The entrapment of the Ca^{2+} indicator arsenazo III in the matrix space of rat liver mitochondria by permeabilization and resealing

Na^+ -dependent and -independent effluxes of Ca^{2+} in arsenazo III-loaded mitochondria

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The permeabilization–resealing technique [Al-Nasser & Crompton, *Biochem. J.* (1986) 239, 19–29] has been applied to the entrapment of arsenazo III in the matrix compartment of rat liver mitochondria. The addition of 10 mM-arsenazo III to mitochondria permeabilized with Ca^{2+} partially restores the inner-membrane potential ($\Delta\psi$) and leads to the recovery of 3.9 nmol of arsenazo III/mg of protein in the matrix when the mitochondria are washed three times. The recovery of entrapped arsenazo III is increased 2-fold by 4 mM- Mg^{2+} , which also promotes repolarization. ATP with or without Mg^{2+} decreased arsenazo III recovery. Under all conditions, less arsenazo III than [^{14}C]sucrose is entrapped, in particular in the presence of ATP. The amount of arsenazo III entrapped is proportional to the concentration of arsenazo III used as resealant, and is equally distributed between heavy and light mitochondria. Arsenazo III-loaded permeabilized and resealed (PR) mitochondria develop $\Delta\psi$ values of 141 ± 3 mV. PR mitochondria retain arsenazo III and [^{14}C]sucrose for more than 2 h at 0 °C. At 25 °C, and in the presence of Ruthenium Red, PR mitochondria lose arsenazo III and [^{14}C]sucrose at equal rates, but Ca^{2+} efflux is more rapid; this indicates that Ca^{2+} is released by an Na^+ -independent carrier in addition to permeabilization. The $\text{Na}^+/\text{Ca}^{2+}$ carrier of PR mitochondria is partially (60%) inhibited by extramitochondrial free Ca^{2+} stabilized with Ca^{2+} buffers; maximal inhibition is attained with 2 μM free Ca^{2+} . A similar inhibition occurs in normal mitochondria with 3.5 nmol of matrix Ca^{2+} /mg of protein, but the inhibition is decreased by increased matrix Ca^{2+} . The data suggest the presence of Ca^{2+} regulatory sites on the $\text{Na}^+/\text{Ca}^{2+}$ carrier that change the affinity for matrix free Ca^{2+} .

INTRODUCTION

In the preceding paper it was shown that matrix Ca^{2+} permeabilizes liver mitochondria to sucrose in a reversible manner (Al-Nasser & Crompton, 1986). Although the process itself is complex, and possibly involves two sites of action of Ca^{2+} , an outstanding feature is that mere removal of Ca^{2+} is sufficient for the mitochondria to reseal spontaneously, so that the experimental procedure of permeabilization and resealing is remarkably simple. Such permeabilized and resealed (PR) mitochondria apparently contain normal amounts of matrix proteins, retain entrapped [^{14}C]sucrose very tightly, and develop high values of $\Delta\psi$ with succinate as respiratory substrate. On the other hand, PR mitochondria lack endogenous matrix Ca^{2+} buffering power and probably lack low- M_r matrix constituents generally, and they are not, therefore, strictly mitochondria. They may be termed 'mitosomes', although the abbreviated term 'PR mitochondria' is used in the present study. PR mitochondria preloaded with appropriate substances may prove to be of great utility in investigating intramitochondrial processes.

Ca^{2+} transport in liver mitochondria is poorly understood in comparison with transport in mitochondria from many other sources, notably heart. One of the outstanding problems is whether liver mitochondria

possess a specific Ca^{2+} carrier that actively extrudes Ca^{2+} in the absence of Na^+ . Although many investigators have proposed such a system (Akerman, 1978; Fiskum & Lehninger, 1979; Tsokos *et al.*, 1980; Nicholls, 1978; Nicholls & Crompton, 1980; Bernardi & Azzone, 1983), others consider the evidence ambiguous [Haworth & Hunter, 1979; Gunter *et al.*, 1983; the preceding paper (Al-Nasser & Crompton, 1986); the present Discussion section]. A second major question is whether the $\text{Na}^+/\text{Ca}^{2+}$ carrier of liver mitochondria possesses external Ca^{2+} regulatory sites, as proposed for heart mitochondria (Hayat & Crompton, 1982, 1985). It has been suggested that these sites markedly influence the relay properties of the Ca^{2+} cycle in heart mitochondria in relation to the regulation of oxidative metabolism by matrix free Ca^{2+} (Crompton, 1985a,b). Recent work indicates that the Ca^{2+} cycle of liver mitochondria may perform an equivalent role in the regulation of oxidative metabolism (McCormack, 1985a,b), and the existence or otherwise of Ca^{2+} regulatory sites in the liver mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ carrier is fundamental to the further examination of this concept. Arsenazo III-loaded PR mitochondria are ideally suited to investigating these two questions. Their use in the present study indicates that liver mitochondria possess both an Na^+ -independent Ca^{2+} -transport system and external Ca^{2+} regulatory sites on the $\text{Na}^+/\text{Ca}^{2+}$ carrier.

Abbreviations used: $\Delta\psi$, mitochondrial inner-membrane potential; PR, permeabilized and resealed; TPP^+ , tetraphenylphosphonium ion; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*NNN*-tri-acetic acid.

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METHODS

Mitochondrial preparation and protein assay

Mitochondria were prepared from female Sprague-Dawley rat liver and their protein content was determined as described in the preceding paper (Al-Nasser & Crompton, 1986). The biuret-based assay for protein was modified slightly in determinations with arsenazo III-loaded PR mitochondria to take account of the contribution by arsenazo III to the absorbance at 540 nm. In this procedure, A_{540} was measured before colour development with CuSO_4 ; an amount of arsenazo III was then added to the blank cuvette to give the same absorbance. CuSO_4 was then added to both cuvettes. In those experiments that required low endogenous Ca^{2+} (Fig. 8 below), 1 mM-EGTA was added to the medium used to wash the first mitochondrial pellet (as described previously; Hayat & Crompton, 1982). This decreased the endogenous Ca^{2+} content of the mitochondrial preparation from 9–14 to 2–3 nmol of Ca^{2+} /mg of protein.

Mitochondrial permeabilization and resealing: preparation of arsenazo III-loaded PR mitochondria

In the standard procedure, mitochondria were incubated in 11–16 ml of standard permeabilization medium (10 mg of mitochondrial protein/ml) containing 120 mM-KCl, 10 mM-Tris/Hepes, 2.5 mM- KH_2PO_4 and 1 μg of rotenone/mg of protein for 5 min at 25 °C; the final pH was 7.0. Sufficient CaCl_2 to yield a total (added plus endogenous) of 35 nmol of Ca^{2+} /mg of protein and 3 mM-succinate were added. In the standard resealing procedure, 10 mM-arsenazo III (potassium salt, pH 7.0) was added 7–8 min later. After a period of 3 min, the incubation mixture was diluted to 80 ml with ice-cold medium containing 210 mM-mannitol, 70 mM-sucrose and 10 mM-Tris/HCl, pH 7.2. The suspension was centrifuged at 7800 *g* for 8 min at 2–4 °C. The pellet was washed twice, each time by resuspension in 80 ml of the mannitol/sucrose/Tris medium followed by centrifugation. After the final centrifugation the mitochondrial pellet was deep purple, characteristic of the arsenazo III- Ca^{2+} complex, and little or no colour was visible in the supernatant. Mitochondria permeabilized and resealed by arsenazo III are designated 'arsenazo-loaded PR mitochondria'. In some experiments, [^{14}C]sucrose was entrapped together with arsenazo III by introducing about 1 μCi of [^{14}C]sucrose into the incubation mixture 20 s before resealing.

'Heavy' and 'light' PR mitochondrial fractions (Table 2 below) were prepared by re-centrifuging the PR mitochondria at 2800 *g* for 3 min (r_{av} , 7 cm) to yield a heavy mitochondrial pellet. The 'light' mitochondria were sedimented from the supernatant by centrifugation at 7800 *g* for 8 min (r_{av} , 7 cm).

Determination of the contents of arsenazo III, [^{14}C]sucrose and Ca^{2+} in PR mitochondria

Arsenazo III-loaded PR mitochondria (containing about 2 mg of protein) were suspended in 1.4 ml of medium containing 120 mM-KCl, 10 mM-Tris/Hepes, pH 7.2, 2% Triton X-100 and 400 μM - CaCl_2 (sufficient to saturate arsenazo III). The arsenazo III content was calculated from the change in absorbance at 650–680 nm on addition of 10 mM-EGTA.

The Ca^{2+} content was determined by substituting

arsenazo III (to give 100 μM total, added plus entrapped) in place of CaCl_2 . These measurements were corrected for the Ca^{2+} present in the assay medium. The Ca^{2+} content of arsenazo III-loaded PR mitochondria sedimented from incubations containing 50 μM -EDTA (Fig. 5 below) were measured in the same way, except that 20 μM - MgCl_2 was included to displace Ca^{2+} from the residual EDTA in the assay (about 1 μM). Absorbances were measured with a Perkin-Elmer model 356 dual-wavelength spectrophotometer.

The [^{14}C]sucrose contents of the cuvettes were determined by liquid-scintillation spectrometry.

Enzyme assays

Succinate oxidation was measured polarographically in 2 ml of medium (pH 7.2) containing 120 mM-KCl, 5 mM-Tris/Hepes, 2 mM- KH_2PO_4 , 3 mM-succinate, 50 μM -cytochrome *c*, 6 μg of rotenone, 1 μM -carbonyl cyanide *m*-chlorophenylhydrazide and 6 mg of mitochondrial protein. Glucose-6-phosphatase was assayed in 2 ml of medium containing 100 mM-KCl, 10 mM-Tris/Hepes, pH 7.0, 2 mM- MgCl_2 and 3 mM-glucose 6-phosphate. The reaction was started by addition of a sample containing about 0.2 mg of protein. Samples were withdrawn at intervals for the measurement of P_i by the method of Stewart (1974). Controls were performed in the absence of glucose 6-phosphate. All assays were conducted at 25 °C.

Measurements of Ca^{2+} fluxes and arsenazo III release in PR mitochondria

Arsenazo III-loaded PR mitochondria were incubated at 25 °C at a concentration of 1–5 mg of mitochondrial protein/ml in standard reaction medium, pH 7.0, containing 120 mM-KCl, 10 mM-Hepes (potassium salt), 0.17 mM- KH_2PO_4 and 1 μg of rotenone/mg of mitochondrial protein. Other particulars are given in the legends. The Ca^{2+} /EGTA and Ca^{2+} /HEDTA buffers used were calibrated as described in the preceding paper (Al Nasser & Crompton, 1986). Changes in absorbance were measured with a Perkin-Elmer dual-wavelength spectrophotometer (model 356).

Na^+ / Ca^{2+} -carrier assay in normal liver mitochondria

Ca^{2+} -depleted liver mitochondria containing 5 mg of protein were incubated at 25 °C in 4.5 ml of standard reaction medium (see above). When the efflux of endogenous Ca^{2+} was complete (6–9 min), CaCl_2 (0–65 nmol), 50 nCi of $^{45}\text{Ca}^{2+}$, 50 nCi of $^3\text{H}_2\text{O}$, 3 mM-succinate and 33 μM -ATP (potassium salt) were added. Ruthenium Red (10 nmol) was added 1 min later when Ca^{2+} accumulation was complete. Four 1 ml samples were transferred to Eppendorf tubes and the following additions were made simultaneously with stirring: none (tube 1), 6 mM-NaCl (tube 2), 2 mM-EGTA (tube 3), and 2 mM-EGTA plus 6 mM-NaCl (tube 4). The tubes were centrifuged simultaneously 20 s later in an Eppendorf bench centrifuge for 2 min. As discussed in the preceding paper (Al-Nasser & Crompton, 1986, Methods section), this centrifugation-stop technique effectively stops transport 14 s after centrifugation has begun. The $^{45}\text{Ca}^{2+}$ and $^3\text{H}_2\text{O}$ contents of the supernatants were determined. The $^3\text{H}_2\text{O}$ contents were used to correct for small errors in pipetted volume. The Na^+ -dependent rates of $^{45}\text{Ca}^{2+}$ efflux in the presence and absence of EGTA were calculated from the supernatant $^{45}\text{Ca}^{2+}$ contents of tube

4 minus tube 3 and of tube 2 minus tube 1 respectively. It is important to note that this procedure gives the Na^+ -dependent Ca^{2+} efflux corrected for Na^+ -independent Ca^{2+} efflux. In all experiments the initial efflux of endogenous Ca^{2+} (before succinate) and the subsequent accumulation of Ca^{2+} were measured with a Ca^{2+} -selective electrode to determine the specific radioactivity of the Ca^{2+} loaded and the extramitochondrial free Ca^{2+} at the beginning of $^{45}\text{Ca}^{2+}$ efflux.

