Phosphate Transport in Rat Liver Mitochondria

PROPERTIES OF A Ca"+-ACTIVATED UPTAKE PROCESS IN INVERTED INNER MEMBRANE VESICLES

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The mechanisms by which Pi and Ca"+ leave the mitochondrial matrix and re-enter the cytoplasm have been studied in inverted vesicles of inner membrane. Such vesicles have the matrix face of the membrane exposed to the external medium and an internal medium of known composition, and so provide a well defined system for the study of transport in the direction of matrix to cytoplasm.

Energy-dependent uptake of Pi into such inverted inner membrane vesicles (IMV) from rat liver mitochondria is stimulated by the presence of either endogenous or added Ca"+. During Ca"+-stimulated Pi uptake, Ca"+ is taken up into an ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid-inaccessible internal space. Ca"+ uptake is absolutely dependent on the uptake of Pi. In the absence of Pi, Ca"+ added to respiring IMV is not taken up and produces no H"+ ejection or respiratory stimulation. Other anions such as acetate, thiocyanate, nitrate, or bicarbonate cannot replace Pi, even at 5-fold higher concentrations. Inhibition of Pi transport with N-ethylmaleimide completely inhibits Ca"+ uptake.

Uptake of Ca"+ by inverted IMV is completely inhibited by externally added ruthenium red under the conditions used, even after passage of IMV over a cytochrome c-Sepharose affinity column to remove any remaining right-side-out vesicles. Uptake is greatly increased by addition of ATP or ADP in the presence of Mg"+, but not by the ATP analog adeny1-5'-yl imidodiphosphate. This stimulation is not prevented by either oligomycin or carboxyatractyloside. Prior uptake of Pi will support uptake of Ca"+ at least as well as simultaneous uptake, but under these conditions no extra Pi uptake is observed. This makes an obligatory symport mechanism unlikely.

A model which is consistent with the data presented depicts the driving force for Ca"+ uptake as being depression of the interior-positive membrane potential by prior electrophoretic uptake of Pi, coupled with precipitation of complexes of calcium phosphate.

Since the first observations of mitochondrial ion transport, a link has been apparent between accumulation of Pi and Ca"+ (1, 2). The minimum possible interaction is defined by the equilibrium constant for complexation and precipitation of the various forms of calcium Pi. Whether there is, in addition, direct or indirect coupling of Ca"+ and Pi transport has been under study for more than a decade.

Uptake of Pi independent of Ca"+ movement has been characterized extensively in mitochondria. All but a small fraction of the total uptake in liver appears to be coupled to influx of protons via an N-ethylmaleimide- and mercurial-sensitive protein carrier (3). In contrast, uptake of Ca"+ appeared at first to be completely dependent on Pi (1, 2). This substantial energy-dependent uptake has been designated “massive loading” (4). The ability of mitochondria to take up a smaller amount of Ca"+ in the absence of added Pi, “limiting loading” (4, 5), has since received extensive study. This is the classical ruthenium red- and La"+-sensitive electrophoretic carrier-mediated Ca"+ uptake (6-8). A controversy currently exists over the net charge of the mobile Ca"+ with certain authors supporting a fully electrical Ca"+ movement accompanied by ejection of an equivalent amount of charge as H"+ (9-13). In contrast, Moyle and Mitchell have suggested that Ca"+ moves only together with Pi (14, 15) or certain monocarboxylate anions (16), accounting for their observation of an apparent Ca"+ transfer.

Until recently the previously characterized pathways for Ca"+ and Pi uptake were considered to be totally responsible for controlling matrix concentrations of these ions (17, 18). However, with the observation that the Ca"+ (or Mn"+) distribution between matrix and cytosol may not be in equilibrium with the membrane potential (19, 29), the movement of Ca"+ exclusively on a single, reversible, electrophoretic carrier has been questioned. Instead it has been suggested that separate influx and efflux pathways may exist (21-25), which maintain Ca"+ distribution at a steady-state, displaced from thermodynamic equilibrium, but necessary for proper cell function. Recently it has been shown by direct measurement in inverted inner membrane vesicles that in the absence of Ca"+ or ionophores Pi can be driven at the expense of respiratory energy from the matrix to the cytosolic surface of the inner mitochondrial membrane (26). The objective of the current studies was to examine the interaction of Ca"+ with this Pi transport mechanism and to gain information on matrix-to-cytosol Ca"+ movement as well.

Uptake of Ca"+ together with Pi into vesicles of mitochondrial inner membrane has been reported previously by Loyter et al. (27, 28) and by Pedersen and Coty (29), but these results have been criticized because of the possibility of contamination of inverted with noninverted vesicles (30). However, since the existence of a second Ca"+ transport system specialized for efflux is now being considered (21-25), it seemed important to re-evaluate whether Ca"+ can, in fact, be taken up by inverted inner membrane vesicles. A preliminary report by Gunter et al. (31) has described uptake of Ca"+ under conditions where the authors indicate that only inverted vesicles...
would be energized. To attack this problem we have used an inner membrane vesicle preparation from rat liver mitochondria which has been shown previously in this laboratory to be essentially completely inverted, using six different criteria (26). We report here that in these vesicles Ca" stimulates the energy-dependent uptake of P. Further, we demonstrate transport of Ca" from the matrix to the cytosolic surface and describe the properties of this uptake system.

EXPERIMENTAL PROCEDURES

Materials-Adult, male CD albino rats, obtained from Charles River Breeding Laboratories, Wilmington, Mass., were fed ad libitum with a Rockland rat diet purchased from Teklad, Winfield, Ohio. The following chemicals were purchased from the indicated sources: ATP, ADP, and Na" in concentrations approximate bovine serum albumin (essentially fatty acid free), AMP-PNP, oligomycin, pyruvate kinase (rabbit muscle, type II), lactic dehydrogenase (beef heart, type III), cytochrome c (horse heart, type III), TMPD, from Sigma Chemical Co.; CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals; digitation from Grand Island Biological Co., CaCl2 and [32P]-phosphoacetic acid from New England Nuclear. [31,32P]Pi was made 1 in HCl, boiled for 1 h, diluted with cold potassium P, to a final concentration of 100 mM, pH 7.4, before use. Ruthenium red was purchased from Polysciences, Inc., recrystallized, quantified by absorbance at 533 nm (molar extinction coefficient = 68,000), and stored in plastic, frozen as an aqueous solution. All other chemicals were of the highest possible grade and were used as obtained.

Methods-The cytochrome c-Sepharose was packed into a small column (3 ml, or until no further protein was released. Then bound membranes were eluted with 1 M KCl, 5 mM Hepes (pH 7.4) KOH.

Mitoplasts (inner membrane plus matrix) were prepared according to the procedure of Schnaitman and Greenawalt (35). Inner membrane vesicles were prepared as described by Wehrle et al. (26), and stored in small aliquots in liquid N,. This initial freezing results in a small loss of Ca" uptake activity and refreezing should be avoided.

Ca" uptake was measured by one of three methods. Experiments using either atomic absorption spectroscopy or Ca" tracer were performed as follows. Incubation media were prepared as described in the figures and tables. IMV were added at zero time with rapid mixing and incubated at 25°C for the indicated times. The reaction was stopped and externally bound Ca" removed by a modification of the EGTA stop technique of Reed and Bygrave (34). Aliquots of a highly buffered EGTA solution were added with rapid mixing, so that the final EGTA concentration equalled the concentration of Ca" in the incubation, and the concentration of Hepes was 10-fold higher. This extra buffering capacity prevents substantial pH lowering upon Ca"/EGTA interaction. The samples were then centrifuged for 10 min at 210,000 × g, the supernatants discarded, and the sediments thoroughly rinsed and drained. Sediments for atomic absorption analysis were extracted with HCl containing La" and the protein-free solutions compared with appropriate standards using an Instrumentation Laboratories spectrometer. Sediments containing 2Ca" were dissolved in 8% formic acid and counted by liquid scintillation as described previously (26). Alternatively Ca" movements were monitored directly by using a Ca"-selective electrode (Radiometer, F2112 Calcium Selectrode).

ATPase activity was measured spectrophotometrically at 340 nm by coupling production of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions essentially as described by Pullman et al. (35). The reaction mixture contained in a final volume of 1 ml at 25°C: 85 mM Tris-Cl, pH 7.4, 4 mM MgCl2, 4 mM ATP, 2.5 mM potassium P, 0.4 mM NADH, 0.6 mM phosphoenolpyruvate, 5 mM KCN, 1 unit of lactic dehydrogenase, and 1 unit of pyruvate kinase. Inner membrane vesicles and other additions were as described in the figure heading.

ATPase inhibitor peptide was prepared as described by Chintron and Perlman (30). For this preparation highly purified fraction with a specific activity of approximately 100 units/mg was used. One unit of inhibitor activity is defined as that amount of inhibitor required to inhibit 0.2 µmol/min of ATPase activity by 50%.

Cytochrome c affinity chromatography of IMV was performed for removal of any contaminating noninverted vesicles (37). Cytochrome c was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Activated Sepharose 4B (3 g dry weight) was washed and suspended with 40 µg of cytochrome c in NaICO3/NaCl buffer and the pH readjusted to 8.3. This slurry was incubated with gentle shaking for 18 h at 4°C. The reacted beads were washed with ethanalamine and washed exactly according to the instructions. The cytochrome c-Sepharose was packed into a small column (7 ml bed volume), maintained in the cold room. The column was washed before each use with 10 ml of elution buffer (225 mM sucrose, 25 mM KCl, 5 mM Hepes) containing 0.5 mM K⁺ absorbate, followed by 25 ml of elution buffer alone. The color change accompanying reduction of cytochrome c was routinely observed. Samples (10 mg of protein) were added, and the column eluted with elution buffer (0.25 ml/min), approximately 15 ml, or until no further protein was released. Bound membranes were eluted with 1 M KCl, 5 mM Hepes, pH 7.4. Mitoplasts (right-side-out) were retained by the column in elution buffer (<95%) and released by 1 M KCl. IMV eluting in the void volume (approximately 70% of applied protein) were judged to have no affinity for cytochrome c or the column itself. Due to the low salt (25 mM KCl) of the elution buffer it was not possible to say whether IMV retained by the column were bound by specific interaction with cytochrome c or by nonspecific interaction.

Protein determinations were by the biuret method (38) in the presence of 0.06% Triton X-100 using bovine serum albumin as standard.

Standard error is not given for each separate value. Each experiment shown is typical of at least two similar experiments. In an experiment shown the number of replicates is indicated. In all cases error is approximately ±1 nmol/mg.

RESULTS

As shown in Fig. 1, the preparation of IMV used for these studies contains a population of vesicles which are at least 98% inverted. Fig. 1A demonstrates, following the method of Wehrle et al. (26), that 96% of the total FCCP-stimulated ATPase activity of IMV can be inhibited by the peptide inhibitor from rat liver, which has a molecular weight of 12,300 (36). Fig. 1B demonstrates, following the method of Hackenberg and Hammon (39), that respiration with succinate is not stimulated by addition of cytochrome c to IMV. In contrast, the mitoplasts from which the IMV were prepared are clearly deficient. Respiration is susceptible to stimulation by cytochrome c. The binding sites for cytochrome c, which are well established as being at the cytoplasmic surface of the inner membrane, are partially vacant but are inaccessible in those vesicles. Five other independent methods have also indicated that the inner membrane vesicles used for this study are essentially completely inverted (26).

In the course of studies on the uptake of P, by inverted IMV, it became clear that P, uptake was substantially increased in the presence of added Ca" (Table 1). In fact, part of the P, uptake observed in the presence of added cation appears to depend on endogenous Ca", as indicated by decreased uptake in the presence of EGTA. Experiments were performed to determine whether Ca" was actually entering the vesicles, or whether Ca" was only acting from the outside, either by precipitating P, externally, or by causing some
Phosphate and Ca\(^{2+}\) Uptake by Inverted Mitochondrial Vesicles

FIG. 1. Demonstration of inverted character of inner membrane vesicles. A, inhibition of ATPase by peptide inhibitor. IMV (50 \(\mu\)g of protein) and varying amounts of inhibitor peptide were incubated for 10 min at 25°C in 250 \(\mu\)M sucrose, 4 \(\mu\)M Tris Hepes, 1 \(\mu\)M FCCP, and 0.5 \(\mu\)M MgATP, pH 6.7, final volume 0.25 ml. Control incubations contained no inhibitor. Aliquots (0.025 ml) were removed and assayed for ATPase activity as described under “Methods.” B, stimulation of respiration by cytochrome c. Mitoplasts (1.9 mg) or IMV (0.4 mg) were added to 3 ml of H medium containing 2.5 mM potassium Pi, 10 \(\mu\)M K\(^+\) succinate, 0.1 \(\mu\)M rotenone, pH 7.4, 25°C. Cytochrome c (2.7 \(\mu\)M) was added where indicated. Control, ---; cytochrome c, ---.

TABLE I

Energy-dependent uptake of P\(_{i}\) and Ca\(^{2+}\) by IMV

IMV (1 mg of protein) were incubated for 5 min at 25°C in 2 ml of H medium, pH 7.4, containing 3 mg of additional bovine serum albumin and other additions as follows: P\(_{i}\) uptake assay included 2.5 \(\mu\)M potassium \(^{32}\)P\(_{i}\), 10 \(\mu\)M K\(^+\) ascorbate, 0.15 \(\mu\)M TMPD, where indicated 0.5 \(\mu\)M CaCl\(_{2}\) or 0.05 \(\mu\)M EGTA, and was terminated with an inhibitor stop as described under “Methods.” Ca\(^{2+}\) uptake assay included 20 \(\mu\)M K\(^+\) succinate, 1 \(\mu\)M MgCl\(_{2}\), 0.5 \(\mu\)M potassium P\(_{i}\), 0.5 \(\mu\)M CaCl\(_{2}\) (+ \(^{45}\)CaCl\(_{2}\) where indicated). No stop indicates that samples were centrifuged without further addition. EGTA stop was as described under “Methods.” Wherever indicated, FCCP was 1 \(\mu\)M. Values are the averages of duplicates.

| Incubation conditions | Ion content | nmol P\(_{i}\)/mg |
|-----------------------|-------------|------------------|
| P\(_{i}\) uptake, inhibitor stop | + FCCP | 5 |
| | + EGTA | 24 |
| | + EGTA + FCCP | 5 |
| | + Ca\(^{2+}\) | 63 |
| | + Ca\(^{2+}\) + FCCP | 7 |

Radiosotope Atomic absorpt-ion assay

| nmol Ca\(^{2+}\)/mg |
|---------------------|
| Ca\(^{2+}\) uptake, no stop |
| Control | 43 |
| + FCCP | 16 |
| Ca\(^{2+}\) uptake, EGTA stop |
| Control | 95 |
| + FCCP | 5 |

change in P\(_{i}\) transport by specific or nonspecific effects on the membrane. The data in Table I also show that Ca\(^{2+}\) is taken up into an EGTA-inaccessible space under conditions similar to those where P\(_{i}\) uptake is stimulated. It should be noted that Ca\(^{2+}\) values after addition of EGTA necessarily represent minimum values for Ca\(^{2+}\) uptake. Values for Ca\(^{2+}\) from atomic absorption agree well with \(^{40}\)Ca values under a variety of conditions, indicating that net uptake, not just exchange of hot for cold Ca\(^{2+}\) is monitored by the radioisotope. The observation of Ca\(^{2+}\) uptake does not rule out the possibility of an external Ca\(^{2+}\) effect, but demonstrates that under certain conditions energy-dependent Ca\(^{2+}\) uptake into inverted vesicles certainly occurs.

The characteristics of Ca\(^{2+}\) uptake by inverted IMV are presented in Table II. Uptake is supported by a variety of substrates and is prevented by uncouplers or inhibitors of respiration. In the absence of P\(_{i}\), energy-dependent uptake is abolished. The improvement in Ca\(^{2+}\) uptake in the presence of bovine serum albumin is consistent with improved energy coupling when free fatty acids are bound.

Although the Ca\(^{2+}\)-selective electrode cannot distinguish between bound and internalized Ca\(^{2+}\), it does monitor Ca\(^{2+}\) movement without the need for stopping and centrifugation.

FIG. 2. Ca\(^{2+}\) uptake by inverted IMV. IMV (3.0 mg of protein) were suspended in 3 ml of a medium containing 120 \(\mu\)M sucrose, 60 \(\mu\)M KCl, 3 \(\mu\)M Hepes, 1 \(\mu\)M MgCl\(_{2}\), 300 \(\mu\)M CaCl\(_{2}\), 15 \(\mu\)g of oligomycin, 2 \(\mu\)g/ml of bovine serum albumin, and where indicated 2 \(\mu\)M K\(^+\) succinate. Ca\(^{2+}\)-selective electrode tracing: (1) potassium P\(_{i}\) (0.1 mM); (2) no potassium P\(_{i}\); (3) plus potassium P\(_{i}\) (0.1 mM), plus ruthenium red (0.4 nmol/mg).
Fig. 2 shows that Ca\textsuperscript{2+} is removed from the medium by IMV in response to respiration and that this occurs only in the presence of P\textsubscript{i}. Uptake is relatively slow (~30 to 40 nmol of Ca\textsuperscript{2+}/min/mg) and linear for several minutes until anaerobiosis, which initiates efflux. As will be demonstrated again later, the Ca\textsuperscript{2+} uptake observed under these conditions is sensitive to ruthenium red in the external medium. That Ca\textsuperscript{2+} efflux, which initiates efflux. As will be demonstrated again later, the Ca\textsuperscript{2+} uptake observed under these conditions is sensitive to ruthenium red in the external medium. That Ca\textsuperscript{2+} efflux, which initiates efflux. As will be demonstrated again later, the Ca\textsuperscript{2+} uptake observed under these conditions is sensitive to ruthenium red in the external medium. That Ca\textsuperscript{2+} uptake by IMV is different from uptake by intact noninverted mitochondria can be seen in Fig. 3. In the absence of added P\textsubscript{i}, rat liver mitochondria take up Ca\textsuperscript{2+}, with a characteristic H\textsuperscript{+} ejection and transient stimulation of respiration (13). In contrast, inverted IMV show neither of these characteristics, and in the absence of P\textsubscript{i}, take up no Ca\textsuperscript{2+} (Fig. 2, Table II).

The dependence of Ca\textsuperscript{2+} uptake on the concentrations of added Ca\textsuperscript{2+} and P\textsubscript{i} are shown in Fig. 4. Although uptake is linear at the time point used (2 min) it is by no means clear what the rate-limiting step is in this complex reaction. Half-maximal Ca\textsuperscript{2+} uptake appears to require about 50 mM calcium added, but the concentration of uncomplexed Ca\textsuperscript{2+} in the presence of the P\textsubscript{i} is undoubtedly much less. The P\textsubscript{i} concentration curve shows a distinct optimum at around 0.1 mM P\textsubscript{i}. This was found in Ca\textsuperscript{2+} electrode studies as well (not shown). Slightly higher P\textsubscript{i} concentrations (0.5 mM) were routinely used, because of the greater reproducibility observed in the “plateau” region.

Under the assay conditions of Table II net Ca\textsuperscript{2+} uptake is specific for P\textsubscript{i}, among the anions tested. Neither permeant anions (NO\textsubscript{3}-, SCN-) nor permeant weak acids (OAc-, HCO\textsubscript{3}-) can replace P\textsubscript{i}, at least at similar concentrations. Identical results were obtained using the Ca\textsuperscript{2+} electrode to avoid EGTA and centrifugation.

ATP and ADP, even in the presence of high levels of oligomycin, substantially increase the amount of Ca\textsuperscript{2+} taken up by inverted IMV (Table III). In contrast, the ATP analog AMP-PNP does not stimulate Ca\textsuperscript{2+} uptake. The stimulation by adenine nucleotides appears to be completely dependent on added Mg\textsuperscript{2+}, and it is insensitive to carboxyatractyloside, a potent inhibitor of the adenine nucleotide translocator. The effect of oligomycin itself is seen in Table IV. For an equal time (5 min) the control and oligomycin-containing assays demonstrate the same amount of energy-dependent Ca\textsuperscript{2+} uptake (in this case 16 to 17 nmol/mg), but this does not take into account the inhibition of vesicle respiration by oligomycin (26). While using the same amount of oxygen as in the oligomycin-containing incubation (in only 2.2 min), IMV without oligomycin could accumulate only 6 nmol of Ca\textsuperscript{2+}/mg. Thus oligomycin does have a significant coupling effect even though no increase in net Ca\textsuperscript{2+} uptake is observed in the 5-min assay.

As can be seen in Table IV, Ca\textsuperscript{2+} uptake is completely sensitive to inhibition by N-ethylmaleimide, an inhibitor of energy-dependent P\textsubscript{i} transport in both mitochondria and inverted IMV (26). In addition, the uptake described here is sensitive to ruthenium red (Fig. 2 and Table IV). This is true even if the IMV are eluted from an affinity column of cytochrome c bound to Sepharose. Any right-side-out vesicles will stick to the column (as verified with mitoplasts; see “Methods”). However, the vesicles which elute in the void volume of the column under low salt conditions still take up Ca\textsuperscript{2+}. Although the uptake capacity is somewhat diminished, per-
indicated. In Experiment 1: oligomycin added was 5 pg, 2.2 min incubation in the absence of oligomycin was accompanied by oxygen consumption equal to that in a 5-min incubation with oligomycin. In Experiment 2: IMV were preincubated with 50 nmol of N-ethylmal- imide/mg of protein for 2 min at 0°C before aliquots were assayed for Ca²⁺ uptake. In Experiment 3: IMV were passed over an affinity column of cytochrome c bound to Sepharose 4B (see “Methods” for details) to remove any right-side-out vesicles before assay for Ca²⁺ uptake in the presence or absence of ruthenium red (1 nmol/mg). Values corrected for nonenergy-dependent uptake and are averages of quadruplicates.

| Incubation conditions | Respiration-dependent increase in Ca²⁺ content (nmol/mg) |
|-----------------------|----------------------------------------------------------|
| Experiment 1: 20 mM K⁺ succinate |                                      |
| 5 min, no oligomycin   | 16                                                       |
| 5 min, oligomycin      | 17                                                       |
| 2.2 min, no oligomycin | 6                                                        |
| Experiment 2: 10 mM K⁺ ascorbate plus 0.15 mM TPPD |                                      |
| Untreated IMV          | 38                                                       |
| N-ethylmaleimide-treated IMV | 1                                                         |
| Experiment 3: 5 mM K⁺ succinate |                                      |
| Untreated IMV          | 29                                                       |
| + Ruthenium red        | 1                                                        |
| IMV from cytochrome c column | 19                                                       |
| + Ruthenium red        | 1                                                        |

Fig. 5. Delayed addition of Ca²⁺ to IMV accumulating P₃. IMV were incubated as described in Table II with 5 mM K⁺ succinate as substrate, except that potassium P₃ was 2 mM and CaCl₂ (0.5 mM) was added initially or after 5 min. In A, P₃ uptake is measured with initial (→) or delayed (→X-) Ca²⁺ addition. In B, Ca²⁺ uptake is measured with initial (→Δ-) or delayed (→L-) Ca²⁺ addition.

The data in Table IV suggested that the previously described P₃ transport mechanism (26) rather than a novel Ca²⁺ plus P₃ translocator is responsible for the Ca²⁺-stimulated P₃ movement observed here. In the experiments shown in Fig. 5 the movements of Ca²⁺ and P₃ are measured in identical incubations. It is obvious (Fig. 5A) that Ca²⁺ added at the beginning of the incubation stimulates P₃ uptake. In Fig. 5B it can be seen that this is accompanied by uptake of Ca²⁺ into the EGTA-inaccessible intravesicular space. In contrast, Ca²⁺ added after a substantial amount of P₃ has already been taken up does not stimulate a sudden burst of P₃ uptake even though the Ca²⁺ itself is taken up rapidly. This is not due simply to the order of addition. In experiments not shown, it was observed that, in a similar incubation under conditions where little net P₃ had been accumulated in the first phase of the incubation (lower P₃ concentration, shorter time), addition of CaCl₂ induces a rapid P₃ uptake whether added initially or later, and in either case Ca²⁺ was taken up as well. From this it appears clear that there is no requirement for simultaneous movement of the two ions. Ca²⁺ uptake can occur whether P₃ has been taken up previous to Ca²⁺ addition or is taken up together with Ca²⁺. The stimulation of P₃ uptake by Ca²⁺, in contrast, occurs only if the vesicles have not previously accumulated a substantial amount of P₃.

**DISCUSSION**

The experiments described here show that energy-dependent uptake of P₃ by inverted inner membrane vesicles can be stimulated by the addition of Ca²⁺. Under these conditions Ca²⁺ is also taken up. It leaves the medium (Fig. 2) and enters an EGTA inaccessible space (Table II) within the vesicle. This is not uptake by vesicles retaining the right-side-out orientation of intact mitochondria. In this paper (Fig. 1) and previously (26) the IMV used here have been shown by a number of independent criteria to be at least 95% inverted. IMV which are not retained on a cytochrome c affinity column still demonstrate Ca²⁺ uptake (Table IV). Furthermore, the Ca²⁺ uptake in IMV differs in several respects from that in intact mitochondria. First, the requirement for P₃ is absolute (Fig. 2, Table II). There is no limited loading (4) of Ca²⁺ in the absence of P₃. Uptake of Ca²⁺ is completely abolished by the P₃ transport inhibitor N-ethylmaleimide, in contrast to the results in mitochondria (13). Second, the H⁺ ejection and transient stimulation of respiration typical of mitochondrial Ca²⁺ uptake is entirely absent in IMV (Fig. 3).

