Uptake of malate dehydrogenase into mitochondria *in vitro*

Some characteristics of the process

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1. It was previously shown [Passarella, Marra, Doonan & Quagliariello (1980) *Biochem. J.* **192**, 649–658] that, when mitochondrial malate dehydrogenase from rat liver is incubated with sulphite-loaded mitochondria from the same source, uptake of the enzyme occurs, as judged by a fluorimetric assay of intramitochondrial enzyme activity. Confirmation of sequestration of the enzyme inside the organelles is provided by its proteinase-resistance after uptake. 2. Enzyme uptake into mitochondria is inhibited by enzyme treatment with mersalyl at concentrations that do not affect its catalytic activity. 3. Enzyme uptake is energy-dependent, as shown by inhibition of the process by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and by antimycin. ATP and oligomycin, on the other hand, both stimulate the process, but stimulation by ATP is inhibited by oligomycin. These results suggest that uptake depends on maintenance of transmembrane ion gradient rather than direct ATP involvement. 4. Measurements of $\Delta\psi$ by means of the 'redistribution signal' probe safranin suggest no dependence of malate dehydrogenase uptake on membrane potential. 5. Comparison of the effects of the ionophores valinomycin, nonactin, gramicidin and nigericin shows that uptake depends on maintenance of a transmembrane pH gradient.

The processes by which mitochondrial proteins synthesized in the cell cytosol are subsequently translocated into mitochondria are currently the subject of intensive investigation (Chua & Schmidt, 1979; Waksman *et al.*, 1980). As one approach to the study of these processes, we have developed model systems in which selective uptake of native mitochondrial isoenzymes into mitochondria *in vitro* can be observed; isoenzymes investigated to date are aspartate aminotransferase (EC 2.6.1.1) (Marra *et al.*, 1977, 1978, 1980) and malate dehydrogenase (EC 1.1.1.37) (Passarella *et al.*, 1980). In the latter case, conditions were established under which the uptake could be observed, but few of the characteristics of the process were reported. The imported malate dehydrogenase is shown here to be proteinase-resistant, uptake into mitochondria is shown to be blocked by modification of the enzyme with mersalyl and the uptake process depends on maintenance of a transmembrane pH gradient. In these respects, the uptake process is identical with that previously reported for aspartate aminotransferase (Marra *et al.*, 1979, 1980; Passarella *et al.*,

1982a), which suggests that the mechanism of transmembrane movement is the same in both cases. This conclusion seems interesting in the light of a possible generalization about the transport mechanism of mitochondrial proteins. In addition we thought it necessary to establish analogies in the transport mechanism of the two enzymes, since studies on competition between the two enzymes for uptake into mitochondria, which may throw more light on the mechanisms involved, may now be carried out.

Materials and methods

Materials

Rat liver mitochondria were isolated and loaded with sulphite as previously described (Passarella *et al.*, 1980). Mitochondrial malate dehydrogenase was purified from rat liver also as described by Passarella *et al.* (1980), and was homogeneous to starch gel electrophoresis at pH 6.2. Oxaloacetic acid, mersalyl (*o*-[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid), ATP, oligomycin, valino-

mycin, nonactin, gramicidin and safranin O were from Sigma; nigericin was a gift from Dr. Rita Casadio (Institute of Botany, University of Bologna, Italy).

Assay of malate dehydrogenase

Enzyme activity was assayed by measurement of the A_{340} decrease in an assay solution (2 ml) containing 0.1 M-triethanolamine/HCl buffer, 2 mM-oxaloacetate and 0.2 mM-NADH at pH 7.5 and 25°C. In all experiments involving use of oxaloacetate, solutions were freshly prepared and kept in ice; no decarboxylation occurred in the time required to complete a set of experiments.

Measurement of uptake of malate dehydrogenase into mitochondria

This was done by a method which measures residual intramitochondrial enzyme activity in sulphite-loaded mitochondria, and the increase in this activity after the mitochondria had been incubated with malate dehydrogenase. Details of the method and conditions under which it provides a reliable measure of uptake of the enzyme are given by Passarella *et al.* (1980). Incubation media used varied, depending on the nature of the experiment. However, repeated controls were carried out during the course of each experiment to verify that no increase in the rate of NADH oxidation occurred owing to oxaloacetate addition in the absence of externally added malate dehydrogenase. Possible leakage of sulphite from the mitochondrial matrix and consequent removal of the endogenous malate dehydrogenase inhibition is therefore ruled out. Constancy of the control was found over 30–90 min.

Details are given in the appropriate Tables and Figures.

Spectrophotometric measurements with safranin

Time-dependent absorbance was recorded with a Beckman DU 8 scanning spectrophotometer at a single wavelength, 520 nm. The safranin response to the malate dehydrogenase addition was also tested with measurements carried out with a dual-wavelength spectrophotometer (Hitachi-Perkin-Elmer 557) at the wavelengths 520–544 nm. The results, however, were the same as those obtained with the Beckman DU 8 spectrophotometer.

Results and discussion

Measurement of intramitochondrial enzyme activity and of enzyme uptake

The method used involves addition of oxaloacetate to a mitochondrial suspension and observation of the rate of decrease of fluorescence of NADH subsequent to reduction of the imported

substrate. Use must be made of mitochondria loaded with sulphite, in which the endogenous malate dehydrogenase is partially inhibited, so that entry of substrate into the organelles is not rate-limiting. A detailed examination of the conditions under which the rate of change of fluorescence gives a true measure of the residual intramitochondrial enzyme activity has been reported by Passarella *et al.* (1980). A typical time course for the reaction is shown in Fig. 1. Addition of purified mitochondrial malate dehydrogenase to the suspension 1 min before addition of oxaloacetate (trace B) resulted in an increase of the rate of change of fluorescence, owing to enzyme uptake into the organelles (Passarella *et al.*, 1980).

Proteinase-resistance of the internalized enzyme

One of the most widely accepted criteria for demonstration of sequestration of proteins into organelles is proteinase-resistance of the imported protein (Maccacchini *et al.*, 1979). Although not done in our previous work, it is reported here to provide further evidence that the assay method used does indeed measure internalized enzyme. Under the conditions described in Fig. 1, addition of enzyme to the mitochondrial suspension resulted in an increase of 54% in rate of change of fluorescence (compare traces A and B). Preincubation of malate dehydrogenase with Pronase for 30 s before addition to the mitochondrial suspension abolished this increase in rate of change of fluorescence (Fig. 1, trace C). This was as expected, since in a control experiment (results not given in detail) incubation of malate dehydrogenase with Pronase in the same medium and at the same concentrations as in Fig. 1 resulted in complete loss of enzymic activity within 30 s. As a further control it was shown (trace E, Fig. 1) that Pronase added alone to the mitochondrial suspension had no significant effect on the rate of change of fluorescence. The central observation is contained in trace D (Fig. 1). When mitochondrial malate dehydrogenase was added to mitochondria 30 s before Pronase and a further 30 s was allowed to elapse before addition of oxaloacetate, the rate of change of fluorescence was then increased by 54% with respect to the control, that is, by the same amount as was observed when malate dehydrogenase but not Pronase was added to the suspension (Fig. 1, curve B). Precisely the same results were obtained when a mixture of trypsin and chymotrypsin was used instead of Pronase (results not shown).

These observations confirm that the increased rate of NADH oxidation observed with mitochondria preincubated with malate dehydrogenase is due to internalization of the enzyme. Had the stimulatory effect been due in any way to remaining external enzyme, the stimulation would have been abolished

Fig. 1. *Pronase-resistance of malate dehydrogenase imported into mitochondria*

Sulphite-loaded mitochondria (4.15 mg of protein) were incubated in 2 ml of a medium containing 0.25 M-sucrose, 1 mM-EDTA, 20 mM-Tris/HCl, pH 7.3, and 2 μ g of rotenone. In all cases, oxaloacetate (OAA) was added (final concn. 2 mM) to start the reaction. The change of fluorescence was recorded and the initial rate of change measured from the tangent (dashed line) drawn to the early part of the trace. Rates of change are expressed as a percentage of that of the control reaction (trace A) taken as 100. Other additions were as follows: B, mitochondrial malate dehydrogenase (mMDH, 9.5 μ g) 1 min before OAA; C, hydrolysed enzyme (mMDH hydrol.); the enzyme (9.5 μ g) was preincubated with Pronase (250 μ g) for 30 s before addition of mitochondria, and oxaloacetate was added 30 s later; D, mMDH (9.5 μ g), then Pronase (PRO, 250 μ g) after 30 s, then oxaloacetate after a further 30 s; E, Pronase (250 μ g), then oxaloacetate after 30 s.

by the addition of Pronase after addition of the enzyme to the mitochondria.

There is now evidence which suggests that rat liver mitochondrial malate dehydrogenase is synthesized as a precursor of higher molecular weight than the native enzyme (Aziz *et al.*, 1981), whereas our results show that the mature enzyme retains the ability to pass through the mitochondrial membrane system. Similarly Waksman *et al.* (1980) have also claimed that the enzyme can pass reversibly through the mitochondrial inner membrane in response to changes in composition of incubation medium. As pointed out by Aziz *et al.* (1981), these results are not necessarily in conflict, but leave open the question of the role of the precursor in import of the protein into mitochondria. Even if uptake of native proteins into the organelles is not strictly analogous to the process occurring *in vivo*, it nonetheless provides a convenient model system for the study of some features of the mechanism of protein import into the organelles.

Inhibition of enzyme uptake by treatment with mersalyl

Mitochondrial malate dehydrogenases from mammals contain seven or eight cysteine residues. For the enzyme from pig heart, reaction with mercurials causes activation at low concentration and inacti-

vation at high concentration (for a review see Banaszak & Bradshaw, 1975). Consistently, with the enzyme from rat liver, mersalyl treatment at high concentration (300 μ M) resulted in progressive inactivation over a period of 10 min (Fig. 2). This inactivation appeared to occur in three distinct phases, but insufficient enzyme was available to allow determination of the number of thiol groups modified in these phases. Treatment of the enzyme with a low concentration of mersalyl (10 μ M for 10 s) did not result in a detectable decrease in enzymic activity, but nevertheless the enzyme thus treated was not imported into mitochondria (Table 1). Under the conditions used in this experiment, malate dehydrogenase added externally to the mitochondria caused an increase of 33% in the rate of change of fluorescence observed. When the enzyme was preincubated with mersalyl and then added to the mitochondrial suspension, no stimulation of the rate of change of fluorescence occurred. There was a slight inhibition, however; in fact, when mitochondria were preincubated with mersalyl alone at the same concentration (0.05 μ M), the same inhibition was found as would have been present as a result of addition of the enzyme-mersalyl mixture. Hence it seems clear that the reaction of one (or a small number of) particularly reactive thiol group(s) with mersalyl effectively blocks uptake of the enzyme into

Fig. 2. *Inhibition of enzyme activity by mersalyl* Enzyme (4.8 μ g) was incubated with mersalyl (300 μ M) in a medium containing 0.25 M-sucrose, 1 mM-EDTA and 20 mM-Tris/HCl, pH 7.3. Samples were removed at the time intervals shown and were assayed for enzyme activity. As control a sample of enzyme was incubated under the same conditions, except that mersalyl was omitted. Results for test samples are expressed as a percentage of the activity in the controls.

Table 1. *Prevention of mitochondrial malate dehydrogenase (mMDH) uptake into mitochondria by enzyme treatment with mersalyl*

Mitochondria (1.06 mg of protein) loaded with sulphite were incubated in 2 ml of a medium containing 0.25 M-sucrose, 1 mM-EDTA, 20 mM-Tris/HCl, pH 7.3, and 2 μ g of rotenone. In all cases, oxaloacetate was added to a final concentration of 2 mM 1 min after addition of substances specified to start the reaction and the rate of change of fluorescence was measured. Rates are expressed as percentages of that of the control reaction.

Sample	Relative rate of change of fluorescence (%)
Mitochondria alone	(100)
Mitochondria + mMDH (3.84 μ g)	133
Mitochondria + mersalyl (0.05 μ M)	95
Mitochondria + mMDH (3.84 μ g) preincubated with mersalyl (10 μ M) for 10 s	95

mitochondria. Precisely the same effect was found with aspartate aminotransferase (Marra *et al.*, 1979), but in that case it was possible to show that only one thiol group was involved. It should be noted that, as reported for aspartate aminotransferase,

blockage of accessible thiol groups in the mitochondrial membrane does not influence uptake of malate dehydrogenase, as demonstrated by lack of inhibition of enzyme uptake by the treatment of mitochondria with mersalyl (for experimental details see Marra *et al.*, 1979). Taken as a whole, the results obtained with malate dehydrogenase and aspartate aminotransferase strongly suggest that a thiol group in each protein is directly involved in uptake into mitochondria, perhaps by transient formation of a disulphide bridge with a membrane protein. This hypothesis receives support from the observation that treatment of the organelles with mercaptoethanol also blocks import (Passarella *et al.*, 1982b).

Energy dependence of malate dehydrogenase uptake into mitochondria

Previous evidence (Passarella *et al.*, 1982a) has shown that uptake of mitochondrial aspartate aminotransferase into mitochondria *in vitro* is energy-dependent, and moreover that the process depends on the transmembrane pH gradient rather than on a direct involvement of ATP or on a cation gradient. A distinction between these possibilities was drawn by studying the effect on this process of various oxidation and phosphorylation inhibitors and ionophores. We carried out similar investigations with malate dehydrogenase, extending the study by using the spectrophotometric probe safranine in order to investigate directly any possible use of $\Delta\psi$ (electric potential) as an energy source.

Safranine is a positively charged dye which has been shown to reveal stacking associated with a large spectral shift in energized mitochondria (Colonna *et al.*, 1973). The spectral shift also occurs in the absence of any protonmotive force and depends only on transmembrane $\Delta\psi$ (Åkerman & Wikström, 1976). A possible decrease of transmembrane $\Delta\psi$ because of the use by the enzyme of this type of energy for transport could be followed by measuring the possible increase in absorbance of safranine plus energized mitochondria, caused by added malate dehydrogenase. As an essential preliminary control we investigated the effect of safranine on the uptake of malate dehydrogenase (Table 2). Safranine, although slightly influencing the decrease of fluorescence caused by the addition of oxaloacetate to the mitochondrial suspension, appears to cause a slight increase in the uptake of added malate dehydrogenase. Thus the experiment reported in Fig. 3 was done in order to follow possible $\Delta\psi$ variation owing to the added enzyme by means of safranine. In agreement with previous findings (Åkerman & Wikström, 1976; Colonna *et al.*, 1973), the addition of succinate to coupled mitochondria produces a decrease in safranine absorbance owing to the formation of $\Delta\psi$. The addition of malate dehydrogenase causes no de-

Table 2. Effect of safranin on uptake of malate dehydrogenase into mitochondria

Mitochondria (1.22mg of protein) loaded with sulphite were incubated in 2ml of a medium containing 0.25M-sucrose, 1mM-EDTA, 20mM-Tris/HCl, pH 7.25, and 2µg of rotenone. In all cases oxaloacetate was added to a final concentration of 2mM 1min after addition of substances specified, to start the reaction, and the rate of change of fluorescence was measured. In the calculation of the fluorescence decrease rate we took into consideration the change in fluorescence owing to the addition of safranin alone. Rates (*v*) are expressed as percentages of that of the control reaction. *v*_{saf} indicates the rate of decrease of fluorescence in the presence of safranin. Abbreviation; mMDH, mitochondrial malate dehydrogenase.

Sample	Relative rate of change of fluorescence (%)	
	<i>v</i>	<i>v</i> _{saf}
Mitochondria alone	(100)	
Mitochondria + mMDH (0.75 µg)	127	
Mitochondria + safranin (12.5 µM)	90	100
Mitochondria + mMDH (0.75 µg) preincubated with safranin (12.5 µM) for 1 min	128	142

Fig. 3. Kinetics of the safranin absorbance change induced by succinate and the effect of malate dehydrogenase addition

Mitochondria (0.87mg of protein) were incubated at 20°C in 2ml of medium containing 0.25M-sucrose, 1mM-EDTA, 20mM-Tris/HCl, pH 7.3, 12.5µM-safranin and, when indicated, 1mM-succinate (SUCC), malate dehydrogenase (mMDH) (1.5µg) and 2µM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added.

crease in $\Delta\psi$: on the contrary, little decrease of absorbance at 520nm, i.e. $\Delta\psi$ increase, is found. As a final control the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to collapse the transmembrane potential. It should be

noted that the addition of succinate to the mitochondria produces an increase in the aspartate aminotransferase uptake (results not shown), whereas investigation could not be made of the effect of this substrate on the uptake of malate dehydrogenase, because succinate strongly inhibits the rate of decrease in fluorescence caused by added oxaloacetate. In another experiment (result not shown) the increase of $\Delta\psi$ was achieved by adding valinomycin to mitochondria in a K⁺-free medium (Åkerman & Wikström, 1976). The decrease in absorbance obtained did not change after the addition of malate dehydrogenase. Results reported clearly show that safranin can be used to follow possible change in $\Delta\psi$ owing to the addition of the enzyme, given that by itself it inhibits neither the activity (results not shown) nor the permeation of malate dehydrogenase (Table 2). The safranin response has been used to measure the transmembrane $\Delta\psi$ either in mitochondria or in *Escherichia coli* inner-membrane vesicles, and various authors have reported the dependence of the spectral shift in safranin on the diffusion potential of potassium (Åkerman & Wikström, 1976; Zanotti & Azzone, 1980; Huttunen & Åkerman, 1980). Under the experimental conditions used we have verified a linear dependence between safranin response and $\Delta\psi$. The slope was 0.008 $\Delta A/mV$, giving quite a good opportunity to measure small variations in potential. It can thus be concluded that no decrease in $\Delta\psi$ in the 1–2 mV range is found after malate dehydrogenase addition. On the contrary, the small but reproducible decrease in absorbance after the addition of malate dehydrogenase may suggest an increase in $\Delta\psi$ produced by a possible ΔpH (proton gradient) decrease, caused by the addition of the enzyme to the mitochondria. Such an effect has been reported for both P_i , known to enter mitochondria along with protons, and nigericin, which catalyses K⁺/H⁺ exchange (Åkerman & Wikström, 1976). The ability of safranin to give a slight but reproducible increase in the malate dehydrogenase uptake (Table 2) also favours a ΔpH -dependent enzyme uptake. In fact, it should be noted that the addition of low concentrations of safranin to mitochondria causes an activation of respiration and an ejection of H⁺ from the matrix (Colonna *et al.*, 1973). Similar results were obtained when measurements were performed of safranin response on addition of aspartate aminotransferase. In the following experiments, we investigated the lack of $\Delta\psi$ involvement and ΔpH -dependent uptake for malate dehydrogenase as well as the role of ATP as an energy source in this process. This was done by using various oxidation and phosphorylation inhibitors and ionophores. The results are summarized in Table 3. In each case, the stimulatory effect on the rate of change of fluorescence caused by externally added enzyme was measured [columns

Table 3. *Effects of oxidation and phosphorylation inhibitors and ionophores on uptake of malate dehydrogenase into mitochondria*

Mitochondria (sulphite-loaded) were suspended in one of the media given below. All media contained 1 μ g of rotenone/ml. Additions of substances were made in the order and at the times shown. Immediately after addition of oxaloacetate (OAA; final concn. 2 mM), the initial rate of change of fluorescence was measured. Results are expressed as a percentage of the rate of the control with only oxaloacetate added [column (1)]. A second type of control was used in which mitochondria were incubated with the test substance before addition of oxaloacetate [column (3)]. When the rate observed in this case differs significantly from that in the control without added test substance, results in columns (4) and (5) are given relative to the controls of both column (1) and column (3), with the latter in parentheses. Abbreviations: mMDH, mitochondrial malate dehydrogenase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Assay media were as follows: medium A, 0.25 M-sucrose/1 mM-EDTA/20 mM-Tris/HCl (pH 7.25); medium B, 100 mM-KCl/1 mM-EDTA/20 mM-Tris/HCl (pH 7.00); medium C, 120 mM-KCl/1 mM-EDTA/5 mM-Tris/HCl (pH 7.25).

Quantity of mitochondrial protein (mg/ml)	Test substance and quantity	Quantity of mMDH (μ g/ml)	Assay medium	Order of additions to mitochondrial suspension and relative rate of change of fluorescence (%)				
				(1) Mit ↓ OAA	(2) Mit ↓ mMDH ↓ 30 s OAA	(3) Mit ↓ test substance ↓ 1 min OAA	(4) Mit ↓ test substance ↓ 1 min mMDH ↓ 30 s OAA	(5) Mit ↓ mMDH ↓ 1 min test substance ↓ 1 min OAA
0.60	FCCP (1 μ M)	1.05	A	100	125	100	100	119
1.60	Antimycin (0.53 μ g/ml)	0.53	A	100	133	144 (100)	156 (108)	180 (125)
0.7	Valinomycin (0.05 μ g/ml)	0.75	B	100	122	90 (100)	167 (185)	146 (161)
0.67	Nonactin (0.28 μ g/ml)	1.85	C	100	130	109 (100)	175 (160)	129 (118)
1.25	Gramicidin (0.25 μ g/ml)	0.93	C	100	112	124 (100)	215 (173)	137 (110)
1.23	Nigericin (0.28 nmol/ml)	0.93	B	100	129	140 (100)	144 (103)	175 (125)

(1) and (2)], as was the effect of the test substance on the assay of intramitochondrial enzyme activity [column (3)]. The values in column (4) then show the effect of the test substance on uptake of mitochondrial malate dehydrogenase into the organelles and, as a final control, the effect of the test substance on the observed rate of change of fluorescence after import of the enzyme was determined [column (5)]. In cases where the test substance affected the rate of change of fluorescence in the absence of external enzyme [that is, where the values in columns (1) and (3) are different], then the values in columns (4) and (5) are expressed as percentages of those in both columns (1) and (3), the latter values being given in parentheses. The origins of the stimulatory effects of some of the test substances on the assay of

intramitochondrial enzyme activity are not clear. They are not due to direct effects on enzyme activity, since assays of malate dehydrogenase in the presence and absence of the test substances under the same conditions as those in Table 1 showed the substances either to be without effect or to be slightly inhibitory (results not shown in detail). However, by use of the values in column (3) as controls, the effects of the test substances on uptake could be assigned with confidence. Controls were performed to exclude any possible increase in NADH oxidation caused by removal of sulphite inhibition of intramitochondrial malate dehydrogenase (see the Materials and methods section). The amount of externally added malate dehydrogenase used was such as not to saturate the uptake process (Passarella *et al.*, 1980), since effects of the test

Table 4. *Effect of oligomycin and ATP on the uptake of malate dehydrogenase into sulphite-loaded mitochondria*
Mitochondria (1.22 mg of protein) were incubated in 2 ml of a medium containing 250 mM-sucrose/1 mM-EDTA/20 mM-Tris/HCl (pH 7.25) and 2 μ g of rotenone. Substances added at the times shown were oxaloacetate (OAA, final concn. 2 mM), ATP (final concn. 1 mM), mitochondrial malate dehydrogenase (MDH, 0.75 μ g) and oligomycin (OLIG, 2 μ g). The rates of change of fluorescence (v) are given relative to that observed when only OAA was added to the suspension, taken as 100 in arbitrary units. The columns labelled v_{OLIG} , v_{ATP} , $v_{\text{ATP+OLIG}}$ and $v_{\text{OLIG+ATP}}$ show the relative rates in the absence and presence of external enzyme in assay systems containing the substances specified added in the order stated.

Additions						v	v_{OLIG}	V_{ATP}	$V_{\text{ATP+OLIG}}$	$v_{\text{OLIG+ATP}}$
+ 1 min	+ 30 s	+ 30 s	+ 30 s	+ 30 s	+ 30 s					
OAA	—	—	—	—	—	100				
MDH	OAA	—	—	—	—	127				
OLIG	—	OAA	—	—	—	82	(100)			
OLIG	—	MDH	OAA	—	—	114	139			
ATP	—	OAA	—	—	—	87		(100)		
ATP	—	MDH	OAA	—	—	122		139		
ATP	—	OLIG	—	OAA	—	79			(100)	
ATP	—	OLIG	—	MDH	OAA	140			177	
OLIG	—	ATP	—	OAA	—	87				(100)
OLIG	—	ATP	—	MDH	OAA	127				145

substances would otherwise have been obscured. It should also be noted that the stimulation of rate of change of fluorescence on addition of external enzyme varied somewhat from one mitochondrial preparation to another.

The uncoupler of oxidation and phosphorylation, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, completely inhibits uptake of malate dehydrogenase into mitochondria (Table 3). This could be interpreted in terms of either depletion of mitochondrial ATP or dissipation of ion gradient (ΔpH and $\Delta\psi$ in the terminology of Mitchell, 1968). Antimycin, an inhibitor of electron transport at coupling site II, is also a consistent inhibitor of uptake. In this case the test substance itself stimulated the rate of change of fluorescence [Table 3, column (3)], but no further stimulation was observed if enzyme was added to the mitochondria after addition of the antimycin [column (4)], but addition of antimycin after malate dehydrogenase [column (5)] resulted in a greater stimulation than with antimycin alone. The inhibition by antimycin in the presence of rotenone-treated mitochondria suggests that site-II-linked endogenous substrates are the effective energy source for the uptake process. Taken as a whole, these results show a nearly complete inhibition of the uptake process by antimycin, which may again be interpreted in terms of inhibition of either ATP generation or transmembrane potential by substrate oxidation.

A distinction between these two possibilities is provided by the results in Table 4. Oligomycin, which inhibits ATP synthesis but not certain other processes which depend on generation of trans-

membrane ion gradient, stimulates enzyme uptake into mitochondria. ATP is also stimulatory, and the effects of ATP addition followed by oligomycin seem to be additive. However, when oligomycin is added before ATP the effect of the latter appears to be abolished, i.e. the stimulatory effect is not greater than when only oligomycin is added. These results argue strongly for ATP involvement in the uptake process via the formation of an ion gradient rather than acting by direct phosphorylation of any component of the system (Passarella *et al.*, 1982a). This observation is in contrast with the conclusion reached by Nelson & Schatz (1979).

To elucidate further the nature of the transmembrane gradient involved in the uptake process, two different types of ionophores were used: valinomycin, nonactin and gramicidin, which dissipate the mitochondrial transmembrane potential, and nigericin, which eliminates the pH gradient but increases membrane potential (Henderson *et al.*, 1969; Quagliariello & Palmieri, 1970; Moore & Pressman, 1964; Laris *et al.*, 1975; Johnson *et al.*, 1981). Results are given in Table 3.

At high concentration of K^+ (100 mM), valinomycin and nonactin increase the ΔpH value, but decrease $\Delta\psi$, resulting in a pronounced stimulation of enzyme uptake. Gramicidin, which similarly increases ΔpH , while decreasing $\Delta\psi$ (Chappell & Crofts, 1965) also stimulated enzyme uptake; that is, the increase in rate of change of fluorescence owing to added external enzyme was much greater in the presence of the ionophores than in their absence.

Nigericin catalyses an electroneutral exchange of

K^+ and H^+ , hence resulting in collapse of the pH gradient while not decreasing $\Delta\psi$. The effect of its presence on enzyme uptake into mitochondria is shown in Table 3. The ionophores had a stimulatory effect on the assay of intramitochondrial enzyme activity, but these stimulations underwent no further increase when enzyme was added externally after addition of nigericin [column (4)], whereas addition of nigericin after the enzyme resulted in additive stimulatory effects. These results show that nigericin effectively blocks enzyme uptake into mitochondria.

Hence the effects of both oxidation and phosphorylation inhibitors and ionophores on the uptake of malate dehydrogenase were precisely the same as those obtained previously with aspartate aminotransferase and, as argued in detail previously (Passarella *et al.*, 1982a), show that uptake of these enzymes is dependent on maintenance of a transmembrane pH gradient.

These conclusions are in agreement with the results of the experiment with safranin.

The uptake process for the two enzymes shows other similarities, in particular that it is inhibited by blockage of one or a small number of reactive thiol groups with mersalyl (the present work and Marra *et al.*, 1979). It seems reasonable to suppose therefore that uptake of these two enzymes occurs by a common pathway and hence that studies of the competition between them for uptake into the organelles should be a valuable approach to elucidating the mechanism involved.

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