Determination of $\Delta\psi$

$\Delta\psi$ was measured and calculated as described in the preceding paper (Al-Nasser & Crompton, 1986), taking into account the binding of TPP^+ to mitochondrial membranes and using $^3\text{H}_2\text{O}$ and $[\text{U-}^{14}\text{C}]\text{sucrose}$ to determine matrix volumes.

RESULTS

Resealing of permeabilized mitochondria with arsenazo III; entrapment of arsenazo III

In Fig. 1 the repolarization of permeabilized mitochondria is used as an index of resealing. The addition of 10 mM-arsenazo III to mitochondria permeabilized by Ca^{2+} in the standard manner (Al-Nasser & Crompton, 1986) caused partial repolarization (Fig. 1a).

Inclusion of 4 mM- MgCl_2 markedly improved arsenazo III-induced repolarization (Fig. 1b, curve i). Other experiments showed that Mg^{2+} was fully effective at 4 mM and half-maximally effective at about 1 mM. The further inclusion of ATP had no observable effect (Fig. 1b, curve ii). Increase in $[\text{P}_i]$ from 2.5 mM (curve i; standard conditions) to 5 mM (curve iii) led to pronounced oscillations in mitochondrial TPP^+ content. A possible explanation is that a fraction of the mitochondria underwent in-phase cycles of resealing and permeabilization (see the Discussion section). The behaviour stresses the fact that $[\text{P}_i]$ is fairly critical in obtaining efficient resealing.

Table 1 reports similar experiments in which the PR

Fig. 1. Repolarization of permeabilized mitochondria by arsenazo III

(a) Standard conditions of permeabilization and resealing. Mitochondria (10 mg of protein) were incubated at 25 °C in 1 ml of standard permeabilization medium containing 2.5 mM- P_i (see the Methods section) and 10 mM- TPP^+ . 3 mM-Succinate and Ca^{2+} to yield a total of 35 nmol of Ca^{2+} /mg of protein were added where indicated. 10 mM-Arsenazo III (A_{III} , K^+ salt, pH 7.0) was added 7 min after Ca^{2+} . (b) As (a), except that 4 mM- MgCl_2 was introduced as indicated and the following modifications were made: curve (i), none; curve (ii), 0.1 mM-ATP; curve (iii), 5 mM- P_i .

mitochondria were washed and the entrapment and retention of arsenazo III was measured. ^{14}C Sucrose retention was also measured, since this is a good indication of the resealing efficiency (Al-Nasser & Crompton, 1986) and provides a reference for arsenazo III entrapment. Resealing with arsenazo III alone led to

Table 1. Retention of arsenazo III and ^{14}C sucrose by PR mitochondria prepared by resealing with arsenazo III under varied conditions

In Expts. 1–7, PR mitochondria were prepared and washed by the standard procedure. In all other experiments the standard procedure was modified by addition as indicated of 4 mM- MgCl_2 , 0.5% bovine serum albumin (BSA; both added 20 s before resealing), and 0.1 mM-ATP [added either 20 s before (b), or simultaneously (s) with, the arsenazo III]. The sucrose and arsenazo III concentrations at the time of resealing were 10 mM. In Expts. 1–20, the ^{14}C sucrose was added 20 s before arsenazo III; in Expt. 21, the ^{14}C sucrose was added simultaneously with arsenazo III. The retention data refer to the contents of arsenazo III and ^{14}C sucrose in PR mitochondria washed three times. These data are expressed as means $\pm$ S.E.M. for experiments with either n separate mitochondrial preparations (Expts. 1–19) or n replicates with the same preparation (Expt. 21); in Expt. 20, duplicate determinations are reported.

Expt. no.	Additions	Retention (nmol/mg of protein)		(a)/(b)	n
		(a) Arsenazo III	(b) ^{14}C Sucrose		
1–7	None	3.9 ± 0.4	4.8 ± 0.4	0.81	7
8–12	Mg^{2+}	7.9 ± 0.4	9.7 ± 0.5	0.81	5
13–19	Mg^{2+} , BSA	7.6 ± 1.2	10.8 ± 1.1	0.70	7
20	Mg^{2+}	7.6, 6.8	8.8, 8.8	0.82	2
	Mg^{2+} , ATP(s)	3.9, 4.4	7.6, 8.0	0.53	2
	ATP(s)	2.9, 4.2	7.4, 7.4	0.48	2
21	Mg^{2+}	5.2 ± 0.1	7.6 ± 0.2	0.68	5
	Mg^{2+} , ATP(s)	2.3 ± 0.1	7.6 ± 0.2	0.32	5
	Mg^{2+} , ATP(b)	0.8 ± 0.1	5.0 ± 0.1	0.16	5

Fig. 2. Arsenazo III entrapment with varied [arsenazo III] as resealant

Mitochondria were permeabilized, resealed and washed according to the standard procedure (see the Methods section) except that the [arsenazo III] used as resealant was varied as indicated on the abscissa.

moderate recoveries of entrapped arsenazo III and [^{14}C]sucrose (Expts. 1–7). The recoveries of both substances were doubled in the presence of Mg^{2+} (Expts. 8–12), and these were unchanged by inclusion of bovine serum albumin (Expts. 13–19). The recoveries in the presence and absence of Mg^{2+} may be correlated with the degree of repolarization.

