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Transport of Calcium Ions by Ehrlich Ascites-Tumour Cells

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Ehrlich ascites-tumour cells accumulate Ca^{2+} when incubated aerobically with succinate, phosphate and rotenone, as revealed by isotopic and atomic-absorption measurements. Ca^{2+} does not stimulate oxygen consumption by carefully prepared Ehrlich cells, but does so when the cells are placed in a hypo-osmotic medium. Neither glutamate nor malate support Ca^{2+} uptake in 'intact' Ehrlich cells, nor does the endogenous NAD-linked respiration. Ca^{2+} uptake is completely dependent on mitochondrial energy-coupling mechanisms. It was an unexpected finding that maximal Ca^{2+} uptake supported by succinate requires rotenone, which blocks oxidation of endogenous NAD-linked substrates. Phosphate functions as co-anion for entry of Ca^{2+}. Ca^{2+} uptake is also supported by extracellular ATP; no other nucleoside 5'-di- or tri-phosphate was active. The accumulation of Ca^{2+} apparently takes place in the mitochondria, since oligomycin and atractyloside inhibit ATP-supported Ca^{2+} uptake. Glycolysis does not support Ca^{2+} uptake. Neither free mitochondria released from disrupted cells nor permeability-damaged cells capable of absorbing Trypan Blue were responsible for any large fraction of the total observed energy-coupled Ca^{2+} uptake. The observations reported also indicate that electron flow through energy-conserving site 1 promotes Ca^{2+} release from Ehrlich cells and that extracellular ATP increases permeability of the cell membrane, allowing both ATP and Ca^{2+} to enter the cells more readily.

Many activities of mammalian cells are regulated by changes in the concentration of free Ca^{2+} in the cytosol. Much evidence indicates that the homoeostasis of cell Ca^{2+} is maintained by two processes, (1) the outward pumping of Ca^{2+} by a Ca^{2+}-dependent cell membrane ATPase (adenosine triphosphatase), opposing passive Ca^{2+} influx from the extracellular fluid, and (2) respiration-dependent pumping of Ca^{2+} from the cytosol into the mitochondrial matrix, from which Ca^{2+} may again be released into the cytosol (Lehninger, 1964, 1970; Gevers & Krebs, 1966; Bygrave, 1966; Rasmussen, 1970; Borle, 1973; Rose & Loewenstein, 1975).

Because cancer often causes aberrations in Ca^{2+} transport or in calcification mechanisms, the dynamics of Ca^{2+} transport by malignant cells and their organelles is of special interest. Mitochondria isolated from Ehrlich ascites-tumour cells transport Ca^{2+} at the expense of respiratory energy (Thorne & Bygrave, 1973*a*,*b*, 1974*a*,*b*). Such mitochondria also have exceptional ability to resist the deleterious action of high concentrations of Ca^{2+} on phosphorylation capacity, and to retain Ca^{2+} once accumulated (see also McIntyre & Bygrave, 1974). Mitochondria from several other experimental tumours also show similar properties (Reynafarje & Lehninger, 1973; A. C. Griffin & A. L. Lehninger, unpublished work).

Several studies on Ca^{2+} transport by suspensions of intact Ehrlich cells have also been reported. Thomason & Schofield (1959) reported that injected $^{45}Ca^{2+}$ rapidly exchanged with the Ca^{2+} of Ehrlich cells growing in the peritoneal cavity of mice. Levinson & Blumenson (1970) demonstrated, using $^{45}Ca^{2+}$, that the Ca^{2+} of Ehrlich cells occurs in three kinetically distinct compartments. However, Bygrave (1966) has reported failure of Ehrlich cells to bring about net accumulation of Ca^{2+} *in vitro* or *in vivo*. Subsequently, Cittadini *et al.* (1973) reported stimulation of respiration by Ehrlich ascites cells by Ca^{2+}, as well as a considerable amount of $^{45}Ca^{2+}$ uptake, which they attributed to mitochondrial activity; whether the $^{45}Ca^{2+}$ uptake reported represented net accumulation of Ca^{2+} was not determined.

The present paper further explores aspects of Ca^{2+} transport in Ehrlich cells. It is shown that substantial net accumulation of Ca^{2+} by isolated respiring Ehrlich ascites cells can take place under very special conditions, namely in the presence of added succinate and phosphate and with rotenone present to block endogenous NAD-linked electron transport. Alternatively, the energy for the uptake of Ca^{2+} can be provided by the mitochondrial hydrolysis of added ATP. The ultimate locus of Ca^{2+} uptake appears to be the mitochondrial compartment. Significantly, Ca^{2+}

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uptake by Ehrlich cells is not supported by glycolysis or by NAD-linked respiratory substrates. A special problem arising in study of Ca^{2+} transport by isolated Ehrlich cells is the possibility that much of the observed transport activity is due to the activity of a relatively small number of cells in which plasma-membrane damage has occurred, allowing Ca^{2+} to enter and gain access to the mitochondria. However, only a small fraction of the total Ca^{2+} uptake takes place in this manner under the conditions used here.

Experimental

Two strains of Ehrlich ascites-tumour cells were used in this study, the Northwestern strain, provided by Dr. Elmon Coe, Department of Biochemistry, Northwestern University School of Medicine, Chicago, and a strain obtained from Dr. A. Clark Griffin of the M. D. Anderson Hospital and Tumor Institute, Houston. Both strains were propagated in the peritoneal cavity of Swiss albino mice (Buckberg Laboratories, Tomkins Cove, NY, U.S.A.). After 7 days, the ascitic fluid and cells were aspirated and immediately diluted in approx. 4vol. of 'saline medium' {150mM-NaCl; 5mM-KCl; and 5mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] (sodium salt) buffer, pH7.4} at 4°C. The cells were centrifuged for 5min at 60*g* at 4°C, washed twice in 3vol. of cold saline medium, and then gently suspended in saline medium to a concentration of approx. 32mg of protein/ml. Protein was determined by the method of Gornall *et al.* (1949). Cell counts showed that 10^6 cells contain 460μg of protein and have dry wt. 875μg, corrected for adhering salt medium. The stock cell suspension was kept at 0°C until used, usually within 3h.

The incubations were carried out in centrifuge tubes immersed in a 25°C water bath. To 4.0ml of saline medium, containing the other components in the final concentrations stated in the legends of the Tables and Figures, 1.0ml of the stock cell suspension was added, and the mixture equilibrated for 2min at 25°C with gentle magnetic stirring. Ca^{2+} was then added at zero time. Samples were taken periodically, filtered quickly on 0.8μm Millipore filters, and washed with 3ml of cold saline medium under vacuum. Alternatively, the cells were recovered by centrifugation for 5min at 60*g* and washing with 3ml of cold saline medium. The washed filters were placed in vials, dried, and counted for radioactivity after addition of scintillation fluid (Bray, 1960). A sample of the unfiltered system was also counted for radioactivity in the same manner to give total $^{45}Ca^{2+}$. Generally a concentration of 200μM-$CaCl_2$ was used, labelled with 2μCi of $^{45}Ca^{2+}$ (ICN Pharmaceuticals, Cleveland, OH, U.S.A.) per tube, equivalent to 36000–40000d.p.m./100μl of the complete system.

Mitochondria were isolated from homogenates of Ehrlich ascites cells (A. C. Griffin, W. V. V. Greenhouse & A. L. Lehninger, unpublished work) and washed three times in saline medium. They showed acceptor control ratios (the quotient rate of oxygen consumption in the presence of 200μM-ADP/rate in the absence of added ADP) exceeding 5.0, with succinate as substrate.

Results

Effect of respiratory substrates and Ca^{2+} on the rate of respiration of various types of tumour cells

Fig. 1 shows typical oxygen-electrode traces indicating the effect of Ca^{2+}, substrates, inhibitors and other agents on the respiratory rate of Ehrlich ascites-tumour cells. Addition of Ca^{2+} alone, in the absence of added substrates, produced no stimulation of the endogenous rate of oxygen consumption in either strain of Ehrlich cells (Fig. 1*a*). When succinate was present in the medium, in the absence or presence of Mg^{2+}, phosphate, or ATP, no increase in oxygen uptake above the endogenous rate was observed. Subsequent addition of Ca^{2+} yielded at most only a very slight stimulation of oxygen uptake (Fig. 1*b*). These properties were always seen in both strains of Ehrlich cells tested. Moreover, we observed no significant respiratory response to the addition of succinate and/or Ca^{2+} in three other types of malignant cells tested (L1210, Walborg AS30D and Novikoff hepatoma cells). Our observations thus differ from those of Cittadini *et al.* (1973), who found substantial stimulation of respiration on addition of succinate and a further large stimulation by Ca^{2+} in their preparations of Ehrlich cells.

Rotenone almost completely inhibited the endogenous oxygen consumption of Ehrlich cells (Fig. 1*c*). If succinate was then added to the rotenone-poisoned cells, a small but significant stimulation of oxygen consumption ensued after a lag period of about 40s; however, subsequent addition of Ca^{2+} evoked little or no increase in oxygen uptake (Fig. 1*c*). Similar results were observed with other types of tumour cells. Thus our observations differ significantly from those of Cittadini *et al.* (1973).

Effect of hypo-osmotic treatment on the respiratory rate of Ehrlich ascites-tumour cells

The question arose as to whether the differences between our experiments and those of Cittadini *et al.* (1973) reflect the degree of intactness of the cell membranes in the Ehrlich-cell preparations used. Although no direct answer can be given to this question, we have found that incubation of our preparations of Ehrlich ascites cells in a hypo-osmotic reaction medium (20mM-NaCl instead of 150mM), resulted in a sub-

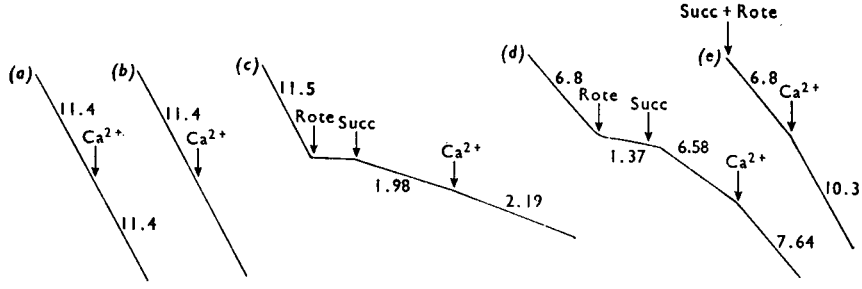

Fig. 1. *Effects of succinate, Ca^{2+} and a hypo-osmotic medium on oxygen consumption by Ehrlich tumour cells*

Oxygen consumption was followed with a Clark-type oxygen electrode in a water-jacketed chamber at 25°C. The basic saline medium (2.5ml) was NaCl, 150mM; KCl, 5mM; Hepes, 5mM, pH7.4, in Expts. *a*, *b* and *c*. In Expt. *b* the saline medium was supplemented with 5mM-ATP, 5mM-succinate, 2mM-Mg^{2+} and 2mM-phosphate. In Expts. *d* and *e*, NaCl and KCl were omitted from the basic saline medium. Freshly prepared cells (500μl of saline medium containing 11.1mg of cell protein) were then added and other additions made as shown. In Expts. *d* and *e* the final NaCl concentration was about 20mM. The additions indicated in the traces were Ca^{2+} (200μM), rotenone (5μM) and succinate (5mM). The numbers on the traces represent the rate of oxygen consumption of the cells in ng-atoms of oxygen/min per mg of protein. Rote, rotenone; Succ, succinate.

stantial increase in oxygen consumption on addition of Ca^{2+} (Fig. 1*d*), simulating the behaviour of the Ehrlich cells described by Cittadini *et al.* (1973). Moreover, in the presence of rotenone, succinate produced a large stimulation of oxygen consumption in the hypo-osmotically treated cells, which was further increased by addition of Ca^{2+} (Fig. 1*e*). It is probable that the permeability of the Ehrlich cells was sufficiently altered by the hypo-osmotic conditions to allow succinate and also Ca^{2+} to enter the cells more readily. The question of damage to the permeability of ascites-tumour cells is discussed further below.

Effect of respiratory substrates and rotenone on Ca^{2+} uptake by Ehrlich cells

Although Ehrlich cells not exposed to hypo-osmotic media show a substantial rate of endogenous oxygen consumption, only a very small uptake of $^{45}Ca^{2+}$ by the cells occurs in the absence of added respiratory substrate, amounting to about 1.0–1.2ng-ions of Ca^{2+}/mg of protein (Fig. 2). When succinate (or succinate plus phosphate) is added, Ca^{2+} uptake is not significantly increased, but when rotenone is present, succinate supports a much larger extent of Ca^{2+} uptake, which is maximal when phosphate is also present, about 7.5ng-ions/mg of protein (Fig. 2). Maximal $^{45}Ca^{2+}$ uptake was observed at about 4min at 25°C in virtually every batch of Ehrlich cells studied. $^{45}Ca^{2+}$ uptake is not accompanied by an increased oxygen uptake under these conditions (Fig. 1*b*); nevertheless, the uptake of Ca^{2+} is energetically coupled to electron transport, since it is completely inhibited by cyanide or antimycin A, as well as by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 3). After 4min, rapid efflux of the newly accumulated $^{45}Ca^{2+}$ occurred.

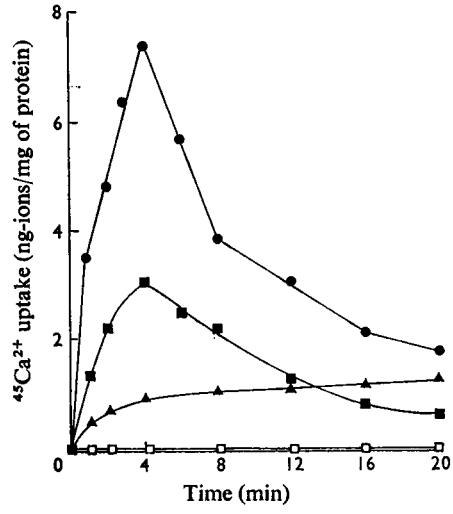

Fig. 2. *Effect of various components on Ca^{2+} uptake by Ehrlich cells*

The basic medium was 150mM-NaCl, 5mM-KCl, 5mM-Hepes, pH7.4, 200μM-$CaCl_2$, and the cells were added at 6.4mg of protein/ml; the total volume was 3.0ml. The additions, as indicated below, were rotenone (4μM), sodium succinate (5mM), and phosphate (2mM). (■) Succinate+rotenone, (□) rotenone alone, (▲) no additions or succinate alone, (●) succinate+rotenone+phosphate.

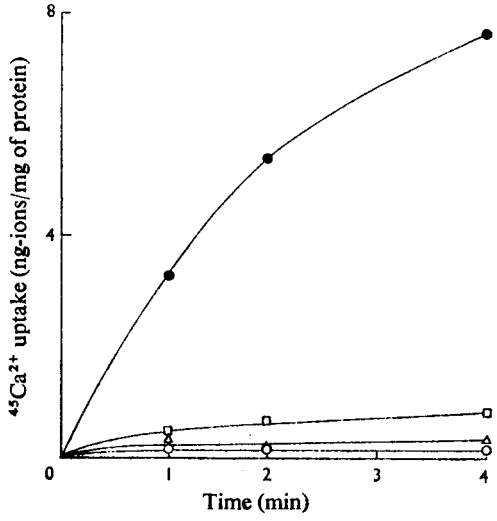

Fig. 3. *Dependence of Ca^{2+} uptake on electron-transport energy*

Conditions were exactly as in Fig. 2, with succinate, phosphate and rotenone present. (●) Complete medium. Cyanide (△) was added at 1.0mM, antimycin A (○) at 1.0μg, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (□) at 3μM.

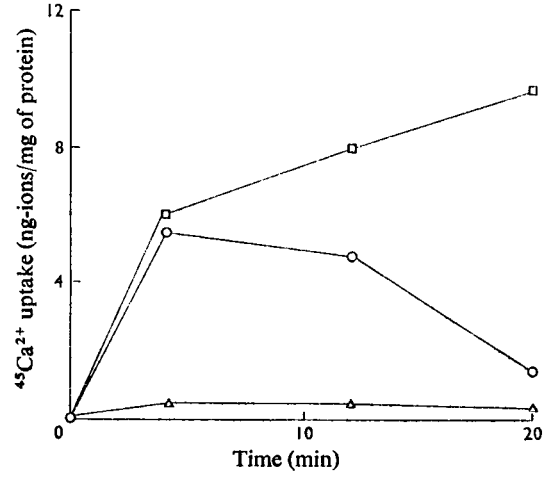

Fig. 4. *Net Ca^{2+} uptake by Ehrlich cells*

The cells (6.4mg of protein, containing 2.9ng-ions of Ca^{2+}/mg) were incubated at 25°C in 150mM-NaCl, 5mM-KCl, 5mM-Hepes (pH7.4), 2.0mM-$MgCl_2$, 2.0mM-phosphate, 4μM-rotenone and 200μM-Ca^{2+}; other additions as shown were 5mM-succinate and 2mM-ATP. The total volume was 2.0ml. (□) Succinate+ATP, (○) succinate, (△) no additions.

The maximum initial rate of Ca^{2+} uptake observed in the presence of succinate, phosphate and rotenone was approx. 3.5ng-ions of Ca^{2+}/min per mg of cell protein (Fig. 2). This was followed by a slower phase, in which the Ca^{2+} uptake was at about one-third of this rate. If it is assumed that the mitochondria are the exclusive locus of uptake of Ca^{2+} and that the mitochondria contain about 10% of the total protein of the Ehrlich cells, then the observed rate of Ca^{2+} uptake is equivalent to about 35ng-ions of Ca^{2+}/min per mg of mitochondrial protein, assuming that all the $^{45}Ca^{2+}$ uptake represents net uptake (but see below). This rate is much lower than the maximal rate of Ca^{2+} uptake in isolated Ehrlich-cell mitochondria supplemented with succinate and phosphate, which is about 250–300ng-ions of Ca^{2+}/min per mg of protein at 25°C (A. C. Griffin & A. L. Lehninger, unpublished work). The maximum amount of $^{45}Ca^{2+}$ taken up by the Ehrlich cells at the 4min peak, equivalent to about 7.5ng-ions per mg of protein (Fig. 2), if taken up only by the mitochondria contained in these cells would represent a Ca^{2+} load of about 75ng-ions of Ca^{2+}/mg of mitochondrial protein. This is considerably higher than the Ca^{2+} content of freshly isolated Ehrlich-cell mitochondria, about 20ng-ions/mg of protein, but much less than the total Ca^{2+} capacity of isolated Ehrlich- or liver-cell mitochondria, which is in excess of 1000ng-ions of Ca^{2+}/mg of protein.

Net uptake of Ca^{2+}

Since the total Ca^{2+} content of the two strains of Ehrlich ascites-tumour cells isolated as described here was in the range 2.6–7.4ng-ions of Ca^{2+}/mg of cell protein, the magnitude of the observed uptake of $^{45}Ca^{2+}$ (for example, 7.5ng-ions of Ca^{2+}/mg of protein in Fig. 2) indicates that net Ca^{2+} uptake occurred, in addition to a possible exchange between Ca^{2+} in the medium and the cells. Net energy-dependent uptake of Ca^{2+} by the Ehrlich cells was in fact shown to take place by direct atomic-absorption measurements (Gochman & Givelber, 1970). Fig. 4 shows that the Ca^{2+} content of Ehrlich cells more than doubled in 4min when they were incubated in the presence of 200μM-Ca^{2+}, succinate, phosphate and rotenone. When succinate and phosphate were omitted, negligible net uptake of Ca^{2+} took place.

Fig. 4 also shows that a much more extensive net accumulation of Ca^{2+} is supported by ATP hydrolysis (discussed below). These observations indicate that most of the observed $^{45}Ca^{2+}$ uptake in the various present experiments actually represents net uptake of Ca^{2+}. Precise determination of the exact proportion of net uptake and isotopic exchange of Ca^{2+} would require more detailed kinetic experiments.

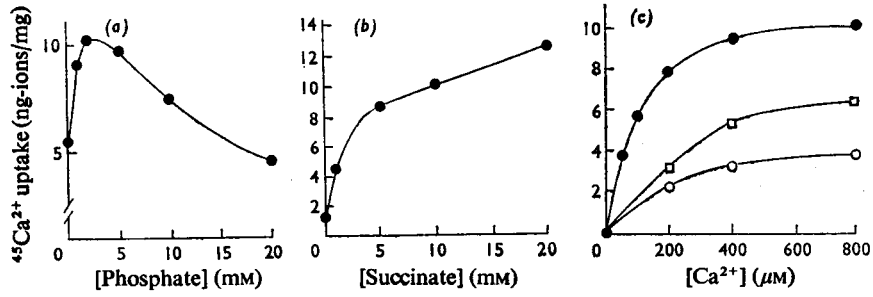

Fig. 5. *Effect of concentration of (a) phosphate, (b) succinate and (c) Ca^{2+} on Ca^{2+} uptake; uptake of P_i*

(*a*) Effect of [phosphate]. Ehrlich cells (7.95mg of protein/ml) were incubated for 4.0min at 25°C in a medium of 150mM-NaCl, 5.0mM-KCl, 5mM-Hepes, pH7.4, 4μM-rotenone and 5mM-succinate with Ca^{2+} at 200μM and succinate at 5mM. The total volume was 2.0ml. The phosphate concentration was varied as shown. (*b*) Effect of [succinate]. Medium as in (*a*), with Ca^{2+} at 200μM and phosphate at 5mM; cells were added at 4.94mg of protein/ml. The total volume was 2.0ml. Succinate concentration was varied as shown. (*c*) Effect of [Ca^{2+}]. Medium as in (*a*). In the experiment shown in the upper curve (●) Ehrlich cells were added at 6.36mg of protein/ml; 5mM-phosphate was present. The total volume was 2.0ml. The Ca^{2+} concentration was varied. In the experiments shown by the two lower curves the P_i concentration was 0.1mM. Curve □ shows the Ca^{2+} uptake and curve ○ the P_i uptake.

Optimal conditions for succinate-supported Ca^{2+} uptake

Fig. 5(*a*) shows that the stimulating effect of phosphate on Ca^{2+} uptake has a sharp optimum at about 2mM; higher concentrations were inhibitory. At all concentrations of phosphate tested, the peak Ca^{2+} uptake was at 4min, followed by Ca^{2+} efflux. The succinate-concentration curve is biphasic (Fig. 5*b*); the steady increase in $^{45}Ca^{2+}$ uptake as succinate is increased above 5mM suggests the occurrence of concentration-dependent unmediated diffusion of succinate into the cells. The Ca^{2+} concentration producing maximum uptake is in the range 400–800μM (Fig. 5*c*), somewhat below the actual concentration of free, ionized Ca^{2+} in blood serum and interstitial fluid.

Uptake of phosphate with Ca^{2+}

The requirement of phosphate for maximal uptake of Ca^{2+} by the Ehrlich cells suggested that phosphate may enter the cells as co-anion with the Ca^{2+}. Direct measurements of the uptake of P_i labelled with ^{32}P were carried out on Ehrlich cells under respiring conditions in which active uptake of Ca^{2+} was occurring. In these experiments, the phosphate concentration in the medium was 0.10mM, which yields about half-maximal $^{45}Ca^{2+}$ uptake (Fig. 5*a*). As shown in Fig. 5(*c*), phosphate labelled with ^{32}P was accumulated by the cells in amounts that were of the same order of magnitude as the $^{45}Ca^{2+}$ uptake, in consonance with the view that phosphate is the major counter-anion entering the cells with Ca^{2+}. In the absence of succinate as energy source, neither Ca^{2+} nor phosphate was taken up to a significant extent.

Effect of other respiratory substrates and glucose on Ca^{2+} uptake

Significant stimulation of $^{45}Ca^{2+}$ uptake is given by the substrates succinate and glycerol 3-phosphate, and the substrate system ascorbate+tetramethylphenylenediamine. The addition of NAD-linked substrates such as malate or glutamate yielded no significant stimulation. Glucose also yielded no stimulation of Ca^{2+} uptake under any conditions, even in the presence of respiratory inhibitors. Although it is well known that Ehrlich cells have a high rate of glycolysis, ATP generated by glycolysis evidently does not support Ca^{2+} uptake.

The failure of malate to stimulate respiration and Ca^{2+} uptake may be due to its inability to enter the cells. However, the fact that the substantial rotenone-sensitive endogenous respiration, which is NAD-linked, is also unable to support Ca^{2+} uptake suggests an alternative conclusion, namely that substrates feeding electrons directly into NAD or energy-conserving site 1 may in general be unable to support Ca^{2+} uptake by intact cells. Moreover, since rotenone is required for maximal Ca^{2+} uptake (Fig. 2), the oxidation of NAD-linked substrates appears to be inhibitory to succinate-supported Ca^{2+} uptake in intact cells. This point is further discussed below.

Effect of various agents on Ca^{2+} efflux

Fig. 6 shows that the addition of cyanide or the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone greatly increases the efflux of $^{45}Ca^{2+}$ from the Ehrlich cells. On the other hand, Ruthenium Red, an inhibitor of Ca^{2+} transport in mitochondria (Moore, 1971), strongly inhibited Ca^{2+} efflux. Mg^{2+} also inhibited Ca^{2+} efflux substantially. Most striking,

Table 1. *Effect of various respiratory substrates on Ca^{2+} uptake*

Ehrlich cells (6.4mg of protein/ml) were incubated for 4min at 25°C in salt medium supplemented with the substrates shown, 2mM-phosphate, and with rotenone either present (+) or absent (−). The concentrations added were malate, succinate, α-glycerol phosphate, ascorbate and glucose at 5mM, tetramethylphenylenediamine at 100μM, and rotenone at 4μM.

Substrate	Rotenone	$^{45}Ca^{2+}$ uptake (ng-ions/mg of protein)
None	−	1.79
Malate	−	1.90
Succinate	−	3.04
None	+	0.95
Malate	+	0.87
Succinate	+	7.79
α-Glycerol phosphate	+	3.99
Ascorbate+tetramethyl-phenylenediamine	+	3.13
Glucose	+ or −	0.95

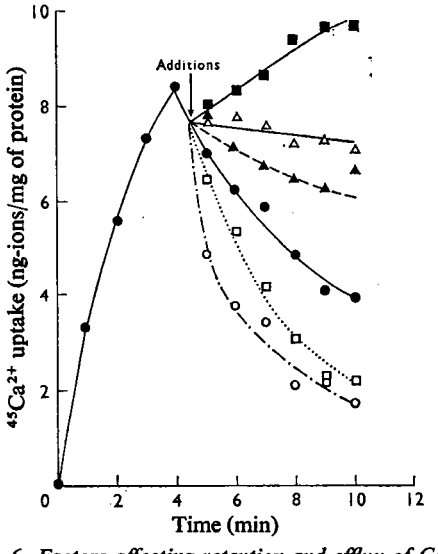

Fig. 6. *Factors affecting retention and efflux of Ca^{2+}*

Cells (6.4mg of protein/ml) were incubated in a medium of 150mM-NaCl, 5mM-KCl, 5mM-Hepes, pH7.4, 4μM-rotenone, 5mM-phosphate, 5mM-succinate and 200μM-Ca^{2+}. The total volume was 3.0ml. At 4.5min, the following additions were made as shown: 2mM-ATP (■), 10mM-Mg^{2+} (△), 50μM-Ruthenium Red (▲), 2μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (□), or 1mM-cyanide (○); (●) no addition.

however, was the effect of added ATP. It not only completely prevented Ca^{2+} efflux but promoted the further accumulation of Ca^{2+} by the cells. This effect

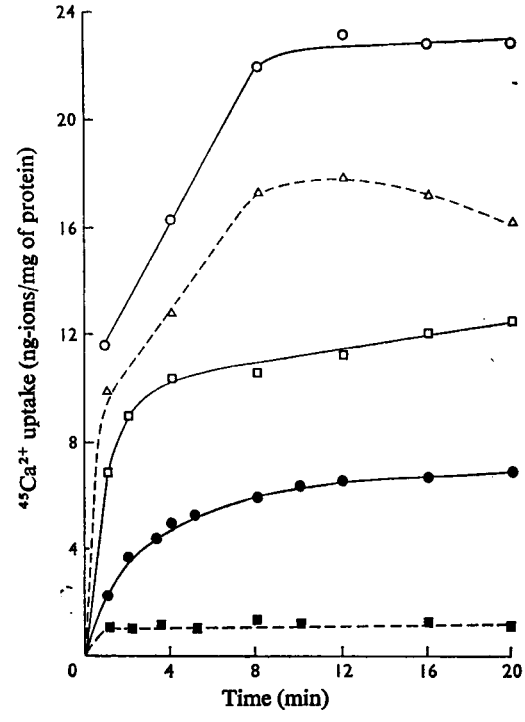

Fig. 7. *Requirements for ATP-supported Ca^{2+} uptake*

The cells (6.36mg of protein/ml) were incubated in a medium of 150mM-NaCl, 5mM-KCl, 5mM-Hepes, pH7.4, 4μM-rotenone, and 200μM-Ca^{2+}, plus additions as indicated of 2mM-ATP, 1mM-$MgCl_2$, 5mM-succinate, and 2mM-phosphate, in a total voulme of 3.0ml. (○) ATP+succinate+P_i+Mg^{2+}, (△) ATP+succinate, (□) ATP+Mg^{2+}, (●) ATP, and (■) no additions.

was rather unexpected, since ATP normally is not present in extracellular fluid and is generally thought to be impermeant through cell membranes.

Support of Ca^{2+} uptake by ATP in the absence of added substrates

Tests were made of ATP and various other additions for their ability to support $^{45}Ca^{2+}$ uptake by rotenone-poisoned cells in the absence of succinate (Fig. 7). Without additions the cells had little or no ability to accumulate Ca^{2+}. However, 2mM-ATP produced a significant rise in $^{45}Ca^{2+}$ uptake, about to the value given by succinate+rotenone+phosphate. The addition of Mg^{2+} together with ATP nearly doubled the amount of $^{45}Ca^{2+}$ taken up, without subsequent efflux. When succinate was added in addition to ATP, there was a further increase in $^{45}Ca^{2+}$ uptake. Maximum $^{45}Ca^{2+}$ uptake was given by ATP+Mg^{2+}+phosphate+succinate, to a value

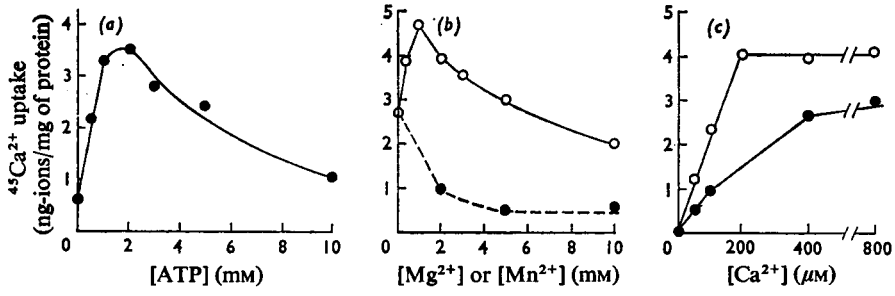

Fig. 8. *Optimal conditions for ATP-supported Ca^{2+} uptake*

(*a*) Effect of [ATP]: Ehrlich cells (7.69mg of protein/ml) were incubated in saline medium containing 4μM-rotenone and 200μM-Ca^{2+} for 12min at 25°C in a total volume of 2.0ml; the ATP concentration was varied. (*b*) Effect of Mg^{2+} (○) and Mn^{2+} (●); the medium was as in (*a*), but contained 2.0mM-ATP; 7.79mg of Ehrlich-cell protein was added/ml. Mg^{2+} and Mn^{2+} were added as $MgCl_2$ and $MnCl_2$. (*c*) Effect of [Ca^{2+}]: Ehrlich cells (6.36mg of protein/ml) were incubated for 12min in saline medium containing 5μM-rotenone, 2mM-ATP, and 1.0mM-Mg^{2+} as indicated.

Table 2. *Effect of inhibitors on ATP-supported Ca^{2+} uptake*

Ehrlich cells (5.8mg of protein/ml in Expt. 1 and 7.31mg/ml in Expt. 2) were incubated for 12min at 25°C in saline medium supplemented as indicated with 2.0mM-ATP, 4μM-rotenone, 5.0mM-succinate, 2mM-phosphate, 20μM-antimycin A, 10μg of oligomycin/ml, or atractyloside as indicated. In Expt. 2, 1.0mM-Mg^{2+} was added.

	System	$^{45}Ca^{2+}$ uptake (ng-ions/mg of protein)	
			Plus oligomycin
Expt. 1.	Endogenous	2.30	2.20
	ATP alone	9.69	3.97
	ATP+rotenone	5.89	0.86
	ATP+succinate+rotenone	15.9	14.6
	ATP+succinate+phosphate+rotenone	20.7	19.8
	ATP+rotenone+antimycin A	3.44	—
	ATP+succinate+rotenone+antimycin A	3.42	0.95
Expt. 2.	ATP+rotenone	12.9	
	ATP+rotenone+2.5μM-atractyloside	4.56	
	ATP+rotenone+5μM-atractyloside	2.38	
	ATP+rotenone+20μM-atractyloside	1.90	

of over 22ng-ions of Ca^{2+}/mg of cell protein, some threefold greater than that supported by succinate in the absence of ATP (Fig. 2). That ATP supports net Ca^{2+} uptake by Ehrlich cells is shown in Fig. 4; in this experiment there was a threefold increase in Ca^{2+} content of the cells.

ATP is completely specific in the support of Ca^{2+} uptake in Ehrlich cells supplemented with nucleotide, Mg^{2+} and rotenone, but no succinate or phosphate. ADP, AMP and cyclic AMP were without significant effect in supporting Ca^{2+} uptake; CTP, GTP, UTP and TTP were also completely inactive. Optimal ATP concentration was approx. 2mM (Fig. 8*a*). Higher concentrations were inhibitory, probably owing to complexing of Ca^{2+}. Fig. 8(*b*) shows that 1mM-Mg^{2+} was optimal when ATP was 2.0mM; Mn^{2+} inhibited Ca^{2+} uptake. Optimal Ca^{2+} concentration for ATP-supported uptake was between 200 and 800μM (Fig. 8*c*), as for succinate-supported Ca^{2+} uptake (Fig. 5*b*).

Action of inhibitors on ATP-supported Ca^{2+} uptake

Oligomycin inhibited ATP-supported Ca^{2+} uptake by about 66% in the absence of rotenone and by nearly 100% in its presence (Table 2). Hydrolysis of added ATP thus can provide all of the energy for Ca^{2+} accumulation by the cells under these conditions. However, when succinate was present together with ATP, oligomycin yielded negligible inhibition of Ca^{2+} uptake, indicating that under these conditions, succinate was providing essentially all of the energy.

Since ATP is still required for maximal uptake and retention of Ca^{2+}, ATP evidently supports succinate-energized Ca^{2+} uptake by some mechanism that is not sensitive to oligomycin.

Atractyloside at 5 μM completely inhibited ATP-supported Ca^{2+} uptake by the cells, down to the value of the endogenous Ca^{2+} uptake (Table 2). Since atractyloside is not known to inhibit cellular processes other than ATP transport by mitochondria, this experiment, together with the inhibitory effect of oligomycin, shows that the ATPase of mitochondria is involved in ATP-dependent support of Ca^{2+} accumulation by the Ehrlich cells, rather than an ATPase of the plasma membrane.

Are free mitochondria present in suspension of Ehrlich cells responsible for Ca^{2+} uptake?

From the preceding experiments the question naturally arises as to whether some or all of the Ca^{2+} uptake observed by the preparation of Ehrlich ascites-tumour cells may actually have taken place in mitochondria that had been physically released from damaged cells. Attempts were made to isolate free mitochondria from suspensions of Ehrlich ascites-tumour cells that had been allowed to accumulate $^{45}Ca^{2+}$ in the presence of succinate, rotenone and phosphate in a preceding incubation at 25°C. After removal of the intact cells by centrifugation at low speeds, the supernatant medium was either centrifuged at 10000*g* for 10 min or filtered through 0.45 μm Millipore filters to recover whatever free mitochondria may have been present. However, no significant $^{45}Ca^{2+}$ could be detected on counting the radioactivity on the filters or the pellets collected.

Examination of Ehrlich cells with Trypan Blue

Uptake of Trypan Blue has often been used to estimate the proportion of damaged cells in a given population. About 5% of the Ehrlich cells in the preparations used in this study were found to absorb Trypan Blue. The proportion of cells that were stained did not increase on storage of the cells for periods as long as 4 h at 0°C. Moreover, there was little or no increase in the fraction of cells taking up the dye after incubating them at 25°C with several of the test media used in this investigation, over periods of 4–20 min.

Is Ca^{2+} uptake limited to Ehrlich cells capable of Trypan Blue uptake?

The question now arises as to whether the Ca^{2+} uptake by the Ehrlich cell preparations reported in the present paper can be accounted for, if it is assumed that all of the observed Ca^{2+} uptake occurs into the mitochondria of the 5% of the cell population that is damaged sufficiently to absorb Trypan Blue. Presumably the cytosol of such cells, and thus their mitochondria, are readily accessible not only to external

Table 3. *Maximal rate and extent of Ca^{2+} uptake by Ehrlich-ascites-cell preparations*

These are calculated on the assumption that all uptake of Ca^{2+} takes place in the mitochondria of 5% of the cells, compared with the observed rate and extent of Ca^{2+} uptake by mitochondria isolated from Ehrlich cells. The values in the first vertical column represent the maximal observed rates and extents on Ehrlich cell preparations, as given by experiments such as those in Figs. 3, 5, 7 and 8. The values in the second vertical column assume that the mitochondria of only 5% of the cells (stained by Trypan Blue) are responsible for all of the Ca^{2+} uptake of the Ehrlich-cell preparations and that 10% of the protein content of the Ehrlich cells is due to mitochondria. The values in the third column represent the actual observed values for Ca^{2+} uptake by mitochondria isolated from Ehrlich cells (see the text). The test systems contained 150 mM-NaCl, 5 mM-KCl, 5 mM-Hepes, pH 7.4, as well as 5 mM-succinate, 2 mM-ATP, 2 mM-phosphate, and 1 mM-Mg^{2+} as shown; rotenone (4 μM) was present in all vessels. Ca^{2+} was always added at 200 μM. The temperature was 25°C.

Rate of Ca^{2+} uptake ... Energy source	Observed Ca^{2+} uptake by Ehrlich cells (ng-ions/min per mg of cell protein)	Ca^{2+} uptake required if the mitochondria of 5% of the cells are responsible for all observed uptake (ng-ions of Ca^{2+}/min per mg of mitochondrial protein)	Observed Ca^{2+} uptake by isolated Ehrlich mitochondria equivalent to 5% of the cells (ng-ions/min per mg of mitochondrial protein)
Succinate+P_i	1.5	300	220
ATP+Mg^{2+}	3.0	600	100
Succinate+ATP+Mg^{2+}+P_i	5.5	1100	150
Maximal extent of Ca^{2+} uptake ...	(ng-ions/mg of cell protein)	(ng-ions/mg of mitochondrial protein)	(ng-ions/mg of mitochondrial protein)
Succinate+P_i	3–5	600–1000	200–250
ATP+Mg^{2+}	10–12	2000–2400	700–800
Succinate+ATP+Mg^{2+}+P_i	20–24	4000–4800	1100–1200

Ca^{2+} but also to added respiratory substrates, ATP, and other components of the medium (see the Discussion section). To test this question, a comparison has been made of the rate and extent of Ca^{2+} uptake of the isolated Ehrlich cells with that in mitochondria isolated from such cells, by using data collected in this laboratory (A. C. Griffin & A. L. Lehninger, unpublished work). Table 3 shows this comparison.

The observed rate and extent of Ca^{2+} uptake by the Ehrlich-cell population cannot be accounted for by the rate and extent of Ca^{2+} uptake of isolated mitochondria equivalent to the mitochondrial content of 5% of the cells. In the experiment in which both succinate and ATP were present, and maximum Ca^{2+} uptakes were observed, the mitochondria of 5% of the cells could account for no more than about one-sixth of the observed Ca^{2+}-uptake rate by the entire cell population. Similarly, the mitochondria of 5% of the Ehrlich cells would be unable to account for the large extent of Ca^{2+} uptake shown by the entire cell population.

Further evidence supporting these conclusions is developed in the Discussion section.

Discussion

Requirements for Ca^{2+} accumulation

Our observations indicate that Ehrlich ascites-tumour cells have the capacity, when properly supplemented, for net accumulation of Ca^{2+}, but the maximal rate and extent of Ca^{2+} uptake are observed only under rather special and possibly unphysiological experimental conditions. Ehrlich cells suspended in an aerobic medium, containing glucose but no succinate or other (site-2-linked) respiratory substrates, transport Ca^{2+} inward at very low rates. It is particularly significant that although glucose is utilized via glycolysis at a high rate in Ehrlich cells, the ATP so generated does not appear to support Ca^{2+} uptake, in confirmation of Cittadini *et al.* (1973). $^{45}Ca^{2+}$ uptake by Ehrlich cells can, however, proceed at significantly high rates, with net accumulation of Ca^{2+}, in media containing components not normally found in extracellular fluid of tumour-bearing animals. For example, when supplemented with succinate and the inhibitor rotenone, Ehrlich cells take up Ca^{2+} at a rate of about 3.5 ng-ions/min per mg of protein, and in media containing ATP as well, Ca^{2+} uptake rates exceeding 12 ng-ions/min per mg of protein have been observed. Under these sets of conditions net increases in the Ca^{2+} content of the cells occurred; moreover, in both conditions Ca^{2+} uptake was dependent on metabolic energy generated by the mitochondria. