Calcium-Ion Transport by Intact Ehrlich Ascites-Tumour Cells

ROLE OF RESPIRATORY SUBSTRATES, P_i AND TEMPERATURE

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1. The interaction of intact Ehrlich ascites-tumour cells with Ca^{2+} at $37^\circ C$ consists of Ca^{2+} uptake followed by efflux from the cells. Under optimum conditions, two to three cycles of uptake and efflux are observed in the first 15 min after Ca^{2+} addition. 2. The respiratory substrates malate, succinate and ascorbate plus *p*-phenylenediamine support Ca^{2+} uptake. Ca^{2+} uptake at $37^\circ C$ is sensitive to the respiratory inhibitors rotenone and antimycin A when appropriate substrates are present. Ca^{2+} uptake and retention are inhibited by the uncoupler S-13. 3. Increasing extracellular P_i (12 to 30 mM) stimulates uncoupler-sensitive Ca^{2+} uptake, which reaches a maximum extent of 15 nmol/mg of protein when supported by succinate respiration. Ca^{2+} efflux is partially inhibited at 30 mM- P_i . 4. Optimum Ca^{2+} uptake occurs in the presence of succinate and P_i , suggesting that availability of substrate and P_i are rate-limiting. 5. Ca^{2+} uptake occurs at $4^\circ C$ and is sensitive to uncouplers and oligomycin. Ca^{2+} efflux at this temperature is minimal. These data are consistent with a model in which passive diffusion of Ca^{2+} through the plasma membrane is followed by active uptake by the mitochondria. Ca^{2+} uptake is supported by substrates entering respiration at all three energy-coupling sites. Ca^{2+} efflux appears to be an active process with a high temperature coefficient.

Ca^{2+} transport has been extensively studied in isolated mitochondria (Lehninger *et al.*, 1967; Chance, 1965), but relatively few studies have been performed on intact cells. We wished to investigate Ca^{2+} transport in a cell that does not specialize in the transport of Ca^{2+} , namely the Ehrlich–Lettré hyperdiploid ascites-tumour cell. These tumours, grown in suspension in the peritoneal cavities of mice, produce large numbers of genetically uniform cells. Several parallel studies can be performed with the same cell batch, which minimizes variability. These tumour cells have been well-characterized in studies of the regulation of metabolism. Since Ca^{2+} is involved in the regulation of glycolysis (Bygrave, 1967) and respiration (Lehninger *et al.*, 1967) we decided to study the interaction of Ca^{2+} with these cells. Because of the above-mentioned qualities of these cells, they provide an excellent system for the development of a model for the cellular dynamics of Ca^{2+} movement in intact non-specialized cells.

Studies by ourselves and others have shown that Ca^{2+} uptake by intact Ehrlich ascites-tumour cells can be observed at $25^\circ C$ during the first few minutes after the addition of Ca^{2+} (McCoy *et al.*, 1976;

Cittadini *et al.*, 1971, 1973; Landry & Lehninger, 1976). Further, Thorne & Bygrave (1973) reported that mitochondria from Ehrlich ascites-tumour cells, in contrast with liver mitochondria, did not release Ca^{2+} that had been actively accumulated. By contrast, Cittadini *et al.* (1973) reported that Ca^{2+} accumulated by intact Ehrlich ascites-tumour cells was subsequently released. The purpose of these studies was to clarify the time course of Ca^{2+} uptake and efflux and to determine which cell components participate in cellular Ca^{2+} transport by the use of various substrates and specific inhibitors.

Cittadini *et al.* (1973) had reported that 5 mM-glutamate plus 5 mM-malate supported Ca^{2+} uptake by Ehrlich ascites-tumour cells. However, Landry & Lehninger (1976) failed to observe Ca^{2+} uptake supported by malate. We set out to resolve this discrepancy, and thus to determine whether substrates feeding electrons into the respiratory chain at each of the three energy-coupling sites were capable of supporting Ca^{2+} uptake. Since glutamate can give rise to succinate via the tricarboxylic acid cycle, we used malate alone. Permeant anions, such as P_i , are known to be involved in mitochondrial Ca^{2+} transport, so the effect of P_i on cellular Ca^{2+} transport was also studied. Another parameter of interest was the temperature-dependence of Ca^{2+} transport, in view of the fact that mitochondrial uptake has been found

Abbreviations used: Mops, 4-morpholinepropanesulphonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene.

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to be less temperature-dependent than the phosphorylation of ADP and other enzymic reactions (Chance, 1965). Preliminary reports of some of these observations have been published (Hines *et al.*, 1976; Charlton & Wenner, 1977).

Experimental

Ehrlich (Ehrlich-Létré hyperdiploid) ascites-tumour cells were maintained and harvested as reported by Wenner *et al.* (1958). The harvested cells were centrifuged at 100g for 5 min at room temperature (21°C) in a Clay-Adams (Parsippany, NJ, U.S.A.) clinical centrifuge, then washed twice and resuspended in an ice-cold Mops physiological buffer consisting of 100 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂ and 10 mM-Mops (pH 6.5 or 7.4). We found that the 2 mM-MgCl₂ did not inhibit cellular Ca²⁺ uptake, in contrast with the findings of Cittadini *et al.* (1973). In addition, it prevented the cells from becoming leaky when stored in ice for a short time. Protein concentration was measured by a biuret method (Gornall *et al.*, 1949), in the presence of 0.5% sodium deoxycholate. The cells were kept in an ice bath and used as soon as possible.

Ca²⁺ uptake was measured with ⁴⁵Ca²⁺ (New England Nuclear Corp., Boston, MA, U.S.A.) as a tracer at a specific radioactivity of 3.1 Ci/mol. The reaction mixture contained ascites cells (5–11 mg of protein/ml), 0.1 mM-CaCl₂, labelled with ⁴⁵Ca²⁺, other reactants (made up in Mops physiological buffer where possible), and Mops physiological buffer to a final volume of 1.0 ml (pH 6.5 or 7.4). The reaction was started by adding cells preincubated for 10 min at the desired temperature. Reactions were carried out in a Dubnoff metabolic shaking water bath (Precision Scientific, Chicago, IL, U.S.A.). The reaction was terminated by vacuum filtration of a 50 µl sample on a nitrocellulose filter (pore size 0.45 µm) followed immediately by washing with 10 ml of 0.15 M-NaCl. The reaction is stopped in approx. 1 s, and the washing, to remove extracellular Ca²⁺, takes approx. 5 s. The filters were air-dried and counted for radioactivity in a Beckman LS-230 liquid-scintillation counter (Fullerton, CA, U.S.A.) in 10 ml of a scintillation mixture consisting of 4.0 g of PPO and 0.1 g of POPOP/litre of toluene. The ⁴⁵Ca²⁺ samples were counted with a totally open window. Vacuum-filtration methods have been used by ourselves and others to measure rapid Ca²⁺ transport in intact cells and in isolated mitochondria (McCoy *et al.*, 1976; Landry & Lehninger, 1976). This method is superior to centrifugation methods because of the shorter time intervals possible between samplings and because of the minimal Ca²⁺ efflux during washing as opposed to centrifugation. Methods using Murexide, a dye whose spectrophotometric properties change on binding Ca²⁺, have been used to continuously monitor

extracellular Ca²⁺ (Cittadini *et al.*, 1973). However, J. Cheney (unpublished work) of this laboratory has found that there is overlap between the spectra of Ca²⁺ and Mg²⁺ measured with this dye.

Hines & Wenner (1977) reported that Ca₃(PO₄)₂ precipitates do not form at 25°C in solutions of ionic strength less than 0.21 with the Ca²⁺ and P_i concentrations used in the present studies. All of these experiments were carried out at ionic strengths of 0.16 or less, and no precipitates were ever observed.

For all Figures [except 1c, 2b and 5 (open circles)] duplicate samples were taken. The lines are drawn through the arithmetic mean of the duplicate points. When the duplicates were too close to be drawn in, only the mean is shown. The magnitude of error in those Figures in which duplicates are not shown was the same or less than in those Figures in which duplicates are shown. All Figures represent typical results of repeated experiments.

Mops was from the United States Biochemical Corp., Cleveland, OH, U.S.A., and oligomycin from Sigma Chemical Co., St. Louis, MO, U.S.A. Rotenone was from S. B. Penick and Co., New York, NY, U.S.A., and *p*-phenylenediamine was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. The uncoupler S-13 (3-*t*-butyl-5-chloro-2'-chloro-4'-nitrosalicylanilide) was kindly given by Dr. P. Hamm (Monsanto Chemical Co., St. Louis, MO, U.S.A.). L-Malic acid was from the Wisconsin Alumni Research Foundation, Madison, WI, U.S.A. All other chemicals were of reagent grade.

Results

Dependence of Ca²⁺ transport on cell concentration

Cell concentrations of 5–16 mg of protein/ml gave highly reproducible results. A slight increase in Ca²⁺ uptake (nmol/mg of protein) was observed with increasing cell concentration within this range (results not shown). Some day-to-day variability was found in the absolute magnitude of Ca²⁺ uptake. This was presumably due to differences in the number of days between inoculation and harvest of cells (7–9), differences between mice in the progress of the ascites tumour and other biological variations. The values presented are typical results of repeated experiments. Ca²⁺ uptake was measured after preincubation with no CaCl₂ or 0.5 mM-CaCl₂ for 10 min or 45 min after isolation of cells from the mice. No significant differences in magnitude of Ca²⁺ uptake were observed, indicating that the cells preincubated without Ca²⁺ were not significantly more permeable to Ca²⁺ than cells preincubated with Ca²⁺.

Dependence of Ca²⁺ transport on energy state

The respiratory inhibitor rotenone inhibits endogenous substrate-supported Ca²⁺ uptake by 60% at

Fig. 1. Ca²⁺ uptake supported by respiratory substrates at 37°C

The reaction mixture was as described in the Experimental section. (a) Protein concentration was 10.3 mg/ml, the pH was 6.5 and 10 μM-rotenone was present in both cases. ●, Control; ▲, 10 mM-succinate. (b) Protein concentration was 9.5 mg/ml, the pH was 7.4 and 10 μM-rotenone was present in both cases. ●, Control; ▲, 1.5 mM-ascorbate plus 1 mM-*p*-phenylenediamine. (c) Protein concentration was 8.7 mg/ml, the pH was 7.4 and 5 mM-malate was present in both cases. ●, Control; ▲, 10 μM-rotenone.

Fig. 2. Effect of malonate and the uncoupler S-13 on succinate-supported Ca²⁺ uptake at 37°C

The reaction mixture was as described in the Experimental section, with 10 μM-rotenone and 10 mM-succinate present in all cases. (a) Protein concentration was 9.4 mg/ml and the pH was 7.4. ●, Control; ○, 1 mM-malonate; ▲, 10 mM-malonate. (b) Protein concentration was 8.3 mg/ml, the pH was 6.5 and 1.88 μM-uncoupler S-13 was added when indicated. ●, Control I; ▲, uncoupler S-13 present from zero time; ○, Control II; △, uncoupler S-13 added at 1.25 min.

37°C, and antimycin A is also inhibitory (results not shown). Rotenone was used to establish a baseline for experiments where exogenous substrates were present. In Fig. 1(a) the time course of Ca²⁺ transport is shown in the presence of succinate and rotenone.

The peak in accumulation of Ca²⁺ occurs at 2–3 min, followed by efflux from the cell. The maximum accumulation of Ca²⁺ ranged between 2.5 and 7.0 nmol/mg of cell protein, depending on the factors outlined above. Small peaks at about 7 and 12 min

were often observed. Succinate penetrates these cells more readily at pH 6.5 (Mazumder & Wenner, 1977), so this pH was used for all succinate experiments.

Ascorbate plus *p*-phenylenediamine, a substrate that feeds electrons into the respiratory chain at energy-coupling site 3, also supports Ca^{2+} uptake by ascites cells (Fig. 1*b*). In contrast with observations by Landry & Lehninger (1976), we find that the NAD^+ -linked substrate malate does support Ca^{2+} uptake by these cells (Fig. 1*c*). The time course and extent of uptake are similar to those observed with succinate as substrate. We found malate-supported Ca^{2+} uptake to be more dependent on cell concentration and freshness than succinate-supported uptake. The uptake observed with malate is sensitive to rotenone (Fig. 1*c*), indicating that it is NAD^+ -linked.

