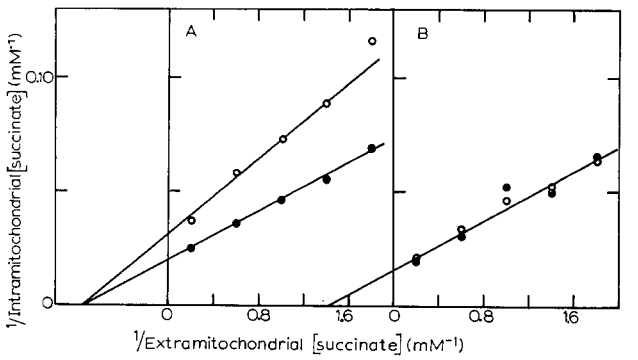

Fig. 3. Effect of tonicity and of valinomycin-induced swelling on the accumulation of succinate by mitochondria. A. Rat-liver mitochondria were incubated at room temperature at a concentration of 5.9 mg protein per ml in a medium containing 50 (O—O) or 150 mM (●—●) sucrose, 50 mM Tris–HCl, 10 mM KCl, 1 mM EDTA and 2 μg/ml antimycin; the final pH was 7.4. The medium contained the same amount of radioactivity and was treated as in Fig. 2. B. Rat-liver mitochondria were incubated at room temperature at a concentration of 9.3 mg protein per ml in a medium containing 150 mM sucrose, 20 mM Tris–HCl, 5 mM Tris acetate, 10 mM KCl, 1 μg/ml rotenone and 0.55–5.0 mM succinate with (O—O) or without (●—●) 40 ng/ml valinomycin; the final pH was 7.4. The medium contained the same amount of radioactivity and was treated as in Fig. 2. The values given in the presence of valinomycin are for the situation where no more swelling occurred as indicated by the constancy of the sucrose-permeable space in parallel experiments.

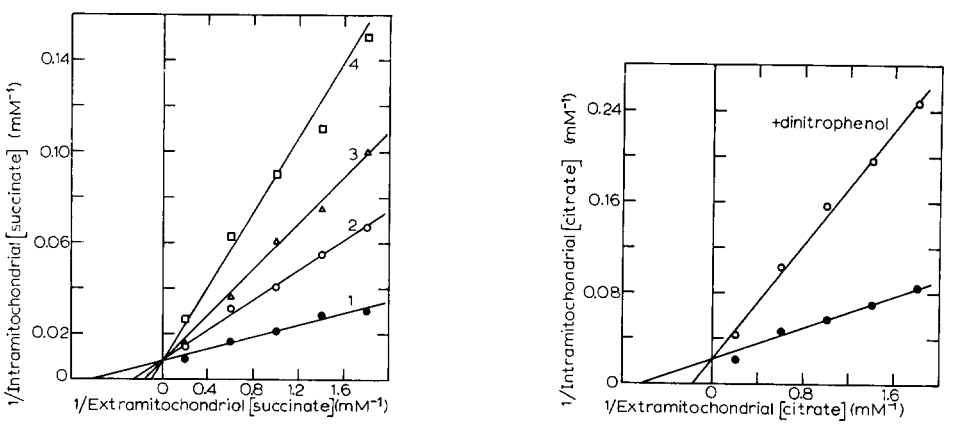

Fig. 4. Effect of other anions on the accumulation of succinate by mitochondria. Experimental conditions as in Fig. 2. Protein concentration 8.8 mg/ml. Additions: 1, none; 2, 5 mM glutamate; 3, 5 mM citrate; 4, 5 mM butylmalonate.

Fig. 5. Effect of 2,4-dinitrophenol on the accumulation of citrate by mitochondria. Experimental conditions as in Fig. 2 except for the use of citrate instead of succinate. Protein concentration 4.6 mg/ml. 2,4-Dinitrophenol was added in a concentration of 0.5 mM.

concentration constant, the intramitochondrial substrate concentration does not change (Fig. 3B).

When the effect of unlabelled substrates on the accumulation of labelled substrates was tested a purely competitive inhibition of the accumulation was observed. A few examples of this are given in Fig. 4. A list of 'K_i' values, demonstrating the effects of a number of substrates on succinate accumulation, and of succinate on the accumulation of a number of other substrates, is given in Table II. In all cases the inhibition was competitive within experimental error.

Since it has been suggested that uncouplers inhibit substrate utilization by inhibition of their entry into the mitochondrion[14,15,7] the effect of 2,4-dinitrophenol on the uptake of the different substrates was determined. Fig. 5 demonstrates that uptake of citrate is indeed inhibited competitively by the uncoupler. The K_i for 2,4-dinitrophenol is 0.18 mM in this experiment. Similar experiments were performed with malate, malonate, pyruvate, α-oxoglutarate and succinate. The K_i for 2,4-dinitrophenol was similar in all cases, varying between 0.10 and 0.18 mM.

In a following publication it will be shown that also the uptake of labelled uncoupler by mitochondria (*cf.* ref. 16) is inhibited competitively by several substrates[17].

TABLE II

COMPETITION BETWEEN DIFFERENT SUBSTRATES FOR ACCUMULATION IN RAT-LIVER MITOCHONDRIA

Incubation conditions as described under Fig. 2. The competing substrate was added at a concentration of 5 mM.

