Mechanism of Sodium Independent Calcium Efflux from Rat Liver Mitochondria†

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ABSTRACT: On the basis of primarily two types of observations, it has been suggested that the Na^+-independent Ca^{2+} efflux mechanism of rat liver mitochondria is a passive Ca^{2+}–$2H^+$ exchanger. First, when a pulse of acid is added to a suspension of mitochondria loaded with Ca^{2+}, a pulse of intramitochondrial Ca^{2+} is often released, even in the presence of the inhibitor of mitochondrial Ca^{2+} influx, ruthenium red. Second, at a pH near 7, the stoichiometry of Ca^{2+} released to H^+ taken up by Ca^{2+}-loaded mitochondria, following treatment with ruthenium red, has been observed to be 1:2. This evidence for a Ca^{2+}–$2H^+$ exchanger is reexamined here by studying the release of Ca^{2+} upon acidification of the medium by addition of buffer, the dependence of liver mitochondrial Ca^{2+} efflux on external medium pH and intramitochondrial pH, and the Ca^{2+}–Ca^{2+} exchange properties of the Ca^{2+} efflux mechanism. These studies show no pulse of mitochondrial Ca^{2+} efflux when pH is abruptly lowered by addition of buffer. The stoichiometry between Ca^{2+} and H^+ fluxes is found to be highly pH dependent. The reported 1:2 stoichiometry between Ca^{2+} efflux and H^+ influx is only observed at one pH. Furthermore, the rate of Ca^{2+} efflux from mitochondria is found to increase only very slightly at most as suspension pH is decreased. The rate of Ca^{2+} efflux is not found to increase with increasing intramitochondrial pH. Finally, no Ca^{2+}–Ca^{2+} isotope exchange can be demonstrated over the Na^+-independent efflux mechanism (i.e., in the presence of ruthenium red). It is concluded that these data do not support the hypothesis that the Na^+-independent Ca^{2+} efflux mechanism is a passive Ca^{2+}–$2H^+$ exchanger.

Mitochondrial Ca^{2+} transport is mediated by separate mechanisms for influx and efflux of Ca^{2+} (Sordahl, 1974; Puskin et al., 1976; Crompton et al., 1976; Nicholls, 1978). Opposing Ca^{2+} fluxes through these separate mechanisms set up a true steady-state balance of mitochondrial to medium [Ca^{2+}] in the period following rapid Ca^{2+} uptake by energized mitochondria, demonstrating the mitochondrion's ability to act as a regulator of external Ca^{2+} (Puskin et al., 1976; Nicholls, 1978).

The Ca^{2+} efflux system of liver and kidney mitochondria appears to differ from that found in mitochondria from heart and other excitable tissue (Crompton et al., 1977; Puskin et al., 1976; Nicholls & Crompton, 1980). The primary efflux mechanism of heart mitochondria appears to be a Ca^{2+}–$n$$Na^+$ exchanger (Crompton et al., 1977), while the primary efflux mechanism of liver mitochondria is Na^+-independent (Fiskum & Lehninger, 1979). Nevertheless, some recent evidence does suggest the existence of Na^+-independent Ca^{2+} efflux in heart mitochondria (Rosier et al., 1981) and Na^+-dependent Ca^{2+} efflux in liver mitochondria (Haworth et al., 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982), but not all published data support this latter conclusion (Zoccarato & Nicholls, 1982).

The separate mechanisms of Ca^{2+} influx and efflux provide a number of advantages for the cell over mechanistically simpler systems, particularly in providing more sensitivity for precise Ca^{2+} regulation (Nicholls, 1978; Nicholls & Crompton, 1980; Rosier et al., 1981). The observation of these complex mitochondrial systems for precise Ca^{2+} regulation then attests to the importance of the mitochondrion, and particularly of the mitochondrial Ca^{2+} efflux mechanisms, in the vital function of cytosolic Ca^{2+} regulation (Fiskum & Lehninger, 1980).

In past work, the efflux of Ca^{2+} from liver mitochondria has been measured either by the addition of the Ca^{2+} chelator, EGTA,[1] or by the addition of the mitochondrial Ca^{2+} influx inhibitor, ruthenium red, to a suspension of mitochondria previously loaded with Ca^{2+}. These two techniques for measuring Ca^{2+} efflux have been found to be in fair agreement (Gunter et al., 1978a), and this has provided the strongest evidence that such rates are free from artifactual effects of either EGTA or ruthenium red. Strong corroboration of this conclusion is provided by new evidence presented below.

Since the suggestion that the Na^+-independent Ca^{2+} mechanism of liver mitochondria might be a passive Ca^{2+} for nH^+ exchanger, as one of several alternative possibilities (Puskin et al., 1976), evidence has been presented supporting the Ca^{2+}–$n$$H^+$ exchanger hypothesis (Åkerman, 1978; Fiskum & Lehninger, 1979). First, Åkerman (1978) has reported a release of Ca^{2+} upon lowering of the pH of the suspending medium, either with or without ruthenium red. If the Na^+-independent Ca^{2+} efflux mechanism passively exchanges Ca^{2+} for H^+, an increase in medium H^+ concentration (acidification) might be expected to drive more of the exchanger to the inside surface of the transport membrane (loaded with protons), where its increased concentration would stimulate a faster rate of Ca^{2+} efflux. However, other explanations for the H^+-induced release of Ca^{2+} are possible. Divalent cations are known to compete with H^+ for intramitochondrial binding (Gunter & Puskin, 1975). Acidification of the medium would be expected to lower the pH of the

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; F_1, the "head group" of the mitochondria ATPase complex; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide; OSCP, oligomycin-sensitivity-conferring protein; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TPP^+, tetraphenylphosphonium; ATPase, adenosinetriphosphatase; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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mitochondrial matrix also and thus to increase the ratio of free to bound Ca^{2+} in the matrix. This could result in an increase in the rate of Ca^{2+} release because of an increase in the intramitochondrial Ca^{2+} chemical potential, independently of any Ca^{2+} for H^+ exchange. Also, if a small percentage of mitochondria were made leaky by the change of conditions (addition of acid), a rapid release of some of the intramitochondrial Ca^{2+} followed by reuptake of this Ca^{2+} (in the absence of ruthenium red) by healthier mitochondria might occur. Release of Ca^{2+} upon acidification of the medium is reexamined in experiments described below. Clearly, were the release of Ca^{2+} from energized mitochondria upon addition of acid a real indication of Ca^{2+} for H^+ exchange, a marked increase in the rate of Ca^{2+} efflux from energized mitochondria as medium pH is decreased would be expected.

Also supporting the Ca^{2+} for $2H^+$ exchange hypothesis is the finding of a 1:2 stoichiometry between Ca^{2+} efflux and H^+ influx, at a pH near 7, after depletion of inorganic phosphate (Fiskum & Lehninger, 1979). This suggests that the exchange is electrically silent (one Ca^{2+} for two H^+). Independent corroborative evidence for a lack of response of the Na^+-independent mitochondrial Ca^{2+} efflux mechanism to large transient changes of transmembrane potential is presented below. To infer from the measured 1:2 stoichiometry that the Na^+-independent Ca^{2+} efflux mechanism of liver mitochondria is a passive Ca^{2+}–$2H^+$ exchanger, however, it must be shown that Ca^{2+}–$2H^+$ exchange is the dominant mechanism of H^+ permeability through the inner mitochondrial membrane, under these conditions. Although this would be difficult to prove conclusively, constancy of the Ca^{2+} to H^+ stoichiometry at a value near 1:2 over a range of pH around the physiological pH could be taken as prima facie evidence that a single mechanism (probably a Ca^{2+}–$2H^+$ exchanger) catalyzed the linked leakage of Ca^{2+} outward and H^+ inward. Results of experiments which test this hypothesis are presented below.

Use of procedures which might modify the system under investigation was avoided in these studies. Use of oxaloacetate or acetoacetate to activate the Na^+-independent mechanism might represent an example of such a procedure, since these reagents have been shown under some conditions to lead to major changes in the mitochondrial transport membrane (possibly by inducing leakiness) (Nicholls & Brand, 1980; Beatrice et al., 1980; Jurkowitz & Brierley, 1982).

Furthermore, as was recently pointed out by Zoccarato & Nicholls (1982), the presence of millimolar amounts of inorganic phosphate can be responsible for alterations in the behavior of Na^+-independent Ca^{2+} efflux, attributable to the formation of a calcium phosphate precipitate in the mitochondrial matrix, and not necessarily to characteristics of the efflux mechanism itself. An attempt was made to avoid the formation of this precipitate in the study described below by the addition of a small amount of acetate but no phosphate to the mitochondrial suspensions.