Malonate, a competitive inhibitor of succinate dehydrogenase, inhibits succinate-supported Ca^{2+} uptake (Fig. 2*a*). In the presence of 1 mM-malonate, Ca^{2+} uptake was 40% of the control. When 10 mM-malonate was added, almost complete inhibition was observed. The uncoupler S-13 causes a substantial inhibition of succinate-supported Ca^{2+} uptake when it is added at zero time (Fig. 2*b*, solid symbols). In addition, when the uncoupler S-13 is added after Ca^{2+} has been accumulated, i.e. at 1.25 min, it causes a rapid release of the accumulated Ca^{2+} (Fig. 2*b*, open symbols). It was observed in this experiment, with fresh cells, that cycles of Ca^{2+} uptake and efflux continued. This had not been observed in studies done with cells kept in ice for periods of 30–60 min (Fig. 1*a*). We decided to attempt to restore continuous cycles of Ca^{2+} uptake and efflux to cells that had been kept on ice.

Effect of P_i on Ca^{2+} transport

The concentrations of succinate and malate used in our experiments were high enough to support more than one cycle of Ca^{2+} uptake and efflux, so it was of interest to learn why further cycles did not occur. Permeant anions, such as P_i , are known to be necessary for optimum Ca^{2+} uptake by isolated mitochondria (cf. Lehninger *et al.*, 1967). Hines & Wenner (1977) found that P_i was required for optimum Ca^{2+} uptake by intact ascites-tumour cells in the presence of glucose. They found that, in contrast with earlier studies (Cittadini *et al.*, 1971, 1973; Landry & Lehninger, 1976), glucose is capable of supporting Ca^{2+} uptake by these intact ascites-tumour cells if P_i is added to the medium. They also noted that 20–30 mM-extracellular P_i maintained the intracellular P_i at control concentrations, and proposed that glycolysis competes with the Ca^{2+} uptake mechanism for intracellular P_i . We therefore tested the effects of extracellular P_i to see whether it could restore further cycles of Ca^{2+} uptake and efflux in the presence of succinate.

The striking stimulation of Ca^{2+} uptake by

Fig. 3. Effect of P_i on respiration-supported Ca^{2+} transport at 37°C

The reaction mixture was as described in the Experimental section. Protein concentration was 5.5 mg/ml, the pH was 6.5 and 10 μM -rotenone and 10 mM-succinate were present in all cases. ●, Control; ▲, 12 mM- P_i (neutralized with Tris); ○, 21 mM- P_i (Tris); △, 30 mM- P_i (Tris).

exogenous P_i is shown in Fig. 3. Succinate-supported Ca^{2+} uptake (Fig. 3) is increased 3-fold in the presence of 21 mM- P_i (neutralized with Tris). In addition, the second and third peaks (see also Fig. 1*a*), small or absent without P_i present, are quite large when P_i is added. Most of the accumulated Ca^{2+} is still available for efflux. However, with 30 mM- P_i present, there is very little Ca^{2+} efflux, and the shape of the time course approaches a hyperbolic curve. Ca^{2+} uptake supported by ascorbate and *p*-phenylenediamine is also stimulated by the addition of extracellular P_i , but to a lesser extent than when succinate is the substrate.

One possible mechanism for the P_i stimulation of succinate-supported Ca^{2+} uptake was as follows: P_i enters the mitochondria in exchange for OH^- , then exits from the mitochondria in exchange for succinate,

Fig. 4. Effect of temperature on endogenous substrate-supported Ca²⁺ transport in the presence and absence of P_i. The reaction mixture was as described in the Experimental section, and the pH was 6.5. (a) Protein concentration was 5.4 mg/ml. ●, 4°C; ▲, 21°C; ○, 30°C; △, 37°C. (b) Protein concentration was 8.0 mg/ml and 21 mM-P_i (neutralized with Tris) was present in all cases. ●, 4°C; ▲, 20°C; ○, 30°C; △, 37°C.

via the P_i/dicarboxylate antiporter. Thus more succinate would be available to support Ca²⁺ uptake. We found that n-butylnalonate, a specific inhibitor of the P_i/dicarboxylate antiporter, did not inhibit the P_i stimulation of succinate-supported Ca²⁺ uptake, even at a concentration of 2 mM. This mechanism, then, cannot explain the effects of P_i. The fact that P_i was stimulatory with other substrates suggests a more basic role for P_i.

The P_i stimulation of succinate-supported Ca²⁺ uptake is completely uncoupler-sensitive. Uncoupler S-13 also inhibits the P_i stimulation of ascorbate- and *p*-phenylenediamine-supported Ca²⁺ uptake. The effect of another permeant anion, acetate, was also tested. Under our conditions, 30 mM-acetate (neutralized with Tris) did not substitute for 30 mM-P_i, having no effect on succinate-supported Ca²⁺ uptake (results not shown).

Effect of temperature on Ca²⁺ transport

Fig. 4(a) shows the effect of temperature on Ca²⁺ uptake supported by endogenous substrates. Ca²⁺ uptake at 4°C is 2-fold that at 37°C under these conditions. The last peak of Ca²⁺ uptake, seen after 10 min at 37°C, is delayed when the temperature is lowered to 30°C, and is not seen when the experiment is conducted at 21°C. With only endogenous substrates present, P_i has almost no effect on the maximum amount of Ca²⁺ uptake at 4°, 20° and 30°C (Fig. 4b). A 25% stimulation is observed at 37°C. The last peak in Ca²⁺ uptake occurs at the same time at the three higher temperatures.

The energy-dependence of Ca²⁺ uptake at 4°C is

Fig. 5. Effects of uncoupler S-13 and oligomycin on Ca²⁺ uptake at 4°C in the presence of endogenous substrates. The reaction mixture was as described in the Experimental section. ●, ▲, Protein concentration 9.4 mg/ml, pH 6.5; ○, protein concentration 9.9 mg/ml, pH 7.4. The control curves of the two experiments were essentially the same, so only one is shown in the Figure. ●, Control; ▲, 1.88 μM-uncoupler S-13; ○, 2 μg of oligomycin/ml.

Fig. 6. Effects of temperature, substrate availability and P_i on Ca^{2+} transport

The reaction mixture was as described in the Experimental section and the pH was 6.5 in all cases. (a) Protein concentration was 10.0 mg/ml. ●, 4°C; ▲, 4°C with 21 mM- P_i (neutralized with Tris); ○, 37°C; △, 37°C with 21 mM- P_i (Tris). (b) Protein concentration was 7.8 mg/ml, and 10 μM -rotenone and 10 mM-succinate were present in all cases. ●, 4°C; ▲, 4°C with 21 mM- P_i (Tris); ○, 37°C; △, 37°C with 21 mM- P_i (Tris).

shown in Fig. 5. Ca^{2+} uptake in the presence of the uncoupler S-13 is only about 10% of the control at 15 min. Since respiration does not occur at 4°C, and the amount of energy available from the hypothetical high-energy intermediate or conformation, or electrochemical gradient, is limited, we decided to try to find the energy source of this Ca^{2+} uptake. Oligomycin (2 $\mu\text{g}/\text{ml}$) inhibits Ca^{2+} uptake at 4°C by 50%. This would suggest that ATP can support Ca^{2+} uptake at this temperature.

In Fig. 6(a) the effects of temperature and P_i are tested in the same experiment with endogenous substrates present. Maximum Ca^{2+} uptake with endogenous substrates at 4°C was approx. 2.5 times that at 37°C, both with and without added P_i . At both temperatures, P_i had almost no effect until 7 min, after which a decrease in uptake occurred. Fig. 6(b) contains the results of the same experiment carried out in the presence of succinate. Ca^{2+} uptake at 15 min at 4°C is not significantly greater with succinate than with endogenous substrates (cf. Figs. 5 and 6b). P_i has no effect on Ca^{2+} uptake at 4°C. The marked stimulatory effect of P_i on Ca^{2+} uptake at 37°C is the same as that observed earlier (Fig. 3). Also as observed above, maintenance of the oscillating pattern is seen at 37°C with added P_i . The maximum Ca^{2+} uptake at 37°C with succinate is no less than that at 4°C. This finding is in contrast with observations in the absence of added substrate. Thus it is apparent that temperature, P_i availability and substrate availability markedly influence the amount of Ca^{2+} transport and the type of response observed.

Discussion

Oscillatory behaviour of Ca^{2+} transport

The interaction of intact Ehrlich ascites-tumour cells with external Ca^{2+} consists of Ca^{2+} uptake by the cells followed by efflux of accumulated Ca^{2+} back into the medium. Under optimum conditions there is more than one cycle of alternate uptake and efflux in the first 15 min after the addition of Ca^{2+} to the medium. These repeated oscillations have not been reported before. The present study is an attempt to clarify the factors that contribute to Ca^{2+} uptake and efflux processes.

Mitochondrial substrate site specificity of Ca^{2+} uptake: evidence for NAD^+ -linked Ca^{2+} uptake

It is generally held that Ca^{2+} uptake by intact Ehrlich ascites-tumour cells is due to mitochondrial Ca^{2+} uptake (McCoy *et al.*, 1976; Cittadini *et al.*, 1971, 1973; Landry & Lehninger, 1976). Consistent with this idea, we have observed Ca^{2+} uptake to be inhibited by rotenone, antimycin A and the uncoupler S-13. However, there has been some disagreement as to which substrates are capable of supporting Ca^{2+} uptake. Glycolysis, which produces both NADH and ATP, was found not to support Ca^{2+} uptake by these cells (Cittadini *et al.*, 1973; Landry & Lehninger, 1976). This was surprising, since cells made permeable by dextran sulphate were capable of accumulating large amounts of Ca^{2+} when ATP was added to the medium (McCoy *et al.*, 1976). Hines & Wenner (1977) found that, if intracellular P_i

concentrations were maintained by adding P_i to the medium, glycolysis can support Ca²⁺ uptake by Ehrlich cells. Apparently, glycolysis competes with Ca²⁺ uptake for P_i.

Ehrlich cells have little or no glycogen deposits (Nirenberg, 1959), and their endogenous substrates are thought to be mainly fatty acids (Weinhouse, 1955). Cittadini *et al.* (1973), using a Murexide spectrophotometric technique, have observed low Ca²⁺ uptake supported by these endogenous substrates. We have also observed Ca²⁺ uptake supported by endogenous substrates, usually from 1 to 2 nmol/mg of protein maximum accumulation. This uptake is inhibited by rotenone, indicating that it is NAD⁺-linked.

Studies of Ca²⁺ uptake have been carried out with other substrates that give rise to NADH via the tricarboxylic acid cycle. However, these studies did not provide unambiguous evidence for NAD⁺-linked uptake. For example, Cittadini *et al.* (1973) reported that a mixture of 5 mM-glutamate and 5 mM-malate would support Ca²⁺ uptake by ascites-tumour cells. However, glutamate can give rise to succinate via the tricarboxylic acid cycle. R. Charlton (unpublished work) has found that the Ca²⁺ uptake observed with this mixture was inhibited 50% by 5 mM-malate, a competitive inhibitor of succinate dehydrogenase. Therefore it could not be established from these studies that NAD⁺-linked substrates can support Ca²⁺ uptake by Ehrlich cells.

Landry & Lehninger (1976) found low Ca²⁺ uptake in the presence of endogenous substrates or malate, measured at 4 min after Ca²⁺ addition. They have also observed an increase in succinate-supported Ca²⁺ uptake when rotenone was present. They have suggested that substrates that feed electrons into respiration at energy-coupling site 1 may not be able to support Ca²⁺ uptake, and may actually inhibit succinate-supported Ca²⁺ uptake. In the studies reported here, it was shown that malate can support Ca²⁺ uptake, and that the extent and time course of this uptake are similar to those observed with succinate as substrate. It has also been shown here that malate-supported Ca²⁺ uptake is inhibited by rotenone, indicating that it is NAD⁺-linked. The failure of Landry & Lehninger (1976) to observe malate-supported Ca²⁺ uptake may have been due to its measurement at 4 min, when Ca²⁺ efflux may have already begun. However, other variations in experimental conditions may account for these differences between our observations.

Landry & Lehninger (1976) proposed that the enhancement of succinate-supported Ca²⁺ uptake by the addition of rotenone suggests an inhibitory role for electron flow through energy-coupling site 1. However, an alternate explanation is possible. Succinate can reduce NAD⁺ by reverse electron flow through the respiratory chain (Chance & Hollunger,

1961). This reduction is inhibited by rotenone. Since electrons flowing backward in the respiratory chain do not contribute to Ca²⁺ uptake, it might be expected that succinate-supported Ca²⁺ uptake would be increased in the presence of rotenone. Endogenous substrate-supported Ca²⁺ uptake is small, and so its loss in the presence of rotenone might be overcome by the increased efficiency of the respiration of succinate. The extent of initial Ca²⁺ uptake is proportional to the rate of respiration, as shown by the inhibitory pattern of malonate.

On the basis of these observations, it is considered unnecessary to postulate a special role for NAD⁺-linked substrates or for energy-conserving site 1.