Substrate	K_m* (*mM*)	K_i *of substrate on succinate accumulation* (*mM*)	K_i *of succinate on substrate accumulation* (*mM*)
Malate	1.7	1.1	3.0
Malonate	1.3	1.1	2.6
α-Oxoglutarate	2.5	2.2	1.9
Butylmalonate	—	1.1	—
Fumarate	—	1.5	—
Citrate	1.3	1.5	5.5
Isocitrate	—	1.0	—
β-Hydroxybutyrate	4.5	3.5	—
Glutamate	4.5	3.5	—
Aspartate	—	3.3	—
ATP	—	8.8	—
Phosphate	—	1.0	—

* See also Table I.

DISCUSSION

The results presented here show that all anions that can penetrate into mitochondria are accumulated in such a way that, at equilibrium, the internal concentration shows a hyperbolic relationship with the external concentration. At all concentrations tested the internal concentration was much higher than the external concentration, but the extrapolated value of the internal concentration at infinite external concentration is finite. Again, it should be stressed that these results were obtained in the absence of energy by blocking endogenous respiration with rotenone

or antimycin. The simplest explanation for this behaviour is found in a model of a mitochondrion with a fixed number of positive charges available to neutralize the mobile negative charges. This model fits in with the known property that the intramitochondrial K^+ exchanges very slowly with externally added K^+ (ref. 18). It also is supported by the fact that loss of K^+ upon aging is accompanied by the loss of intramitochondrial anions[18,19].

In most cases the intramitochondrial K^+ concentration is somewhat larger than the intramitochondrial substrate concentration, expressed in equiv/per l, at infinite external substrate concentration. This can be interpreted to mean that there are some immobile negative charges inside the mitochondrion, possibly those of proteins, phospholipids and nucleotides[19].

The mutual effects of penetrating anions are also understandable if all anions have to compete for the available pool of positive ions inside the mitochondria. Indeed, the fact that the maximum uptake of mono-, bi- or trivalent anions is approximately the same if expressed as the number of negative charges accumulated is very suggestive for limitation of these uptakes by a common counterion.

It should be kept in mind that the values for intramitochondrial concentrations of substrates mentioned in this paper are all equilibrium values since no difference was found between samples taken at 30 sec or at 90 sec. This may explain why some substrates (*e.g.* phosphate) compete with succinate for accumulation while they have no inhibitory effect on succinate oxidation. Under conditions where succinate is oxidized its steady-state intramitochondrial concentration will be lower than that measured here (*cf.* Fig. 3A of ref. 7 with Fig. 2 of this paper) and will be determined by the balance between the actual rate of entry and the rate of utilization. Moreover, under oxidizing conditions, the metabolic products will act as competitors for the pool of positive ions and thereby further decrease the intramitochondrial substrate concentration.

Under conditions different from those used to obtain the results reported in Table II, QUAGLIARIELLO AND PALMIERI[7] found also that the accumulation of succinate was inhibited by a number of bivalent anions, but, in contrast with our results, no effect of monovalent anions nor any effect of succinate on citrate accumulation was found.

As to the actual rate of uptake of the different anions very little can be said except that it is extremely high in comparison with the rate at which these determinations can be carried out. This was to be expected since the rate of metabolism of substrates, representing a minimum value for their rate of entry, would require the accumulations to be finished in a few seconds at room temperature. Even when measured at 0° the steady-state equilibrium was reached within the shortest experimental time (approx. 10 sec). To obtain true initial rates of uptake, therefore, another technique will have to be developed.

Information concerning the rate of uptake may be obtained indirectly by measurement of steady-state levels of metabolites in respiring mitochondria. In the steady state, the rate of uptake of a substrate will be equal to its rate of oxidation. QUAGLIARIELLO AND PALMIERI[7] have found that, under these conditions, 2,4-dinitrophenol, which both inhibits succinate oxidation[20,21,14] and, as shown above, decreases the accumulation of succinate by the mitochondria, causes a lowering of the steady-state concentration of succinate. This shows convincingly that the inhibition of

succinate oxidation is due to inhibition of the rate of entry of succinate and not of its metabolism (*cf.* ref. 14).

The process of accumulation of anions by mitochondria looks very analogous to an anion-exchange process, where the fixed positive charges are represented by the intramitochondrial cations (mainly K^+). In the experiments described above the mitochondria are initially 'loaded' with endogenous substrates and phosphate, which exchange for the added anions. This exchange was already described by GAMBLE[1], who demonstrated that the accumulation of citrate and malate was accompanied by a release of phosphate from the mitochondria. Originally, energy has been invested to bring these endogenous substrates and K^+ into the mitochondria but since in the steady state the 'leak' of K^+ is very small there is also very little net loss of energy by bringing other anions in. However, by introducing an artificial permeability to K^+, for example through addition of valinomycin, one can either increase or decrease the amount of substrate accumulated depending on the availability of energy (*i.e.* by addition of respiratory substrate or uncoupler, respectively). Since changes in K^+ content are always accompanied by equivalent changes in the volume of the matrix space no changes in the concentration of accumulated substrate can be found (Fig. 3B).

It seems, then, that mitochondria are able to take up anions in such a way that at ordinary external concentrations the intramitochondrial concentration of the anion is higher than that in the surrounding medium. This process involves no readily measurable expenditure of energy in the form of high-energy compounds generated from ATP or respiration. However, the fact that energy is required to increase the internal pool of K^+ *plus* anions (in the presence of valinomycin[22]) lends support to the hypothesis that the pool of intramitochondrial anions, normally fixed by the immobility of the cations, is originally formed and maintained by an energy-requiring process.

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