The fact that the Ca²⁺ uptake observed in inverted IMV is sensitive to the inhibitor ruthenium red (Fig. 2, Table IV) may indicate that under the conditions used (the precise internal milieu, external medium) the classical electrogenic carrier is responsible for Ca²⁺ movement. This carrier does appear to promote a ruthenium red-sensitive efflux of Ca²⁺ from mitochondria under a variety of conditions, including addition of EGTA (19) or phosphoenolpyruvate (40). However, it is important to note that in the present studies ruthenium red is added to the matrix surface of the membrane, a situation that never occurs in studies of Ca²⁺ efflux from intact mitochondria. Efflux of Ca²⁺ under other conditions does appear insensitive to ruthenium red added at the cytosolic surface (8, 41). This has suggested to some authors (21–25, 40) the involvement of a second, and somewhat different, Ca²⁺ carrier in efflux. However, it is impossible to say a priori what effect ruthenium red may have on any carrier or carriers when added to the matrix surface. It might also be significant to note that in these studies (as opposed to studies of efflux from mitochondria) that Ca²⁺ and ruthenium red are added at the same surface, or that ruthenium red is added to a membrane surface at which a negative potential is found (see, for instance, Reference 19).

Concentrations of Ca²⁺ and P₃ found optimal for Ca²⁺ uptake into inverted IMV (Fig. 4) are high compared to cytosolic Ca²⁺ concentrations, or compared to the Kᵥ for movement of Ca²⁺ into intact mitochondria (4 μM, Reference 18). However,
these concentrations could be attained easily within the matrix space depending on the degree to which Ca\(^{2+}\) is bound. Given the small internal volume of the IMV (1 µl/mg), internal complexation process undoubtedly also is occurring and probably provides the main driving force for net accumulation (uptake minus release) of Ca\(^{2+}\) and of Ca\(^{2+}\)-stimulated extra Pi. Although complexation of calcium and phosphate may not be a physiological driving force for Ca\(^{2+}\) or Pi, extrusion from the mitochondrion, it is a convenient aid for revealing the existence of a potential efflux pathway. The inability of SCN\(^{-}\) or NO\(^{3-}\) to support Ca\(^{2+}\) accumulation emphasizes the importance of Ca\(^{2+}\) complexation in the overall Ca\(^{2+}\) uptake process measured here, as anions have been shown to enter inverted IMV in response to respiration (26). The inability of HCO\(^{3-}\) to support uptake indicates again that a process different from mitochondrial uptake is occurring in inverted IMV.

Stimulation of Pi-dependent Ca\(^{2+}\) uptake by ATP and ADP is substantial (Table III). Although the similar effect on mitochondrial calcium plus Pi uptake has been ascribed to the ability of ATP to stabilize calcium-Pi granules (42), such an effect can be ruled out in the present studies. ADP cannot be phosphorylated to ATP in the presence of high levels of oligomycin. Carboxyatractylate, a powerful inhibitor of the adenine nucleotide translocator, has no effect on the stimulation. The site involved is clearly on the matrix surface of the membrane. Because it is the Mg\(^{2+}\)-complexes which are active, nonspecific permeability increase due to chelation of necessary Mg\(^{2+}\) by nucleotide can also be ruled out.

That Ca\(^{2+}\) uptake by inverted IMV requires transported Pi, as opposed to Pi, in the external medium or externally bound Pi, is indicated by the complete abolition of Ca\(^{2+}\) uptake by N-ethylmaleimide (Table IV). Although a direct effect of N-ethylmaleimide on Ca\(^{2+}\) transport cannot be ruled out, inhibition of Pi transport both by Pi plus H\(^{+}\) electroneutral symport (3) and by electrophoretic Pi uniport (26) are known to be sensitive to this SH group reagent. That Ca\(^{2+}\) transport is not directly coupled to cotransport of Pi is suggested by Fig. 5. Pi accumulated in the absence of Ca\(^{2+}\) can support Ca\(^{2+}\) uptake at least as well as Pi; taken up at the same time as Ca\(^{2+}\). Uptake of Ca\(^{2+}\) into Pi-preloaded vesicles does not stimulate further Pi uptake. These data, together with the classical inhibitor sensitivities of Ca\(^{2+}\) uptake to ruthenium red and N-ethylmaleimide, permit construction of a tentative model to describe the matrix-to-cytosol directed transport observed in IMV.

The simplest possible model for the observed uptake of Ca\(^{2+}\) and Pi, which is consistent with the data is shown in Fig. 6, which also includes the classical model of Ca\(^{2+}\) and Pi uptake by mitochondria (43). The present results in no way rule out the presence of other transport systems or more complex regulation or both.

In the intact mitochondrion (Fig. 6A) electron transport generates an interior-negative membrane potential. Ca\(^{2+}\) is taken up electrochemically, probably with two positive charges. The resulting decrease in the membrane potential causes increased respiration and protons are ejected, compensating for the positive charge introduced by Ca\(^{2+}\), until the matrix becomes excessively alkaline. Endogenous CO\(_2\), as HCO\(^{3-}\), may provide some internal acidification (44). Added Pi, taken up by electroneutral proton symport, neutralizes interior alkalinity and supports the uptake of large additional amounts of Ca\(^{2+}\) (5).

In contrast, in inverted IMV (Fig. 6B) respiration induces an interior-positive membrane potential and Ca\(^{2+}\) cannot enter at all in the absence of anion. Pi is free to enter electrophoretically (26). This causes a decline in the membrane potential and Ca\(^{2+}\) may enter, fully charged, or perhaps in exchange for internal proton, which would simultaneously provide a chemical gradient for Ca\(^{2+}\) accumulation and partial charge compensation. A completely electroneutral Ca/2 H\(^{+}\) exchange might be expected to support some Ca\(^{2+}\) uptake in the absence of Pi, but other constraints might apply to prevent observable levels of uptake, so the data here cannot be taken to rule out such exchange. In any case precipitation of calcium-Pi is clearly necessary to pull Ca\(^{2+}\) uptake. Other anions are ineffective.

The presence of two independent Ca\(^{2+}\) transport mechanisms, considered necessary to explain the apparent displacement of the matrix/cytosol Ca\(^{2+}\) gradient from equilibrium (23, 45) is not inconsistent with the present data. The carrier functioning under the conditions used here is sensitive to ruthenium red at its matrix face. Whether this represents the action of the classical electrophotoretic carrier or a novel carrier has not been determined. What carrier is involved in physiological efflux is equally unclear. The possible involvement of an N-ethylmaleimide-insensitive Ca\(^{2+}\) plus Pi, symporter (14, 15) can, however, be ruled out by the data (Table IV and Fig. 5). A Na\(^{+}/Ca\(^{2+}\) exchange such as that described for mitochondria from heart and certain other tissues (46, 47) appears not to be present in liver (47) and would therefore appear to have no role in the Ca\(^{2+}\) and Pi movements described here. (Also, Na\(^{+}\) was specifically excluded from all solutions.) The possibility of an electroneutral Ca\(^{2+}\)/2 H\(^{+}\) exchanger has been discussed in connection with Ca\(^{2+}\) efflux from rat liver and Ehrlich ascites cell mitochondria (41). Åkerman (48) has reported an acid-pulse-induced Ca\(^{2+}\) efflux from rat liver mitochondria. Tsuchiya and Rosen (49, 50) have described a Ca\(^{2+}\)/H\(^{+}\) exchange system in inverted bacterial vesicles. The need for further study of the mechanism of Pi, and Ca\(^{2+}\) influx and efflux, and the regulation of steady-state ion levels in mitochondria and whole cells is apparent.

Since the completion of the work reported here, a brief communication has appeared in which the uptake of Ca\(^{2+}\) by IMV is described (51). As in earlier studies (27-29) and in the present work, such uptake requires the uptake of Pi. The IMV...
of Lützker et al. (51), prepared under conditions quite different from those used in the present work, show a ruthenium red-stimulated uptake of Ca\(^{2+}\) with Pi, which supports the notion of a second Ca\(^{2+}\) transporter active under certain conditions, as discussed above.

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