Whenever possible an effort was also made to ensure that the quality of the mitochondria used in these experiments was satisfactory by simultaneous measurement of membrane potential by using either a tetraphenylphosphonium electrode (Kamo et al., 1979; Affolter & Sigel, 1979) or fluorescence of the carbocyanine dye diS-C_3-(5) (Laris et al., 1975). These precautions should ensure that the Ca^{2+} efflux system under investigation in these experiments was modified as little as possible by the experimental procedures used.

Materials and Methods

Media. Media used were made up as follows: E medium contained 6 mM Hepes (pH 7.2 at 22 °C), 3.0 mM potassium succinate, 1 mM $MgCl_2$, and sufficient mannitol/sucrose (3:1) to bring the osmolarity to 300 mOsm (approximately 195 mM mannitol and 65 mM sucrose). R medium contained the same plus 1 g/L defatted BSA. In some experiments low concentrations of Na^+ salts were substituted for the corresponding K^+ salts, for convenience.

Mitochondrial Preparation. Mitochondria were prepared by a modification of the procedure of Schnaitman & Greenawalt (1967), as previously described, and by a modification of the procedures of Bustamante et al. (1977) and of Fleischer et al. (1979). The primary advantage of the latter procedure is higher mitochondrial yield.

^{45}Ca Efflux Measurements. Mitochondria (1 mg/mL) were suspended in E medium (22 °C) to which varying amounts of potassium acetate were added. After 4 min was allowed for thermal equilibration of the suspension, 80 nmol of $^{45}CaCl_2$/mg of protein was added, while the sample was stirred or vortexed. The samples were constantly stirred or periodically vortexed. Ruthenium red (4 nmol/mg of protein) and/or other substances were added to the sample at preselected times after addition of Ca^{2+}, as indicated in the figure captions. Immediately prior to each addition, 1.6-mL aliquots of the suspension were rapidly filtered by using Millipore Swinnex filter holders containing a Whatman 934-AH glass prefilter and a Millipore HA 0.45-μm filter. Following addition of ruthenium red and/or other substances of interest to each sample, aliquots (1.6–2.3 mL) were withdrawn periodically and rapidly filtered. Equal volumes of filtrates or unfiltered aliquots were added to scintillation fluid in separate vials and counted to yield sample or standard counts respectively for each sample.

Intramitochondrial Water Volume and Measurement of pH Gradient. Intramitochondrial water volume was measured by a lyophilization technique, similar to that used previously (Puskin et al., 1976), and also by a dual label technique similar to that described by Rottenberg (1979). The pH gradient was measured by using the [^{14}C]acetate technique described previously (Puskin et al., 1976) except for a modification involving the use of tritiated water in the measurement of total pellet water.

Electrode Measurements. Electrode measurements were performed in a 7-mL Lucite chamber, water jacketed for cooling, and fitted with a clear Lucite top with holes to support the electrodes. Air was eliminated, and a small positive pressure of O_2 was maintained at the top of the chamber during experiments. Samples were gently stirred during the experiments by using a small magnetic stir bar in the electrode chamber.

pH. H^+ chemical activity was measured with a Markson 2885 Helimark pencil electrode and a Markson Model 90 pH/temperature meter. pH electrodes were calibrated with standard commercial pH buffers. The buffering power of each of the mitochondrial suspensions used in the electrode experiments was determined under conditions similar to operating conditions by additions of carefully calibrated amounts of acid (HCl) and base (NaOH) to the suspension of interest and noting the change of pH. In this way a buffering curve was obtained, which allowed changes of pH to be interpreted in terms of the amount of H^+ or OH^- necessary to be introduced to the suspending medium in order to evoke the change of pH.

Ca^{2+}. Ca^{2+} chemical activity was measured by using a Radiometer F2002 Ca^{2+} electrode and a Ag–AgCl reference electrode using a 3% agar-saturated KCl salt bridge. The output was amplified by using a Keithley 616 digital electrometer, and both [Ca^{2+}] data and pH data were simulta-

neously recorded on a Hewlett-Packard 7100 BM strip chart recorder. Dilutions of Orion Calcium Standard Solution 92-20-06 were used in calibrating the response of the Ca^{2+} electrode. This system gave a linear Nernstian response (slope 29 mV/decade) from over 10^{-3} to 3×10^{-6} M Ca^{2+} with significant but nonlinear electrode response down to 2×10^{-7} M Ca^{2+} in the absence of mitochondria. The buffering power of the mitochondrial suspension to changes in medium Ca^{2+} was measured in a calibration procedure similar to that used with the pH electrode described above. In this case, 4 nmol of the Ca^{2+} uptake inhibitor ruthenium red per mg of mitochondrial protein was used to inhibit Ca^{2+} uptake.

Effects of Ca^{2+} on the output of the pH electrode, effects of H^+ on the output of the Ca^{2+} electrode, and competition between H^+ and Ca^{2+} for binding to the outwardly accessible surfaces of the mitochondrial membrane system were studied, and small corrections were made to the electrode data where necessary. Ethanol rinses and uncouplers such as CCCP strongly interfered with the Ca^{2+} electrode and were avoided. Some substances such as tetraphenylphosphonium and Mg^{2+} interfered at higher concentrations and were used at lower concentrations.

TPP^+. A tetraphenylphosphonium (TPP^+) electrode and reference electrode (Kamo et al., 1979; Affolter & Sigel, 1979) were constructed by Mr. Bruce Jensen of this laboratory, and the TPP^+ electrode was filled with a 10^{-3} M solution of tetraphenylphosphonium.

The output of this electrode system was amplified by using a Keithley 616 digital electrometer and recorded on a Hewlett-Packard 7100 BM strip chart recorder. The TPP^+ electrode shows linear Nernstian response with a slope of around 59 mV/decade between 10^{-3} and 10^{-7} M TPP^+.

Measurement of Fluorescence. The fluorescence of the dye 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] was measured during one set of the experiments described below. The dye was the generous gift of Dr. Alan Waggoner of Carnegie-Mellon University. Although the change in fluorescence intensity of this dye, as used here, was dependent upon the membrane potential of energized mitochondria, the dependence is nonlinear over at least part of the range of interest (Laris et al., 1975). The use of the dye rather than the use of a tetraphenylphosphonium electrode was necessitated by the use of valinomycin in these experiments. Valinomycin can cause major changes in the permeability characteristics of the membrane of the tetraphenylphosphonium electrode.

Techniques used in the carbocyanine dye measurements were similar to those described by Hoffman & Laris (1974) except that the sample volumes were only 1.3 mL.

Acetate Flux Measurements. Acetate flux was measured in a separate aliquot with [^{14}C]acetate, under conditions otherwise identical with those of the electrode experiment on the same mitochondrial preparations used in the electrode experiments. As with the electrode measurements, air was also eliminated above the samples used for acetate measurements, and a small positive pressure of O_2 was maintained at the top of the chamber during experiments. Techniques were similar to those used during the pH gradient measurements. Measured acetate flux was based on changes of pellet acetate with time. The correction to proton flux was made by using the usual assumption that the acetate only crossed the membrane in the protonated form.

Inorganic Phosphate Flux Measurements. While inorganic phosphate was not added in any of the electrode experiments reported here, inorganic phosphate usually increased in concentration with time in a suspension of mitochondria due to hydrolysis reactions such as ATP hydrolysis.

Phosphate flux measurements were carried out on the same aliquots used for acetate measurements. Aliquots of 4 mL were withdrawn from the chamber periodically and centrifuged at 0 °C for 2 min at 12000 rpm in a Beckman JA20 rotor. The supernatants were withdrawn and after removal of protein by precipitation in 4% trichloroacetic acid were assayed for inorganic phosphate by using the technique of Berenblum & Chain (1938).

It was assumed that as each phosphate crosses the membrane on the P_i–OH exchanger the effect with respect to determining the effective proton flux is the same as if H_3PO_4 had crossed the membrane. The number of free H^+ released per phosphate was determined through use of the Henderson–Hasselbalch equation at each pH.

Results

Measurement of Mitochondrial Ca^{2+} Efflux. In the past, the rate of Ca^{2+} efflux from mitochondria has been measured by addition of the chelator, EGTA, or of the inhibitor of mitochondrial Ca^{2+} influx, ruthenium red, without proof that the addition of either of these agents does not, in itself, artifactually affect the rate being measured.

There is an independent method by which mitochondrial Ca^{2+} efflux can be measured, which involves only the addition of small amounts of $^{45}Ca^{2+}$ to the system; consequently, this method should be free of possible artifacts produced by EGTA or ruthenium red. In this approach, it is noted that under steady-state conditions the rates of Ca^{2+} influx and efflux are equal. If in the steady state an amount of $^{45}Ca^{2+}$ could be added to the suspension, which is negligibly small compared to the amount of external Ca^{2+} present, and if the total external Ca^{2+} is known, then the influx rate (and by inference the efflux rate) could be determined by measuring the rate of internalization of labeled Ca^{2+}. Unfortunately, because carrier-free $^{45}Ca^{2+}$ is very difficult to obtain, addition of enough $^{45}Ca^{2+}$ to provide adequate counting statistics dictates a Ca^{2+} addition equivalent to more than 50% of the external Ca^{2+} under the conditions of greatest interest, perturbing the steady state and giving rise to a period of net Ca^{2+} influx. Furthermore, the rates of internalization of the external label are too rapid, at room temperature, to allow kinetically reliable filtration experiments to be carried out.

These problems can be ameliorated by working over a range of added $^{45}Ca^{2+}$ concentration and by working at lower temperature. It would be expected that as the amount of added $^{45}Ca^{2+}$ is decreased, the net influx, brought about by the increased external Ca^{2+} concentration, would decrease. This decrease would probably be linear in the concentration range just above the steady-state concentration, and the measured influx rate should approach the true steady-state efflux rate at lower amounts of added Ca^{2+}. Decreasing the temperature (to 4 °C in the data shown) slows transport rates so that accurate rates of internalization of isotope can be determined.

As can be seen in Figure 1, extrapolation of the Ca^{2+} flux rate to zero added ionized Ca^{2+} does indicate a rate of $^{45}Ca^{2+}$ flux not significantly different from that measured by addition of EGTA, under similar conditions. This strongly suggests that, under these conditions, no significant artifactual inhibition or activation of mitochondrial Ca^{2+} efflux accompanies the use of EGTA.

It has been observed that the rate of mitochondrial Ca^{2+} efflux measured by EGTA addition is similar to that measured by ruthenium red addition (Gunter et al., 1978a). If the Ca^{2+} efflux rate measured by EGTA addition is valid, as implied by the data and arguments presented above, then this should

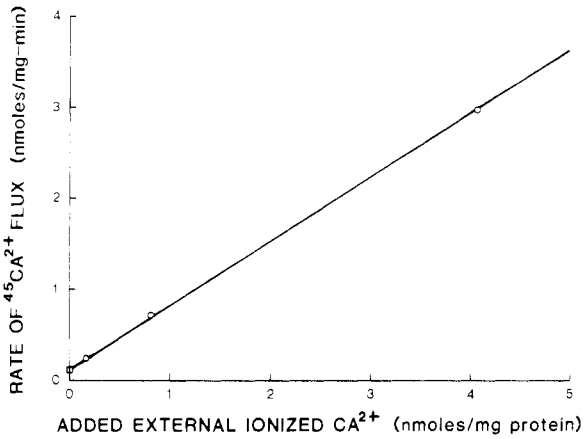

FIGURE 1: Rate of $^{45}Ca^{2+}$ flux in the presence of varying amounts of added external ionized Ca^{2+}. The rate of $^{45}Ca^{2+}$ flux was measured by two different techniques under similar experimental conditions. Mitochondria (1 mg/mL) were suspended in R medium containing 4 mM acetate. This suspension was subsequently subdivided into separate tubes for the "steady-state isotope exchange" experiments (○), for efflux measurement by "EGTA addition" (□), and for the control experiment. After thermal equilibration of the mitochondria at 4 °C, 80 nmol of $^{45}CaCl_2$/mg of protein was added (while rapidly vortexing) to the control and EGTA addition samples, and a like amount of unlabeled Ca^{2+} was added similarly to the steady-state isotope exchange samples. Volumes of medium equivalent to the volume of EGTA to be added to the EGTA addition sample were added to each of the nonradioactive steady-state isotope exchange samples 7 min after Ca^{2+} addition and then to the control sample. Immediately following this, an aliquot of the control sample was filtered by using a Millipore Swinnex holder and filters as discussed under Materials and Methods. EGTA was added (while rapidly vortexing) 8 min after Ca^{2+} addition to the sample termed "EGTA addition sample" at a final concentration of 0.5 mM, and an aliquot of the EGTA addition sample was filtered, immediately after mixing. Aliquots were withdrawn periodically from the control and EGTA addition samples, over the next 22 min, and filtered. Meanwhile, 8 min after addition of cold Ca^{2+} to a steady-state isotope exchange sample, 0.181 nmol of $^{45}CaCl_2$/mg of protein was added to an exchange sample while vortexing, followed by rapid sequential withdrawal and filtration of four aliquots from this sample in less than 30 s. Thirteen minutes after addition of cold Ca^{2+}, 0.181–4.156 nmol of $^{45}CaCl_2$/mg of protein was added to separate isotope exchange samples and the rapid withdrawal and filtration process repeated. Equal volumes of filtrate (sample) and unfiltered sample (standard) were added to scintillation fluid and counted. Corrections were made for Ca^{2+} binding to succinate and BSA in the suspending medium based upon dissociation constants found in the literature (Neuman & Neuman, 1958; Gelles & Hay, 1958), permitting calculation of "ionized Ca^{2+}". This allowed plotting the measured rates of influx of the Ca^{2+} against added ionized Ca^{2+}.

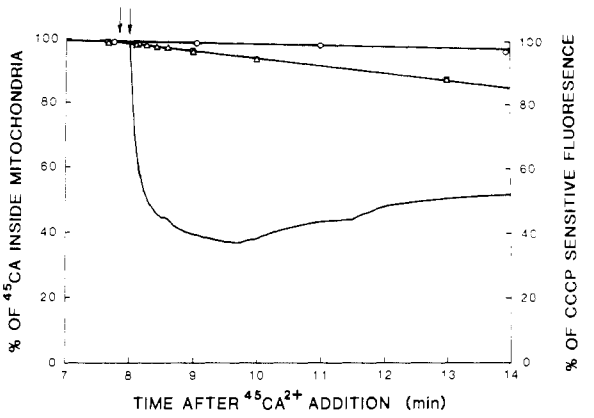

FIGURE 2: Intramitochondrial Ca^{2+} and percent change of CCCP-sensitive fluorescence of diS-C_3-(5) vs. time. Mitochondria were suspended at 1 mg/mL in medium, identical with that used in the experiments shown in Figure 1, except that 5.77 ng of valinomycin/mg of mitochondrial protein was added to each sample. After several minutes of equilibration of the mitochondria in the suspending medium (22 °C), 10 nmol of labeled Ca^{2+}/mg of mitochondrial protein was added to the suspension at "zero" time. To the control sample, indicated by open circles (○), were made no further additions; 5 nmol of ruthenium red/mg of mitochondrial protein was added at the first arrow to the samples indicated by open squares (□) and open triangles (△). KCl (31 mM) (final concentration) was added at the second arrow to the sample (△). Aliquots were periodically withdrawn from all samples and filtered to determine intramitochondrial Ca^{2+}, as described in the caption to Figure 1 and under Materials and Methods. To another sample, similar to the one for which the data are indicated by open triangles, except for having only cold Ca^{2+} (10 nmol/mg) and 4.8 μM (final concentration) diS-C_3-(5) added, was again added ruthenium red followed by 31 mM KCl, as for the sample indicated by open triangles. The fluorescence of this sample was recorded as a function of time by using an Aminco-Keirs fully corrected spectrometer as described under Materials and Methods. At the end of the experiment, the uncoupler, CCCP, was added to deenergize the mitochondria and to determine the level of fluorescence associated with deenergized mitochondria under the conditions of this experiment. One hundred percent change in CCCP-sensitive fluorescence should correspond to a change of membrane potential of approximately 160 mV as mentioned under Materials and Methods. The membrane potential change, observed during the course of this experiment, for those samples to which 31 mM external K^+ was added, was approximately 100 mV.

also be true for Ca^{2+} efflux rates measured after addition of enough ruthenium red to block mitochondrial Ca^{2+} influx.

Na^+-Independent Mitochondrial Ca^{2+} Efflux Is Not Affected by Change in Membrane Potential. Changes of transmembrane potential can be induced by adding large concentrations of K^+ to the suspending medium of respiring mitochondria treated with the ionophore valinomycin. As shown in Figure 2, there is no effect of such change of membrane potential on the rate of Ca^{2+} efflux, as determined by the ruthenium red addition method.