The recovery of entrapped arsenazo III was consistently slightly less than that of [^{14}C]sucrose (19% less in Expts. 1–12). This disparity was increased when ATP was added simultaneously with arsenazo III, irrespective of the presence or absence of Mg^{2+} (Expt. 20). Further experiments were conducted, however, in which [^{14}C]sucrose and arsenazo III were added simultaneously. Under these conditions, also, the recovery of arsenazo was markedly less than that of sucrose when ATP was also added at the instant of resealing (Expt. 21). Introduction of ATP before resealing did decrease

Fig. 3. Accumulation of Ca^{2+} by arsenazo III-loaded PR mitochondria

Arsenazo III-loaded PR mitochondria (50 mg of protein/ml) prepared by the standard procedure were preincubated with 0.5 mM-EGTA at 0 °C for 15 min. A sample (0.14 ml, containing 7 mg of protein) was mixed with 7 ml of standard reaction medium (see the Methods section) containing 2 mM- MgCl_2 and Ca^{2+} /EGTA (5 mM) buffer, yielding 0.15 μM -free Ca^{2+} . The solution was divided equally between test and blank cuvettes. (a) The absorbance difference at 660 nm was measured; 3 mM-succinate was added to the test cuvette. (b) The difference spectra of the following: curve (i), the cuvettes of (a) 3 min after succinate when the absorbance change was complete; curve (ii), 2.5 μM -arsenazo III in standard reaction medium, with 200 μM - Ca^{2+} and 1 mM-EGTA in the test and blank cuvettes respectively; curve (iii), the final supernatant after preparation of the arsenazo III-loaded PR mitochondria (0.14 ml of supernatant/7 ml of standard reaction medium) with 200 μM - Ca^{2+} and 1 mM-EGTA in the test and control cuvettes respectively; curve (iv), the instrument baseline.

[^{14}C]sucrose entrapment (34%), but decreased arsenazo III entrapment to a greater extent (89%).

In summary, it appears that arsenazo III enters permeabilized mitochondria less rapidly than sucrose. Nevertheless, the procedure of permeabilization and resealing with arsenazo III does lead to the entrapment and retention of large amounts of arsenazo III, i.e. 2–8 nmol of arsenazo III/mg of protein with 10 mM-arsenazo II as resealant. It would be predicted that the

Table 2. Distribution of entrapped arsenazo III and [^{14}C]sucrose between 'heavy' and 'light' PR-mitochondrial fractions

'Heavy' and 'light' PR-mitochondrial fractions were prepared, washed and assayed as described in the Methods section. The mitochondria were resealed with 10 mM-arsenazo III in the presence of 10 mM-sucrose and 4 mM- Mg^{2+} . The data are expressed as means $\pm$ S.E.M. of replicates (*n*) from a single mitochondrial preparation.

Parameter	(a) 'Heavy' fraction	(b) 'Light' fraction	(b)/(a)	<i>n</i>
Protein (% of total)	51.7	48.3	—	1
Glucose-6-phosphatase activity (nmol of P_i /min per mg of protein)	4.7 $\pm$ 0.4	16.5 $\pm$ 0.6	3.50	3
Succinate oxidation (nmol of O_2 /min per mg of protein)	71 $\pm$ 2	83 $\pm$ 2	1.17	3
[^{14}C]Sucrose content (nmol/mg of protein)	9.3 $\pm$ 0.6	10.2 $\pm$ 0.8	1.10	5
Arsenazo III content (nmol/mg of protein)	7.3 $\pm$ 0.3	8.4 $\pm$ 0.2	1.15	5

Fig. 4. Retention of arsenazo III and $[^{14}\text{C}]$ sucrose by PR mitochondria under varied conditions

Arsenazo III and $[^{14}\text{C}]$ sucrose were entrapped simultaneously by the standard procedure (see the Methods section). The washed loaded PR mitochondria were incubated (as described below) and, at the time intervals indicated on the abscissae, samples containing 1 mg of protein were centrifuged in an Eppendorf bench centrifuge for 2 min. The pellets were suspended in 50 μl of water and assayed for arsenazo III and $[^{14}\text{C}]$ sucrose as described in the Methods section. The incubation conditions were as follows. Closed symbols (a): the PR-mitochondrial suspension was retained on ice; samples were diluted 50-fold with ice-cold medium containing 120 mM-KCl and 10 mM Tris/Hepes, pH 7.2, and immediately centrifuged as described above. The zero-time samples were diluted and centrifuged 25 min after PR-mitochondrial preparation; subsequent samples were diluted and centrifuged 1 h and 2 h later (lower abscissa); ●, Arsenazo III; ▲, $[^{14}\text{C}]$ sucrose. Open symbols: the PR-mitochondrial suspension was diluted 50-fold with standard reaction medium (KCl/Hepes/rotenone/ P_i ; see the Methods section) plus additions as stated below at zero time. At intervals thereafter (upper abscissa), samples were centrifuged and assayed as described above. The additions were as follows: (a) 2 nmol of Ruthenium Red/mg of protein and 3 mM-succinate; ○, arsenazo III; △, $[^{14}\text{C}]$ sucrose. (b) ▽, Arsenazo III, no addition; □, arsenazo III, 2 nmol of Ruthenium Red/mg of protein and 3 mM-succinate. (a) and (b) were obtained with different PR-mitochondrial preparations.

amount of arsenazo III entrapped could be varied by varying the concentration of arsenazo III used as resealant. This is borne out in Fig. 2, which shows an approximately linear relationship.

Entrapment of arsenazo II in mitochondria

Liver mitochondrial preparations are known to be contaminated with endoplasmic reticulum (Katz *et al.*, 1983) and, although there are no reasons to suppose that the procedure would introduce arsenazo III into endoplasmic reticulum, this aspect was investigated by examining the distribution of recovered arsenazo III between 'heavy' and 'light' subfractions of the mitochondrial fraction (Table 2). The ratio of arsenazo III retained by these two fractions was essentially the same as the ratios of retained $[^{14}\text{C}]$ sucrose and the rates of succinate oxidation, a mitochondrial marker. On the other hand, glucose-6-phosphatase, an endoplasmic-reticulum marker, was distributed largely in the light-mitochondrial fraction. These data are consistent with homogeneous entrapment into mitochondria.

Further evidence that arsenazo III was entrapped in mitochondria is reported in Fig. 3. The addition of succinate to Ca^{2+} -depleted arsenazo III-loaded PR mitochondria induced a progressive increase in absorbance at 660 nm (Fig. 3a) consistent with mitochondrial

Ca^{2+} uptake in response to succinate-generated $\Delta\psi$. When the increase in absorbance was complete (3 min), the difference spectrum ($\pm$ succinate) showed an absorbance peak at 655–660 nm (Fig. 3b, curve i) characteristic of the Ca^{2+} -arsenazo III complex (curve ii). Fig. 3(b) also shows that the difference spectrum ($\pm \text{Ca}^{2+}$) of the supernatant obtained after the final PR mitochondrial wash did not differ detectably from the baseline, which confirms the visual observation that essentially all the arsenazo III in the final pellet was contained within the PR mitochondria.