Effect of P_i on the magnitude and time course of Ca²⁺ transport

Exogenous P_i dramatically increased the magnitude of Ca²⁺ uptake. The primary site of action is probably the mitochondria, since no P_i effect is observed in the presence of uncoupler S-13 or in cells with low substrate concentrations (Fig. 6). In addition, it can be seen by examining Fig. 3 that, in the presence of 21 mM-P_i, cycles of uptake and efflux continue for at least 15 min. Without exogenous P_i present, only one cycle of Ca²⁺ transport occurs (Fig. 1a). Since succinate is present in an excess, this is probably due to insufficient P_i availability in the cell. As stated above, Hines & Wenner (1977) have established that 20–30 mM-extracellular P_i can maintain the intracellular concentration of P_i at control values in the presence of endogenous substrates or added glucose. Without exogenous P_i, intracellular P_i concentrations decreased by one-half within 10 min after addition of glucose, and Ca²⁺ uptake by the ascites-tumour cells was also decreased. In the present studies, conducted at high concentrations of P_i (30 mM), very little efflux was observed. This may be due, in part, to intramitochondrial sequestration of Ca₃(PO₄)₂, making part of the accumulated Ca²⁺ unavailable for efflux. A direct inhibition of efflux at the level of the mitochondrial membrane or the cell membrane would also be consistent with our data. At 21 mM-P_i, Ca²⁺ uptake is greatly increased, and significant efflux also occurs. Therefore the action of P_i cannot be explained solely by irreversible sequestration of Ca²⁺ or inhibition of Ca²⁺ efflux. In normal rat liver mitochondria, it has been generally thought that Ca²⁺ uptake is directly coupled to electron transport, with P_i or other permeant anions passively following Ca²⁺ for electrochemical balance (Rasmussen *et al.*, 1965; Mela & Chance, 1968). More recently, Lehninger (1974) has proposed that entry of a proton-yielding anion, such as P_i, is the first event occurring in response to the inside-alkaline proton gradient generated by electron transport. Then this inside-negative P_i gradient is the source of

motive force for Ca^{2+} uptake and other processes. Thus Ca^{2+} can bind to the mitochondrial membrane without P_i , but P_i is necessary for transport into the matrix. Our studies cannot distinguish between these two possible mechanisms of the action of P_i .

Temperature-dependence of the uptake and efflux phases of Ca^{2+} transport

Ca^{2+} uptake by isolated rat liver mitochondria at 0°C has been reported by Chance (1965). It was shown here that intact Ehrlich cells take up Ca^{2+} at 4°C . This uptake is inhibited by the uncoupler S-13, indicating that it is active mitochondrial uptake. The inhibition of this uptake by oligomycin indicates that its energy source is ATP. Ca^{2+} uptake at 4°C is a slow process that is not dependent on substrates, as would be expected since no respiration occurs at this temperature. P_i also is not limiting under these conditions.

Ca^{2+} efflux from intact ascites-tumour cells does not occur at 4°C , and therefore it appears to be an active process with a higher temperature coefficient than that of Ca^{2+} uptake. Since most of the Ca^{2+} taken up by the cell enters the mitochondria, total cellular efflux must include efflux from the mitochondria followed by efflux across the plasma membrane. Either or both of these processes may be the temperature-dependent process that is inhibited at 4°C . Borle (1972, 1973), working with intact monkey kidney cells grown in cell culture, found that efflux of Ca^{2+} from the mitochondria into the cytosol is passive. He has found efflux from the cytosol to the extracellular medium to be energy-dependent. Schatzmann & Vincenzi (1969), in their studies of human erythrocytes, have reported a $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -activated adenosine triphosphatase in the plasma membrane. This adenosine triphosphatase is responsible for net Ca^{2+} efflux from the cell, and is highly temperature-dependent. Drahota *et al.* (1965) found that Ca^{2+} efflux from preloaded isolated rat liver mitochondria did not occur at 0°C . Studies with isolated mitochondria have the disadvantage of separating the mitochondria from possible regulatory molecules present in the cytoplasm. Nonetheless, the possibility that Ca^{2+} efflux from Ehrlich cells is regulated at the level of the mitochondria must be considered. The results presented here would be consistent with any of the above mechanisms of Ca^{2+} efflux.

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