The results show that during a period in which the membrane potential changes (by approximately 100 mV) in the samples to which KCl was added, no significant difference can be detected in the rate of Ca^{2+} efflux between that sample and the other sample to which no KCl was added (and no change of membrane potential occurred). These data provide further support for the hypothesis that the Na^+-independent Ca^{2+} efflux mechanism is not influenced by the membrane potential (Fiskum & Lehninger, 1979).

Release of Ca^{2+} upon Acidification of the Medium. Past experiments describing Ca^{2+} release upon acidification of the suspending medium were performed by adding acid (HCl) to a mitochondrial suspension, preloaded with Ca^{2+} (Åkerman, 1978). These experiments were carried out before recognition of a possible small component of Na^+-dependent Ca^{2+} efflux, in the liver mitochondrial system (Haworth et al., 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982).

From the current prospective, it would seem preferable to perform these experiments by saturating any possible Na^+-dependent efflux, in order to eliminate pH changes in Ca^{2+} efflux due to the Na^+-dependent mechanism induced by acidification. Also, it would be preferable to acidify the suspensions by addition of buffer instead of by addition of acid, in order to minimize possible adverse effects on mitochondria caught in localized very low pH regions before adequate mixing has occurred.

Results of an experiment performed in this way are shown in Figure 3. ^{45}Ca efflux measurements were carried out as described under Materials and Methods. The suspending medium (E medium) was modified by including 40 mM NaCl to saturate any possible Na^+-dependent Ca^{2+} efflux while mannitol/sucrose was reduced to preserve the osmolarity. Solutions of Pipes and Hepes buffers were added to drop the pH from 7.4 to that desired for each part of the experiment.

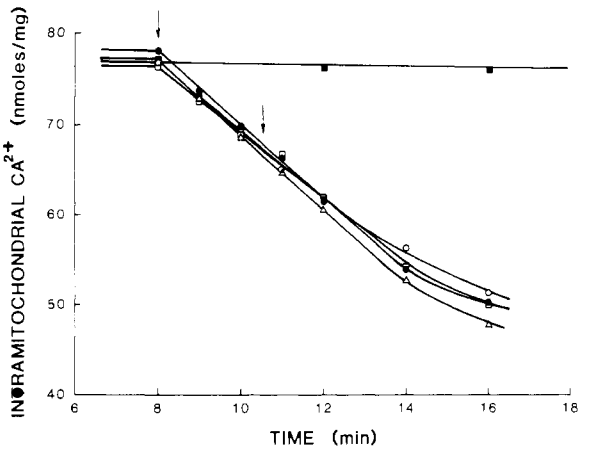

FIGURE 3: Effect of abrupt change of mitochondrial suspension pH caused by addition of buffers on mitochondrial Ca^{2+} efflux. Mitochondria (1 mg/mL) were suspended in 135 mM mannitol, 45 mM sucrose, 6 mM Hepes (pH 7.4), 40 mM NaCl, 3 mM acetate, and 3 mM succinate. Eighty nanomoles of labeled Ca^{2+}/mg of protein was added. Efflux was initiated by addition of 5 nmol of ruthenium red/mg of protein as indicated by the first arrow 8 min after Ca^{2+} addition. At the second arrow Pipes buffer (pH 6.00) was added (and the solution stirred) in amounts so as to abruptly drop the suspension pH to 7.2 (○), 7.0 (□), 6.8 (△), and 6.6 (●). There was no observable effect of this abrupt pH change on the rate of Ca^{2+} efflux.

The results show no observable release of Ca^{2+} upon acidification of the medium by addition of buffer. Similar results were obtained from an otherwise identical experimental in which K^+ was substituted for Na^+.

In control experiments in which HCl was added to the mitochondrial suspensions instead of buffer, it was possible to see a pulse of Ca^{2+} released from the mitochondria as reported by Åkerman (1978) particularly in those cases where the acid was added rapidly.

Simultaneous Measurement of Ca^{2+} Efflux and H^+ Influx. Because significant variability in the rates of ion flux between parallel experiments cannot be completely eliminated, it is preferable when the rates of Ca^{2+} efflux and proton influx are compared to measure both simultaneously in the same aliquot. To guard against artifacts due to altered states of the mitochondrial membrane (e.g., spontaneous decrease in activity of electron transport or leakiness of the membrane), simultaneous measurement of membrane potential is also desirable. Simultaneous measurement of these parameters was carried out with a set of Ca^{2+}, pH, and TPP^+ electrodes along with the necessary reference electrodes. Calibration of these three types of electrodes is described under Materials and Methods.

Rates of Ca^{2+} efflux measured by using the calibrated electrode technique were compared with rates of Ca^{2+} efflux measured with $^{45}Ca^{2+}$ and Millipore filtration, as described under Materials and Methods. In general, there was good agreement between efflux rates measured by these two techniques (i.e., the efflux rates measured by using ^{45}Ca fall within the observed experimental variance of similar data obtained by using the electrode technique) when allowance was made for the slower response time of the electrode technique.

Mitochondria often rapidly change the volume of their matrix compartments upon Ca^{2+} uptake. Depending on the concentration in the suspending medium of anions such as acetate, which can permeate the inner mitochondrial membrane in the uncharged (protonated) form, mitochondria can either shrink or swell as Ca^{2+} is sequestered. If the concentration of acetate-like anions is low and a barely permeant anion such as Cl^- is the dominant anion species, the mitochondria will shrink upon uptake of Ca^{2+}. If, on the other hand, the concentration of acetate-like anions is high, the mitochondria will swell upon uptake of Ca^{2+}. At an acetate concentration near 3 mM, minimum change of volume occurs (T. E. Gunter and K. K. Gunter, unpublished observations). It is convenient to measure ion fluxes under these latter conditions, in order to minimize the number of volume measurements necessary and to maximize the accuracy of volume estimation. Details of the volume measurement techniques used are given under Materials and Methods.

In measuring the relative stoichiometry of Ca^{2+} efflux and proton influx, proton flux due to phosphate and acetate movement occurs and complicates the interpretation of the experimental results. In earlier work (Fiskum & Lehninger, 1979) this problem was attacked by using neither acetate nor added phosphate in the media. While this is an effective way to minimize these undesired proton fluxes, mitochondrial water volume changes markedly under these conditions, and phosphate flux still occurs because of phosphate-generating reactions occurring in the mitochondrial matrix. In the experiments discussed below, approximately 3 mM acetate, but no added phosphate, is used, and the fluxes of [^{14}C]acetate and of phosphate are directly measured in the same or in a duplicate aliquot, under identical conditions, as described under Materials and Methods. This allows corrections to be made for those components of proton flux due to acetate and phosphate fluxes, while also allowing the measurements to be made under conditions of minimum volume change. The presence of acetate should also minimize formation of calcium–phosphate precipitates in the mitochondrial matrix.

Typical Ca^{2+} and OH^- efflux data from liver mitochondria, obtained after addition of ruthenium red to a mitochondrial suspension which had been maintaining [Ca^{2+}] in a steady state, are shown in Figure 4. OH^- efflux data corrected for the measured fluxes of acetate and phosphate, as described below, are also shown. The measurement techniques are as described under Materials and Methods, and the conditions of the experiment are as described in the figure caption. The data are shown as relative change of Ca^{2+} and of OH^- per milligram of mitochondrial protein in nanoequivalents from a zero point chosen to best represent the steady-state values of Ca^{2+} and OH^- following uptake. The data obtained within the period of rapid Ca^{2+} uptake and corresponding acidification of the suspending medium are suppressed in this figure to emphasize Ca^{2+} and OH^- efflux (or H^+ influx) following ruthenium red addition. This method of displaying the data allows perfect 1:2 Ca^{2+} for OH^- efflux or Ca^{2+} efflux for H^+ influx stoichiometry to show up as regions of parallel slope. Membrane potential measurements made by using the TPP electrode techniques, as described under Materials and Methods, are also shown at the top in each part of the figure.

It can be seen that the Ca^{2+}:H^+ stoichiometry can deviate quite significantly from 1:2. It was possible to observe a Ca^{2+}:H^+ stoichiometry of close to 1:2 at a pH value near 7, as previously reported (Fiskum & Lehninger, 1979; Fiskum & Lehninger, 1980), at a lower concentration of endogenous phosphate than that found for the mitochondria used in the experiment shown in Figure 4. No experiments have yielded a constant Ca^{2+}:H^+ stoichiometry over a range of pH.

Corrections to H^+ flux due to flux of phosphate and acetate were measured at each pH point studied under conditions similar to those used in Figure 4. Movement of both acetate and phosphate should be in response to the pH gradient (ΔpH) as it changes during Ca^{2+} release. It would be expected that both acetate and phosphate are released from the mitochondrial matrix with time during Ca^{2+} release, and this was indeed

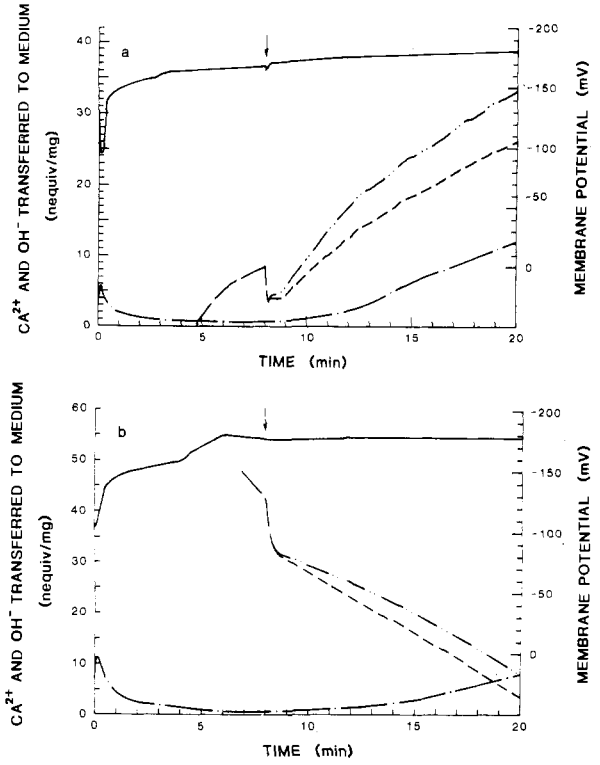

FIGURE 4: Ca^{2+} (-·-) and OH^- transfer into medium and membrane potential (—) vs. time. Uncorrected OH^- data are shown by (- - -), and OH^- data corrected for acetate and phosphate movement are shown by (-··-). OH^- efflux is treated as the equivalent of H^+ influx. Mitochondria (2 mg/mL) were suspended in 5 mL (total volume) of 195 mM mannitol, 65 mM sucrose, 0.1 mM $MgCl_2$, 3 mM potassium succinate, 3 mM potassium acetate, and 5 μM TPP-Cl. pH was adjusted to the desired range by addition of hydroxide ion prior to addition of mitochondria. If further pH adjustments were necessary after addition of mitochondria, the process was carried out by very slow addition of hydroxide ion, while the mitochondrial suspension was stirred. Calibration of the three electrodes used was carried out as described under Materials and Methods. Ca^{2+} (60 nmol/mg of protein) was added to the suspension at a time designated as time = 0. Ruthenium red (4 nmol/mg of protein) was added at the arrow at time = 8 min to inhibit influx over the uniport mechanism. pH at the time of ruthenium red addition was 7.47 (a) and 8.47 (b). The results are plotted as transfer of Ca^{2+} and OH^- to the medium in nanoequivalents per milligram, so that a 1:2 Ca^{2+} for OH^- stoichiometry would be indicated by parallel lines on these figures. The OH^- data were corrected by directly compensating for the effects on OH^- transfer to the suspending medium by the transfer of acetate and phosphate. The corrections were made as described under Materials and Methods. The error in the Ca^{2+} data are approximately ±7% and in the OH^- data approximately ±10% based on the variance of duplicate samples.

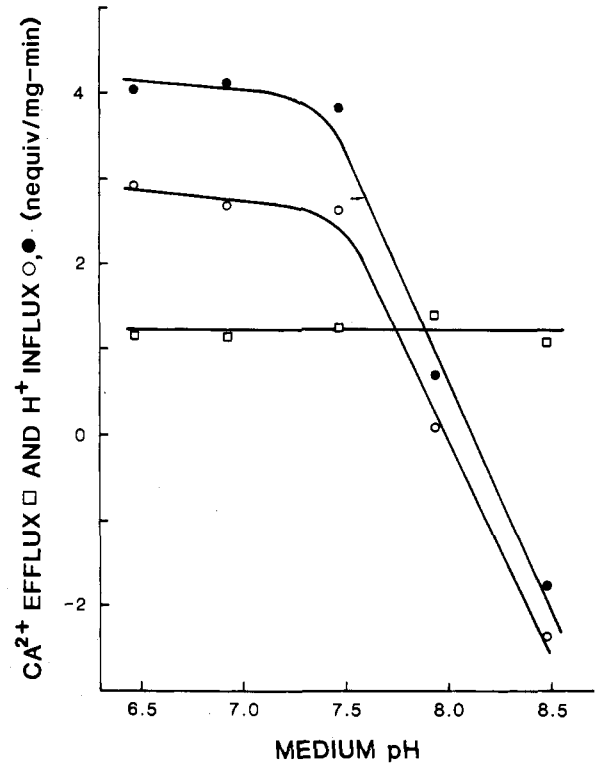

FIGURE 5: Ca^{2+} efflux (rate) and both uncorrected and corrected H^+ influx (rate) after ruthenium red addition as a function of medium pH for energized mitochondria. Fluxes were measured from slopes of a set of data, similar to those shown in Figure 4, for which the pH after Ca^{2+} uptake was as indicated by the data points. Ca^{2+} efflux is indicated by open squares (□). H^+ influx uncorrected for H^+ flux due to AcO^- and P_i movements is indicated by the open circles (○). H^+ influx corrected for H^+ fluxes due to AcO^- and P_i is indicated by the filled circles (●). The data shown represent the averages of the results of duplicate experiments. The values of standard deviations calculated from the variance of duplicate points were approximately 0.27 for the uncorrected OH data, 0.27 for the corrected OH data, and 0.10 for the Ca^{2+} data.

observed. By use of these acetate and phosphate data, rates of OH^- efflux (H^+ influx), such as those shown in Figure 4, can be corrected for effective H^+ or OH^- flux due to acetate and phosphate movement. Examples are shown in Figures 5 and 6.

Figure 5 shows typical results from simultaneous measurements of Ca^{2+} efflux and H^+ influx after ruthenium red addition, as a function of suspension pH, under energized conditions. H^+ influx data, corrected for the effects of acetate and phosphate flux, are shown as well. The conditions and techniques used in these measurements are similar to those used in the measurements shown in Figure 4. Figure 6 shows typical flux (rate) data from similar measurements carried out following deenergization of the mitochondria with antimycin A and subsequent fall of the membrane potential. Corrections for acetate and phosphate fluxes were also measured during these experiments, carried out under deenergized conditions in the same way as described for the energized samples. The observed acetate and phosphate fluxes were larger under these conditions, as can be seen from the size of the corrections to the OH^- flux data (Figure 6).

The ratios of H^+ influx to Ca^{2+} efflux in units of [nmol of H^+/(mg·min)]/[nmol of Ca^{2+}/(mg·min)], obtained from the data obtained by using energized mitochondria, range from around 7 at pH 6.5 to around −3.5 at pH 8.4 for the corrected data and from around 4.7 at pH 6.5 to around −4.5 at pH 8.4 for the uncorrected data. For the data obtained by using deenergized mitochondria the range is from 3.7 to −1.2 for the corrected data and from 2.0 to −1.5 for the uncorrected data over the same pH range.

Several observations are of interest with regard to the data shown in Figures 5 and 6. First, coincidence of the lines fit to the data, representing 1:2 Ca^{2+} efflux to H^+ influx stoichiometry, only occurs at one point in each figure. The pH value at which this coincidence occurs depends on the endogenous phosphate present in the preparation, as would be expected from the dependence of Ca^{2+} efflux (rate) on phosphate concentration as described by Zoccarato & Nicholls (1982). There is no tendency for the lines to overlie one another over any range of pH, as would be expected if the experimentally observed stoichiometry represented a 1:2 Ca^{2+} for H^+ exchange. These data suggest that the 1:2 Ca^+ or H^+ exchange stoichiometry, obtained at one value of pH, was coincidental and should not, therefore, be used as support for the passive exchanger hypothesis. Simple leakiness of the

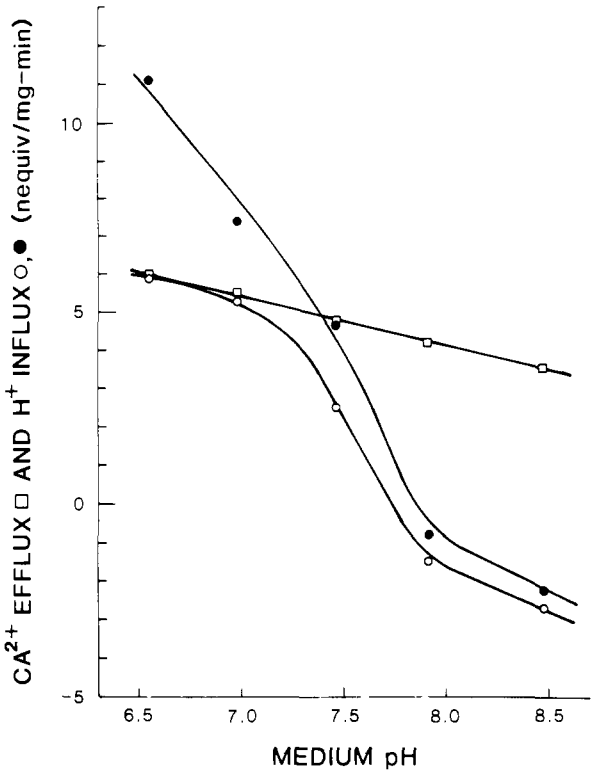

FIGURE 6: Rate of appearance of Ca^{2+} in and disappearance of H^+ from the medium as a function of medium pH for deenergized mitochondria in the presence of ruthenium red. Ruthenium red was added 30 s prior to addition of antimycin A. Fluxes were measured from slopes of a set of data, similar (except for deenergization) to those shown in Figure 4, immediately after the membrane potential reached zero. The pH after antimycin A addition was as is indicated by the data points on these experiments. Ca^{2+} efflux is indicated by open squares (□). H^+ influx uncorrected for H^+ flux due to AcO^- and P_i movements is indicated by open circles (○), and H^+ influx corrected for H^+ flux due to AcO^- and P_i movements is indicated by the filled circles (●). The data shown represent the averages of the results of duplicate experiments. The values of standard deviations calculated from the variance of duplicate points were larger for the rates of higher absolute value than for the lower rates. The average values for these standard deviations converted to percentages of the average were approximately 16.0% for the uncorrected OH data, 17.6% for the corrected OH data, and 14.3% for the Ca^{2+} data.

membrane to H^+ is likely responsible for a component of the measured H^+ flux. These data demonstrate that the situation is more complex than has been heretofore suggested. Since without specific inhibitors of the various mechanisms of H^+ and of Ca^{2+} flux it is not possible to associate a given amount of H^+ flux with Na^+-independent Ca^{2+} efflux, it should likewise not be claimed that these data, per se, disprove the passive Ca^{2+}–$2H^+$ exchange hypothesis.

Second, in the data shown here, the rate of unidirectional, ruthenium red insensitive Ca^{2+} efflux under energized conditions does not show a dependence on the pH of the suspending medium. $^{45}Ca^{2+}$ efflux measurements (data not shown), performed as described under Materials and Methods, were used to corroborate the electrode measurements of Ca^{2+} efflux. The range of variation of Ca^{2+} efflux observed by using these two techniques was similar and was thought to be due primarily to variation in levels of endogenous phosphate (Zoccarato & Nicholls, 1982). The range of variation of Ca^{2+} efflux with pH for energized samples was between zero and a maximum of about a 2-fold increase in Ca^{2+} efflux with a 400-fold increase in suspension H^+ activity, measured with both electrode and isotope techniques. For deenergized samples, the dependence of Ca^{2+} efflux on suspension H^+ activity was slightly larger, as is shown in Figure 6. Whether or not the very small dependence of Ca^{2+} efflux on suspension pH with energized samples is real, even the maximum dependence observed in any set of data is very small indeed. Some increase in the rate of Ca^{2+} efflux with decreasing pH might be expected, since with decreasing pH, intramitochondrial divalent cation binding decreases and the amount of free intramitochondrial divalent cation increases rapidly (Gunter & Puskin, 1975). This variation in intramitochondrial free Ca^{2+} with pH could easily explain the small dependence of Ca^{2+} efflux on medium pH, observed in some cases with energized samples. In view of the weakness of this dependence, it is very unlikely that the explanation is passive Ca^{2+}–nH^+ exchange.

Similarly, if liver mitochondria responded to a pulse of acid by rapid release of intramitochondrial Ca^{2+} through a Ca^{2+}–nH^+ exchanger, a larger dependence of Ca^{2+} efflux on medium pH would be expected than was observed in the data of Figure 5. These observations then corroborate the results shown in Figure 3 and suggest that one of the alternative explanations for the acid pulse data, discussed in the introduction, is valid instead of the passive Ca^{2+}–nH^+ exchanger hypothesis.

Third, given the large buffering power of the mitochondrial matrix (Gear et al., 1967), the observed H^+ fluxes, particularly in the nonenergized case, behave as expected for leakage down the electrochemical potential gradient.

Since the problems caused by electron transport and subsequent outward transport of protons are eliminated by metabolically inhibiting the mitochondria, the use of deenergized conditions (as in Figure 6) might represent a better protocol in experiments seeking to measure transport stoichiometry. Unfortunately, the data shown in Figure 6 demonstrate that even under these simplified conditions, the situation is still too complex to allow any firm conclusion with regard to transport stoichiometry to be drawn.

Over 60 experiments of the type shown in Figure 4 were performed in the course of this work. While much of the relevant information obtained from those experiments is shown in Figures 4–6, there is an additional observation to be made concerning these data, taken as a whole. Measurement of initial rates of efflux under both active and passive conditions over a range of external pH were primary goals of this work. Consequently, deenergization (with antimycin A) was usually carried out under conditions where most of the Ca^{2+} initially sequestered by the mitochondria was still in the mitochondrial matrix at the time of deenergization. This left a high (internal to external) gradient of Ca^{2+} chemical potential at the time of deenergization. Under these conditions, the rate of Ca^{2+} efflux usually increased upon deenergization. On those few occasions, however, when most of the Ca^{2+} initially in the mitochondria had already been released by the time of deenergization, the Ca^{2+} efflux rate often decreased upon deenergization. In other words, where the Ca^{2+} chemical potential gradient was large, the rate of Ca^{2+} release from the matrix increased upon deenergization; where the Ca^{2+} chemical potential gradient was not as large, the rate of Ca^{2+} release from the matrix decreased.

Response of Ca^{2+} Efflux to Changes in Intramitochondrial pH. Experiments were also carried out to investigate the response of the ruthenium red insensitive mitochondrial Ca^{2+} efflux system to changes in intramitochondrial pH. One object was to keep conditions as similar as possible within the set of experiments except for variation of the pH gradient over a range of over 1 pH unit. In the experiments described here, it was decided to keep the amount of Ca^{2+} taken up constant,

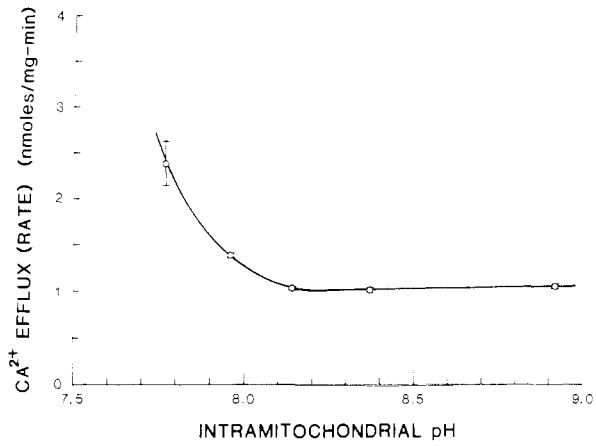

FIGURE 7: Rate of Ca^{2+} efflux vs. intramitochondrial pH. Mitochondria (4 mg/mL) were suspended in 195 mM mannitol, 65 mM sucrose, 20 mM Hepes (pH 7.4), and 3.0 mM succinate. Varying amounts of acetate were used. Ca^{2+} (80 nmol/mg of protein) was added, 8 min was allowed for complete uptake to occur, and ruthenium red (5 nmol/mg of protein) was added to inhibit Ca^{2+} uptake over the uniport. Aliquots of each sample were periodically taken and filtered to determine the rate of Ca^{2+} efflux from the mitochondria. Mitochondrial volume measurements (using [^{14}C]sucrose) and pH gradients (using [^{14}C]acetate) were carried out on parallel samples containing the isotopic label of interest as is described under Materials and Methods.

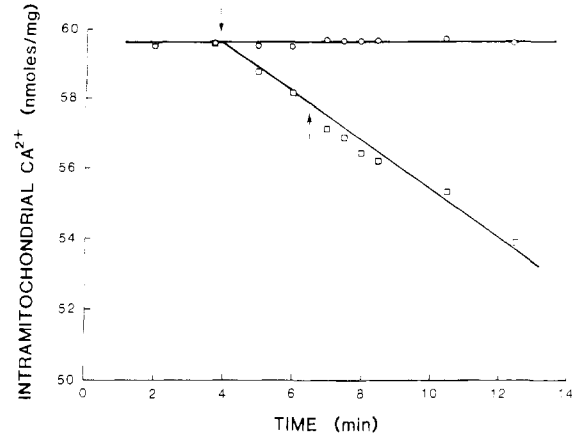

FIGURE 8: Intramitochondrial Ca^{2+} vs. time. Mitochondria (1 mg/mL) were suspended in 195 mM mannitol, 65 mM sucrose, 6 mM Hepes (pH 7.4), 6 mM acetate, and 3 mM succinate. Efflux was induced by addition of (4 nmol/mg of protein) ruthenium red, as indicated by the arrow at 4 min after addition of 60 nmol/mg of protein of labeled Ca^{2+}. Addition of 8000 nmol of cold Ca^{2+}/mg of protein (□) occurred at the second arrow (6.5 min after initial addition of the labeled Ca^{2+}). (○) indicates control points to which no further additions were made. Aliquots were sequentially withdrawn and filtered to determine the amount of labeled Ca^{2+} in the suspending medium.

prior to initiation of net efflux by ruthenium red addition, and to vary the pH gradient by varying the amount of acetate present in each aliquot. External pH was kept constant by use of an adequate concentration of Hepes buffer (20 mM at pH 7.4) and by adjusting pH prior to ruthenium red addition to each aliquot.

pH gradient was determined by measuring the intramitochondrial and external concentrations of [^{14}C]acetate in a triplicate set of aliquots, identical with those used in the Ca^{2+} efflux experiments, except for containing small amounts of [^{14}C]acetate (see Materials and Methods). Multiple intramitochondrial water volume measurements were performed on another triplicate set of aliquots, during the course of each experiment, as described under Materials and Methods. $^{45}Ca^{2+}$ efflux was measured at a series of times following ruthenium red addition, as described under Materials and Methods.

Figure 7 shows the rate of Ca^{2+} efflux plotted against intramitochondrial pH just before ruthenium red addition measured as described above. As is seen in the figure, Ca^{2+} efflux appears to decrease as intramitochondrial pH increases.

A passive exchanger such as the putative Ca^{2+}–$2H^+$ exchanger can be driven by the gradients of either of the transported ionic species. Consequently, if the Na^+-independent efflux mechanism under consideration here is indeed a passive exchanger, Ca^{2+} efflux would be expected to increase with increases in the pH gradient. As has been seen in Figure 5 above, it does not respond to decreases in medium pH; therefore, it might be expected to increase with increasing intramitochondrial pH (at constant external pH). Such an effect might be caused for example by a decrease in competition for binding and transport between Ca^{2+} and H^+ on the matrix side of the membrane due to the increased pH. Figure 7, however, shows no such increase of Ca^{2+} efflux with an increase in internal pH.

Two effects could complicate the interpretation of these results. First, whether or not the Na^+-independent efflux mechanism functions as a passive Ca^{2+}–$2H^+$ exchanger, there could be competition for binding between Ca^{2+} and H^+ to the mechanism with a resulting increase in Ca^{2+} efflux with internal pH. Such competition would be expected for a Ca^{2+}–$2H^+$ exchanger but could not be ruled out even if the H^+ were not transported over this mechanism.

Second, it is known that intramitochondrial divalent cation binding increases with increasing matrix pH (Gunter & Puskin, 1975). This effect could very well account for the decrease in Ca^{2+} efflux with increasing intramitochondrial pH observed in Figure 7.

Exchange Properties of the Na^+-Independent Ca^{2+} Efflux System. Those passive Ca^{2+}–$2H^+$ exchangers which have been best characterized heretofore, namely, the bacterial ionophores A23187 and X537A, exchange Ca^{2+} for H^+ across biological membranes because these ions are high in the selectivity series for the respective ionophores and are present in relatively high concentration on their respective initial sides of the membrane. These ionophores can exchange Ca^{2+} for Ca^{2+} or H^+ for H^+ as easily as Ca^{2+} for $2H^+$ under the proper conditions. This suggests an additional method which could be used to test for the presence of a passive Ca^{2+} exchanger, namely, to test for the presence of Ca^{2+}–Ca^{2+} exchange.

While it is not mandatory that every passive Ca^{2+} exchanger be able to exchange Ca^{2+} for Ca^{2+}, it is difficult to conceive of any likely mechanism which would not do so. Hence, a test for Ca^{2+}–Ca^{2+} exchange is relevant to the question of whether the mechanism is a passive Ca^{2+}–$2H^+$ exchanger. The test for Ca^{2+}–Ca^{2+} exchange was carried out similarly to the acid pulse experiments described by Åkerman (1978). $^{45}Ca^{2+}$ (80 nmol/mg of protein) was loaded into the mitochondria with succinate as substrate. After uptake reached the steady-state phase, ruthenium red was added to induce net efflux of the labeled Ca^{2+}. After a steady rate of Ca^{2+} efflux had been attained, cold Ca^{2+} was added in increasing amounts to a set of aliquots. The range of Ca^{2+} concentrations in the additions was chosen so as to cover and exceed the intramitochondrial Ca^{2+} chemical activity. If the mechanism of Ca^{2+} efflux were a passive Ca^{2+} exchanger, it would be expected that addition of Ca^{2+} to the suspending medium would drive transport of Ca^{2+} inward in exchange for labeled intramitochondrial Ca^{2+}.

Figure 8 shows the results of one of these experiments in which 8000 nmol of Ca^{2+}/mg of protein or 8 mM external Ca^{2+} is added. As can be seen even the addition of such a large

pulse causes no significant change in the observed rate of Ca^{2+} efflux.

Discussion

It is reassuring, at the present level of understanding of mitochondrial Ca^{2+} efflux, to find that experiments such as that described and shown in Figure 1 support the validity of the commonly employed techniques for measurement of efflux, i.e., EGTA or ruthenium red addition. While it would have been better if this experiment could have been carried out at a higher temperature, the rate of internalization of label was too fast at higher temperature to permit the necessary experimental accuracy. The mitochondria were energized, however, at the temperature used (4 °C). The membrane potential of mitochondria at this temperature has been found by measurements using the tetraphenylphosphonium electrode to be about the same as at 23 °C (data not shown). Furthermore, artifactual effects of EGTA or ruthenium red on mitochondrial Ca^{2+} influx and efflux would most probably be caused either by chelation of Ca^{2+} associated in a functional way with the transportase or by direct binding of the agent itself with the transport complex, so as to modify the rate of transport. If these effects exist at room temperature, they would also be expected to occur at the lower temperature used.

The lack of a significant effect on the rate of mitochondrial Ca^{2+} efflux caused by large transient variations in transmembrane potential is truly surprising, whatever the mechanism of efflux. If the transport mechanism is active, it might be expected that the greatly increased rate of electron transport induced by valinomycin-mediated K^+ uptake should have some effect. This is not definitive since an active mechanism of energy transduction for Ca^{2+} efflux is at this point hypothetical and the characteristics of such a mechanism are speculative.

If the mechanism of mitochondrial Ca^{2+} efflux is a passive Ca^{2+}–$2H^+$ exchanger, the response is even more difficult to understand. Internalization of K^+ under the conditions of this experiment, mediated by valinomycin, causes rapid alkalinization of the mitochondrial matrix and acidification of the suspending medium. This is because outward proton pumping, energized by electron transport, is used by the mitochondrion to reestablish $\Delta\psi$ under these conditions. Such changes would be expected to lead to an increased rate of Ca^{2+} efflux through a passive Ca^{2+}–$2H^+$ exchanger.

These data suggest that the dependence of Ca^{2+} efflux on both external and internal pH should be investigated more closely.

In an apparent contradiction to data shown above, Zoccarato & Nicholls (1982) found Ca^{2+} efflux through the Na^+-independent mechanism to be substantially increased upon deenergization with uncoupler, while we report no substantial change in efflux upon variation of membrane potential with valinomycin and K^+ (Figure 2). However, we also observe an increase in efflux upon treatment with uncoupler (data not shown), consistent with results of the earlier workers, when sufficient uncoupler to cause a complete breakdown of membrane potential is used. Complete deenergization using the metabolic inhibitors antimycin A or cyanide causes the same effect. It is possible that these observations may be related to a modification of the efflux mechanism or to the membrane itself by complete deenergization.

Jurkowitz & Brierley (1982) and Jurkowitz et al. (1983) have recently published results of studies of the characteristics of ruthenium red insensitive Ca^{2+} efflux from beef heart mitochondria suspended in Na^+-free medium. They found that uncoupler induced rapid ruthenium red insensitive release of Ca^{2+} from heart mitochondria, which had been treated with acetoacetate or in which adenine nucleotides or NADPH had been depleted. This Ca^{2+} release was always accompanied by swelling of the mitochondria and loss of endogenous Mg^{2+} and was associated by the authors with mitochondrial membrane changes observed earlier by others (Beatrice et al., 1980).

It is tempting to speculate that the effects observed by Jurkowitz & Brierley (1982) and by Jurkowitz et al. (1983) are related to those described by Zoccarato & Nicholls (1982) and by our group following addition of uncoupler. Apparently, partial deenergization using valinomycin and K^+ as shown in Figure 2 above is not sufficient to induce these changes. It may also be true that the changes induced by uncoupler in the work of Jurkowitz et al. (1983) are related to the changes induced in our work by complete deenergization with antimycin A (Figure 6), although the conditions of the experiments of Jurkowitz et al. (1983) differ substantially from those reported here.

The observation of no rapid release of Ca^{2+} (Figure 3) when the medium is acidified by addition of buffer suggests that the Ca^{2+} release induced by direct acid addition, observed in past experiments (Åkerman, 1978), resulted from damage to a small fraction of the mitochondria of the aliquot by the acid. If one postulates a Ca^{2+}–$2H^+$ exchange to account for the rapid transient release of Ca^{2+} with a rapid decrease of medium pH, then it must follow that the rate of Na^+-independent Ca^{2+} efflux should increase significantly as medium pH decreases. The observation, in Figure 5, that it does not do so then supports the view suggested by the data of Figure 3 that one of the alternate possibilities is true instead.

The observation that the Ca^{2+} efflux to H^+ influx stoichiometry is near 1:2, at one value of pH, which can be near pH 7, while corroborating the data obtained in earlier work (Fiskum & Lehninger, 1979), likewise invalidates the earlier inference that this transport is mediated by a passive Ca^{2+}–$2H^+$ exchanger under the conditions of these experiments. If stoichiometric data can be said to support the Ca^{2+}–$2H^+$ exchanger mechanism, it must be because no other operative mechanism, contributes significantly to the permeability of either Ca^{2+} or H^+ under the conditions of measurement. If this condition is violated, then the observed stoichiometry may have little to do with the mechanism of interest. If the condition is satisfied, then it would be expected that the 1:2 stoichiometry would be observed over a range of pH near the physiological pH, but as can be seen from Figures 5 and 6, it is not. It must then be concluded that this type of data obtained under the conditions used in these experiments offers no significant support for the passive Ca^{2+}–$2H^+$ exchanger hypothesis. The data in Figures 5 and 6 do not disprove the passive Ca^{2+}–$2H^+$ exchanger hypothesis but imply that much more is occurring, especially with respect to H^+ permeability, than simple Ca^{2+} for H^+ exchange. Oxaloacetate and acetoacetate were used in the work of Fiskum & Lehninger (1979) to "induce" higher activity in the Na^+-independent Ca^{2+} efflux mechanism and not in the work reported here. While the use of oxaloacetate and acetoacetate has been criticized for causing a modification in the mitochondrial membrane, which causes a decrease of membrane potential (Beatrice et al., 1980; Nicholls & Brand, 1980), it is also possible that such agents do induce a change in the mechanism of Ca^{2+} efflux, causing the characteristics of the mechanism to differ under the conditions used by Fiskum & Lehninger (1979) and under those used in this work.

If the Na^+-independent Ca^{2+} efflux mechanism of liver mitochondria were a passive Ca^{2+}–$2H^+$ exchanger, then Ca^{2+} efflux must be driven by some combination of the intramito-

chondrial Ca^{2+} chemical potential, a high proton chemical potential in the suspending medium, or a low intramitochondrial proton chemical potential. The data shown in Figure 5 above, showing that Ca^{2+} efflux does not depend on the pH of the suspending medium, eliminate the possibility that a high proton chemical potential in the suspending medium is a significant driving force under the conditions of these experiments.

It might be argued that the major effect of ΔpH upon Ca^{2+} efflux could be due to the alkalinization of the mitochondrial matrix. The correlation between the rate of Ca^{2+} efflux and pH change might then be sought as an increase in the rate of Ca^{2+} efflux with an increase in matrix pH or with ΔpH at constant external pH. The heart of this argument is that higher matrix pH could lead to less competition for binding to the exchanger between H^+ and Ca^{2+} on the inner surface of the transport membrane and thus lead to more rapid Ca^{2+} efflux. The data shown in Figure 7, however, clearly show that at constant external pH, the rate of release of intramitochondrial Ca^{2+} does not increase with increasing intramitochondrial pH, but it decreases.

The results in Figure 8 show that, under the experimental conditions used, the liver mitochondrial Ca^{2+} efflux system will not catalyze Ca^{2+}–Ca^{2+} exchange. If the liver mitochondrial Ca^{2+} efflux mechanism is a passive Ca^{2+}–$2H^+$ exchanger, it must be an exchanger of a different type than A23187 or X537A or of any other passive exchanger known. Even if such an exchanger has separate obligatory sites for binding Ca^{2+} and H^+, it is difficult to see why it cannot exchange intramitochondrial for external Ca^{2+} under the experimental conditions outlined in the caption of Figure 8. These conditions were chosen to minimize the transmembrane pH gradient and to set up an external to internal free Ca^{2+} gradient. The endogenous Ca^{2+} influx mechanism of mitochondria (passive uniporter) can be induced to rapidly exchange internal and external Ca^{2+}. This is a property of most passive, carrier-like systems (Åkerman, 1978).

In summary, there is no trustworthy data supporting the hypothesis that a passive Ca^{2+}–$2H^+$ exchanger catalyzes the release of Ca^{2+} from liver mitochondria. The behavior of this Ca^{2+} efflux mechanism in energized mitochondria has been seen to be unlike that of any known passive exchanger. The magnitude of the flux of inorganic phosphate measured in order to provide a correction to H^+ flux data is much too small to account for the observed rates of efflux of Ca^{2+} through a simple symport mechanism. This is consistent with the conclusions of Zoccarato & Nicholls (1982).

The work of Gunter et al. (1978b) with submitochondrial particles and of Rosier et al. (1981) with a partially reconstituted mitochondrial Ca^{2+} transport system has suggested that an active Na^+-independent Ca^{2+} transport mechanism may be present in mitochondria. Rosier et al. (1981) discussed this hypothetical active mechanism as if it were electrogenic; however, their experimental results would also be consistent with an active exchanger mechanism.

The results of this past work (Gunter et al., 1978b; Rosier et al., 1981) and of the work reported here leave the concept of an active liver mitochondrial Ca^{2+} efflux mechanism as a possible hypothesis. It may be that the unresponsiveness of this efflux system to transient changes in transmembrane potential reflects a system which is of a nonelectrogenic type (as a 1:2 exchanger would be) and which, as an active system, is practically independent of ΔpH as a thermodynamic driving force.

It is possibe that upon deenergization this transport system is released (at least partially) from its energy transduction mechanism and begins to function passively. This is consistent with the change in the dependence of the rate of Ca^{2+} release upon medium pH upon deenergization (Figures 5 and 6). While the stoichiometry of Ca^{2+} efflux to H^+ influx under deenergized conditions (Figure 6) is still not 1:2 over a significant range of medium pH, a small but significant dependence of Ca^{2+} efflux upon medium pH is apparent. This point of view could also be consistent with the observation that the rate of Ca^{2+} release increases upon deenergization when the Ca^{2+} chemical potential gradient (a passive driving force) is high. Likewise, it could also explain why the rate of Ca^{2+} release might decrease upon deenergization if the Ca^{2+} chemical potential gradient is low, as is sometimes observed.

The energy contained in both the membrane potential and in the proton chemical potential is important in the process of oxidative phosphorylation. Since both oxidative phosphorylation and precise control of cytosolic Ca^{2+} are mandatory for proper function of most eukaryotic cells, it seems plausible that the cell might need more flexible control over the system responding most rapidly to changes in cytosolic Ca^{2+} concentration (the mitochondrion) than could be provided by a combination of passive Ca^{2+} transport mechanisms. The uniporter plus passive Ca^{2+}–$2H^+$ exchanger system would be controlled by precisely the same thermodynamic forces as would oxidative phosphorylation.

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