Retention of arsenazo III by PR mitochondria

In the present study arsenazo III-loaded PR mitochondria were used to assay $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity. It was important, therefore, to assess the capacity of PR mitochondria to retain arsenazo III over the period of time required to conduct all the assays and during the actual assays. Fig. 4(a) (broken lines) shows that arsenazo III was fully retained by PR mitochondria stored on ice for at least 2 h; in the same period negligible loss (7%) of $[^{14}\text{C}]$ sucrose occurred.

Under assay conditions, however (25 $^{\circ}\text{C}$), the contents of both arsenazo III and $[^{14}\text{C}]$ sucrose decreased by 30% in 8 min, but were stable thereafter. Under the same experimental conditions, PR mitochondria resealed with

Fig. 5. Release of Ca^{2+} from arsenazo III-loaded PR mitochondria in the absence of Na^+

Arsenazo III-loaded PR mitochondria were incubated at 25 °C in standard reaction medium (5 mg of mitochondrial protein/ml) containing 20 μM -Ruthenium Red and 3 mM-succinate. After 5 min, 50 μM -EDTA was added and immediately (zero time in the Figure) a sample of the incubation mixture was centrifuged in an Eppendorf bench centrifuge for 2 min. Further samples were centrifuged at intervals thereafter as indicated on the abscissa. The Ca^{2+} and arsenazo III contents of the mitochondrial pellets were determined as described in the Methods section (designated C_0 and A_0 respectively below). The Ca^{2+} release attributable to permeabilization (O) was calculated from the expression:

$$[C_0 - (C_0 \times A_t/A_0)]$$

where the subscripts 0 and t indicate time = 0 and time > 0 respectively. The Ca^{2+} release in excess of that attributable to permeabilization (●) was calculated from:

$$[(C_0 \times A_t/A_0) - C_t]$$

The total Ca^{2+} release was thus the sum of the two curves. The results given are means $\pm$ S.E.M. for 11 separate PR-mitochondrial preparations. The values of C_0 and A_0 were 4.7 ± 0.4 and 3.9 ± 0.4 nmol/mg of protein respectively.

EGTA did not release [^{14}C]sucrose (Al Nasser & Crompton, 1986), which suggests that the release observed under the present conditions was due to some re-permeabilization by intramitochondrial free Ca^{2+} . Other experiments showed that arsenazo III-loaded PR mitochondria incubated under the conditions of Fig. 4(a) developed a $\Delta\psi$ of 141 ± 3 mV (mean $\pm$ S.E.M.; five PR mitochondrial preparations). Arsenazo III release was not detectably affected by coupled respiration (Fig. 4b), indicating that the release was $\Delta\psi$ -independent, consistent with re-permeabilization-dependent release. In practice there was some variation from one preparation to another in the initial rate and extent of arsenazo III release from PR mitochondria. $\text{Na}^+/\text{Ca}^{2+}$ -carrier assays (below) were conducted after a preincubation period when the initial release of arsenazo III was essentially complete.

Na^+ -independent release of Ca^{2+} from arsenazo III-loaded PR mitochondria

A recurring issue (see the Discussion section) is whether Ca^{2+} is released from liver mitochondria via a specific Na^+ -independent transport system or whether non-specific permeabilization is responsible. This question was approached in a novel manner by using arsenazo III release from PR mitochondria as a means to determine how much of the Ca^{2+} released was due to permeabilization. Preliminary experiments established that Ca^{2+} release from arsenazo-loaded PR mitochondria was promoted by introduction of EDTA or EGTA; of these, EDTA was used in preference to allow spectrophotometric determination of PR-mitochondrial Ca^{2+} (see the Methods section). The data (Fig. 5) show that although most of the Ca^{2+} released by PR mitochondria is attributable to non-specific permeabilization, a significant amount of Ca^{2+} was released in excess of this. In addition, the two Ca^{2+} fractions were released according to different time courses. Thus 77% of the extra efflux of Ca^{2+} , but only 45% of the efflux attributable to permeabilization, occurred within 5 min.

It is evident that the Ca^{2+} released via this additional mechanism did not proceed to completion, but ended after 15 min incubation, when about 20% of the total PR-mitochondrial Ca^{2+} had been released, and the reason for this behaviour is unclear. Nevertheless, the efflux of Ca^{2+} in excess of that attributable to permeabilization presumably reflects the existence of an Na^+ -independent Ca^{2+} -transport system. It should be stressed that the extra Ca^{2+} was released from PR mitochondria with a high $\Delta\psi$ (above) and in the presence of sufficient Ruthenium Red to block Ca^{2+} flux via the uniporter in PR mitochondria (Al Nasser & Crompton, 1986).

Effect of external free $[\text{Ca}^{2+}]$ on $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity in PR mitochondria

The capacity of arsenazo III-loaded PR mitochondria to expel Ca^{2+} in an Na^+ -dependent manner was investigated as reported in Fig. 6. The rate of absorbance decreased was increased by EGTA alone and increased further in the presence of Na^+ . The increment in rate induced by Na^+ may be attributed to Ca^{2+} efflux via the $\text{Na}^+/\text{Ca}^{2+}$ carrier (considered below). Since this preparation contained 6.3 nmol of Ca^{2+} /mg of protein, the initial rates of Na^+ -independent and Na^+ -dependent Ca^{2+} efflux may be calculated to be 0.6 and 0.7 nmol of Ca^{2+} /min per mg of protein, which are similar to those of normal mitochondria (Haworth *et al.*, 1980; Goldstone & Crompton, 1982). Fig. 6(a) also reports the rapid release of PR-mitochondrial Ca^{2+} by the Ca^{2+} ionophore A23187 in the presence of EGTA, which would give the maximal observable absorbance change with this particular preparation. In general, the maximal possible changes in absorbance (650–680 nm) with different preparations lay in the range 0.007–0.015 with 2 mg of PR-mitochondrial protein/ml and using PR mitochondria sealed with 10 mM-arsenazo III in the absence of Mg^{2+} .

Fig. 6(b) shows the same experiments in the presence of 1.9 μM extramitochondrial free Ca^{2+} . The Na^+ -independent rate was decreased slightly (about 25%) with respect to the rate observed in the absence of extramitochondrial free Ca^{2+} (Fig. 6a). $\text{Na}^+/\text{Ca}^{2+}$ -carrier

Fig. 6. Na^+ -induced release of Ca^{2+} from arsenazo III-loaded PR mitochondria

Arsenazo III-loaded PR mitochondria were incubated at 25 °C in standard reaction medium (2 mg of mitochondrial protein/ml) containing 4 μM -Ruthenium Red and 3 mM-succinate. After preincubation for 7 min, the following additions were made at the arrows: (a) 5 mM-EGTA $\pm$ 6 mM-NaCl or 5 mM-EGTA + 0.4 nmol of A23187; (b) Ca^{2+} /EGTA (5 mM) buffer yielding 1.9 μM -free Ca^{2+} $\pm$ 6 mM-NaCl.

activity (Na^+ -dependent increment in absorbance change) was inhibited more strongly by 1.9 μM free Ca^{2+} (about 60%). The effects of external Ca^{2+} on the Na^+ -dependent and Na^+ -independent processes need to be interpreted cautiously, however. As discussed above, repermeabilization contributes significantly to Na^+ -independent release. Since PR mitochondria contain Ca^{2+} , it is clear that permeabilization-dependent release of arsenazo III and Ca^{2+} into a medium containing EGTA would produce a decrease in arsenazo III absorbance; the same release into a medium containing Ca^{2+} /EGTA buffer (e.g. with 1.9 μM free Ca^{2+}) would produce less absorbance decrease. For this reason, the apparent small inhibition of the Na^+ -independent process by extramitochondrial Ca^{2+} cannot be taken as evidence that external Ca^{2+} inhibits the Na^+ -independent Ca^{2+} -transport system. However, this consideration would only apply to the Na^+ -induced increment in

absorbance change if Na^+ promoted non-specific permeabilization.

Table 3 investigates the effect of Na^+ on permeabilization-dependent release of arsenazo III under similar experimental conditions to those of Fig. 6. In three separate experiments, Na^+ increased the mean rate of arsenazo III absorbance decrease 1.76-fold. In the same experiments, and over the same time period, the amount of arsenazo III released was not significantly changed by Na^+ . Thus the Na^+ -induced increment in absorbance decrease and its inhibition by external Ca^{2+} may be correlated with Na^+ / Ca^{2+} -carrier activity.

The effect of external free $[\text{Ca}^{2+}]$ in PR-mitochondrial Na^+ / Ca^{2+} -carrier activity is given more fully in Fig. 7. The salient feature is that Ca^{2+} inhibited partially, with maximal inhibition (60%) being attained with 2 μM external free Ca^{2+} . This type of inhibition is quite the same as reported previously for the Na^+ / Ca^{2+} carrier of heart mitochondria assayed conventionally with extramitochondrial arsenazo III (Hayat & Crompton, 1982, 1985). The possible physiological significance of this is considered in the Discussion section. For comparison, the apparent effects of extramitochondrial free Ca^{2+} on the Na^+ -independent rates are also included (Fig. 7, broken lines).

Inhibition by external Ca^{2+} of Na^+ / Ca^{2+} -carrier activity in normal mitochondria

It was important to know whether the sensitivity of the Na^+ / Ca^{2+} carrier to external Ca^{2+} was changed by the repermeabilization–resealing procedure. In the experiment of Fig. 8, the Na^+ / Ca^{2+} carrier in normal liver mitochondria was assayed from the amount of $^{45}\text{Ca}^{2+}$ efflux during the initial 34 s after adding Na^+ (see the Methods section). Since intramitochondrial Ca^{2+} changed during this period, the abscissal values are the average contents during efflux; the percentage change in mitochondrial Ca^{2+} was greatest at the lowest matrix Ca^{2+} contents ($\pm 6\%$ of the values given during the course of efflux). At the lowest matrix Ca^{2+} content, 2–4 μM external free Ca^{2+} inhibited Na^+ / Ca^{2+} -carrier activity by 58%, which is similar to the inhibition observed in PR mitochondria. A notable feature, however, is that the degree of inhibition by Ca^{2+} was not constant, but decreased with increased matrix Ca^{2+} to 17% with 15 nmol of Ca^{2+} /mg of protein. Matrix free Ca^{2+} need not be directly proportional to total matrix Ca^{2+} . Nevertheless, if V_{max} is taken to be about 1.7 nmol

Table 3. Changes in intramitochondrial arsenazo III absorbance and content caused by Na^+

The changes in arsenazo III absorbance at 650 minus 680 nm were measured as in Fig. 6(a). The absorbance changes in 5 min were expressed as a percentage of the maximal absorbance change produced by ionophore A23187. The changes in intramitochondrial arsenazo III content were measured in the same incubations and over the same time period by the method described in Fig. 4 and were expressed as a percentage of the arsenazo III contents at the time of addition of EGTA $\pm$ Na^+

Expt.	Decrease in 5 min of arsenazo content or absorbance (%)					
	Absorbance			Content		
	(a) $-\text{Na}^+$	(b) $+\text{Na}^+$	(b)/(a)	(c) $-\text{Na}^+$	(d) $+\text{Na}^+$	(d)/(c)
1	26	50	1.9	32	31	0.97
2	23	36	1.6	27	31	1.15
3	16	29	1.8	10	8	0.80
Mean $\pm$ S.E.M.			1.76 $\pm$ 0.09			0.97 $\pm$ 0.10

Fig. 7. Dependence of $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity in PR mitochondria on the extramitochondrial free $[\text{Ca}^{2+}]$

The $\text{Na}^+/\text{Ca}^{2+}$ carrier was assayed as in Fig. 6. $5.9 \mu\text{M}$ Free Ca^{2+} was obtained with $\text{Ca}^{2+}/\text{HEDTA}$ (5 mM) buffer; the other concentrations of free Ca^{2+} were obtained with $\text{Ca}^{2+}/\text{EGTA}$ buffers and with EGTA. $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity was calculated from the Na^+ -induced increment in the initial rate of absorbance decrease. $\blacktriangle$, $\bullet$, $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity; $\triangle$, $\circ$, the rate of absorbance decrease in the absence of Na^+ . The results are expressed as a percentage of the $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity in the absence of extramitochondrial Ca^{2+} . The results obtained with two separate PR-mitochondrial preparations are shown ($\bullet$, $\circ$ and $\blacktriangle$, $\triangle$).

of $\text{Ca}^{2+}/\text{min}$ per mg of protein, the data indicate K_m values for matrix Ca^{2+} in the presence and absence of external Ca^{2+} of about 7.8 and $2.6 \text{ nmol of Ca}^{2+}/\text{mg of protein}$ respectively. External Ca^{2+} may inhibit the $\text{Na}^+/\text{Ca}^{2+}$ carrier, therefore, by decreasing the affinity of the carrier for internal Ca^{2+} .

DISCUSSION

In the present study, the permeabilization-resealing technique (Al-Nasser & Crompton, 1986) has been applied to the entrapment of the Ca^{2+} indicator arsenazo III. It was necessary to entrap more than $3 \text{ nmol of arsenazo III/mg of mitochondrial protein}$ to ensure acceptable signal/noise ratios in transport assays (e.g. Fig. 6). Since the entrapment of this amount necessitated

Fig. 8. $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity in normal mitochondria with varied matrix Ca^{2+} and in the presence and absence of extramitochondrial free Ca^{2+}

The assays were conducted as described in the Methods section in the presence ($\circ$) or absence of EGTA ($\bullet$, extramitochondrial free $\text{Ca}^{2+} = 2\text{--}4 \mu\text{M}$). The results were obtained with eight different mitochondrial preparations, and each data point represents the mean $\pm$ S.E.M. of the eight determinations. The lateral bars arise since in practice some variations in Ca^{2+} loading occurred. The abscissa values are the averages of the mitochondrial Ca^{2+} contents at the beginning and the end of $^{45}\text{Ca}^{2+}$ efflux.

the addition to permeabilized mitochondria of more than 3 mM -arsenazo III, which, at such concentrations, chelated Ca^{2+} sufficiently well to cause resealing, in practice arsenazo III was used also as resealant.

Mg^{2+} and ATP are known to oppose Ca^{2+} -induced permeabilization (Beatrice *et al.*, 1980; Leblanc & Clauser, 1974; Nicholls & Brand, 1980; Nicholls & Crompton, 1980; Crompton, 1985a). Interestingly, the effects of these substances were quite different. Mg^{2+} markedly increased the recovery of both arsenazo III and sucrose to equal extents (Table 1). It is reasonable to assume that the amount of sucrose recovered reflects the fraction of PR mitochondria in the impermeable form at the instant in time of dilution (before PR-mitochondrial sedimentation). These forms may be represented as shown below, neglecting for the purpose of this discussion the possibility of intermediate forms (Al-Nasser & Crompton, 1986);

Thus it is not unlikely that Mg^{2+} increased entrapment by displacing the reaction(s) to the left; conceivably Mg^{2+} may have decreased the affinity of the permeabilization reactions for Ca^{2+} or, alternatively, inhibition of

mitochondrial Ca^{2+} reuptake, and therefore repermeabilization may have been responsible. Quite unlike Mg^{2+} , ATP caused a selective (with respect to sucrose) restriction of arsenazo III entrapment (Table 1, Expts. 20 and 21). Moreover, this action of ATP did not require Mg^{2+} (Table 1, Expt. 20). This observation cannot be explained by a changed steady-state distribution of mitochondrial forms at the time of dilution, which would affect arsenazo III and sucrose retention equally, as discussed above. Rather, an effect of free ATP on arsenazo III entry at the instant of resealing is indicated, such that the mitochondria resealed with the normal amounts of sucrose, but much less arsenazo III, entrapped. There would seem to be two possible explanations for this. Firstly, as stated above, it does appear that, even in the absence of ATP, arsenazo III enters rather less rapidly than sucrose. If free ATP simply increased the rate of resealing, a selective restriction of arsenazo III entrapment, as observed, would be anticipated. Secondly, the data could also be explained by assuming that free ATP alters the permeability properties of the permeable form such that arsenazo III permeation is selectively limited. Irrespective of which explanation applies, the data do introduce a new dimension to the phenomenon of permeabilization. Mg^{2+} and ATP are typically considered together as promoters of mitochondrial integrity in the presence of Ca^{2+} , and a common biochemical basis for their actions has been proposed, namely in the reacylation of lysophospholipids arising from phospholipase A_2 activity (Broekemeier *et al.*, 1985). The present data, however, although not excluding a requirement for Mg^{2+} and ATP in esterification, do strongly indicate that Mg^{2+} and ATP may also act in additional capacities independently of each other and on separate target processes.

Whereas sucrose entry into permeabilized mitochondria is complete within a few seconds (Al Nasser & Crompton, 1986), sucrose release from PR mitochondria occurred over a period of several minutes (Fig. 4). The release of sucrose and arsenazo III from PR mitochondria, therefore, was presumably severely rate-limited by the impermeable-to-permeable reaction. This would account for the equal rates of release of these two substances but their unequal rates of entry into the permeable form. It follows that the release of such markers would provide an accurate index of the degree to which the release of other matrix components occurred via repermeabilization, provided that the permeation of such a component across the inner membrane of the permeable form was not severely restricted. In such a case, the release of the marker would overestimate the permeabilization-dependent release of the particular component. On the other hand, there are no grounds for suspecting that the use of arsenazo III, for example, as a marker would underestimate the permeabilization-dependent release of other matrix components.

All mitochondria that have been investigated extrude Ca^{2+} in the absence of Na^+ when influx is inhibited by Ruthenium Red. Na^+ -independent Ca^{2+} efflux was first reported by Crompton *et al.* (1976) for liver mitochondria and subsequently attributed to a system distinct from the $\text{Na}^+/\text{Ca}^{2+}$ carrier (Crompton *et al.*, 1979). In mitochondria from some tissues, e.g. heart, the rate of Na^+ -independent Ca^{2+} efflux is negligible with respect to $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity, whereas in mitochondria from some other tissues, notably liver and kidney,

Na^+ -independent Ca^{2+} efflux is highly significant (Crompton *et al.*, 1978; Nicholls & Crompton, 1980).

Correlation of H^+ influx with Ca^{2+} efflux led several investigators to propose $\text{Ca}^{2+}/\text{H}^+$ antiport (Akerman, 1978; Fiskum & Lehninger, 1979; Tsokos *et al.*, 1980), but Gunter *et al.* (1983) have questioned this interpretation on the basis that their $\text{Ca}^{2+}/\text{H}^+$ stoichiometries were highly dependent on extramitochondrial pH. In addition, it seems to us that since respiration-supported pre-accumulation of Ca^{2+} generates a pH gradient across the inner membrane, acid outside, some apparent H^+ uptake would occur during Ca^{2+} release via permeabilization that would eliminate whatever gradients existed. Also the evidence that the Na^+ -independent release occurs by an electroneutral mechanism (Brand, 1985) does not discriminate between carrier-mediated and permeabilization-dependent release of Ca^{2+} . Indeed, Haworth & Hunter (1979) attributed Na^+ -independent Ca^{2+} efflux to non-specific Ca^{2+} -induced permeabilization, and this concept, somewhat modified, has been reiterated by others (Broekemeier *et al.*, 1985).

However, a number of related, seemingly compelling arguments have been presented in support of active, carrier-mediated efflux of Ca^{2+} by an Na^+ -independent mechanism. In brief, these are based on the apparent preservation of $\Delta\psi$ during Ca^{2+} efflux, and behaviour consistent with steady-state Ca^{2+} cycling, namely Ca^{2+} accumulation ratios far from electrochemical equilibrium, and the maintenance of extramitochondrial free Ca^{2+} at a precise level irrespective of large variations in matrix Ca^{2+} content (Nicholls 1978; Nicholls & Scott, 1980; Nicholls & Crompton, 1980). Yet even these arguments would appear to be questioned in the preceding paper (Al-Nasser & Crompton, 1986). Since Ca^{2+} -induced permeabilization is reversible, and is reversed simply by a decrease in matrix free $[\text{Ca}^{2+}]$, one cannot exclude the possibility that mitochondria *in vitro* become permeabilized, lose Ca^{2+} and $\Delta\psi$, but then require inner-membrane impermeability and $\Delta\psi$, and then reaccumulate Ca^{2+} , and so on, as indicated below:

With relatively low amounts of Ca^{2+} , the permeable fraction at any instant would be very low, thereby allowing $\Delta\psi$ to be maintained in the whole population; for example 1% of the total in the permeable form at any instant would decrease measured $\Delta\psi$ in the whole population by about 0.3 mV. Thus the mere maintenance of a reasonable value of $\Delta\psi$ with time in no way precludes continuous Ca^{2+} efflux via permeabilization. It is relevant here to stress the key observation made in the preceding paper (Al-Nasser & Crompton, 1986) that sucrose enters PR mitochondria continuously despite maintenance of $\Delta\psi$ with time. The model above would account for an apparent steady-state distribution of Ca^{2+} across the inner membrane removed from electrochemical equilibrium, and, on addition of Ruthenium Red, for apparent 'active' extrusion of Ca^{2+} at a rate determined by the rate of the impermeable-to-permeable reaction.

Arsenazo III-loaded PR mitochondria offered a novel approach to clarifying this tissue. Arsenazo III release

was used as a marker of permeabilization, and this showed that PR mitochondria released Ca^{2+} in the absence of Na^+ partly via permeabilization and partly via an additional mechanism. In the first 5 min of assay, the additional mechanism accounted for 40% of the Ca^{2+} released. This would seem to provide unequivocal evidence for an Na^+ -independent transport system for Ca^{2+} in liver PR mitochondria. In this connection, Bernardi & Azzone (1983) made the important observation that Na^+ -independent Ca^{2+} release is promoted by increased $\Delta\psi$, a feature that is more readily considered in terms of active Ca^{2+} transport. The relative contribution of carrier-mediated transport and permeabilization to total Na^+ -independent Ca^{2+} efflux in normal liver mitochondria may well differ from the relative contributions in PR mitochondria noted here. Nevertheless, in general, previous studies of the process in normal mitochondria in which the relative contributions were not resolved cannot be easily interpreted in favour of either mechanism (Crompton, 1985b). Unless means are developed of dealing with this ambiguity in normal mitochondria, PR mitochondria would appear to provide a clear advantage in investigating the Na^+ -independent Ca^{2+} -transport system.

A further experimental advantage of arsenazo III-loaded PR mitochondria is in allowing Ca^{2+} transport to be assayed from changes in matrix Ca^{2+} with extramitochondrial free Ca^{2+} maintained at constant, precisely defined, levels. This feature was exploited in investigating whether the $\text{Na}^+/\text{Ca}^{2+}$ carrier of liver mitochondria is subject to regulation by changes in extramitochondrial free Ca^{2+} . The data clearly showed that the liver mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ carrier is inhibited by increasing external free Ca^{2+} over the range 0–2 μM . The partial nature of the inhibition is similar to that observed in heart mitochondria (Hayat & Crompton, 1982, 1985), and a similar interpretation may be applied, namely the presence of external Ca^{2+} regulatory sites on the carrier. Moreover, it seems that occupation of these external regulatory sites by Ca^{2+} is transmitted into a conformational change of the Ca^{2+} (substrate)-binding sites on the matrix side. Previous studies in this laboratory have shown that the $\text{Na}^+/\text{Ca}^{2+}$ carrier of liver mitochondria is activated in mitochondria isolated from livers perfused with glucagon or β -adrenergic agonists (Goldstone & Crompton, 1982; Goldstone *et al.*, 1983). $\text{Na}^+/\text{Ca}^{2+}$ -carrier activation was due to an increase in the apparent affinity of the carrier for matrix Ca^{2+} . The present study suggests a possible mechanism by which such externally arising stimuli might alter carrier conformation on the matrix side, namely by relieving the inhibition by external Ca^{2+} .

Ca^{2+} cycling across the inner membrane of mitochondria from heart, liver and other tissues (Crompton *et al.*, 1976; Nicholls & Crompton, 1980) is currently interpreted as a mechanism by which increases in cytosolic free Ca^{2+} may be relayed into increases in matrix free Ca^{2+} with consequent activation of matrix dehydrogenase (Denton & McCormack, 1980; McCormack, 1985a,b). In our view the significance of the regulation of the $\text{Na}^+/\text{Ca}^{2+}$ carrier by cytosolic free Ca^{2+} lies in amplification of the relay response such that a change in cytosolic free Ca^{2+}

produces a relatively large change in matrix free Ca^{2+} (Hayat & Crompton, 1982; Crompton & Roos, 1985; Crompton, 1985b). Thus an increase in cytosolic free Ca^{2+} would promote net influx by increasing uniporter activity (unidirectional influx), and by inhibiting $\text{Na}^+/\text{Ca}^{2+}$ -carrier activity (unidirectional efflux), and thereby produce a disproportionately large increase in matrix free Ca^{2+} in the new steady state. This would facilitate a sensitive response of oxidative metabolism to changes in cytosolic free Ca^{2+} .

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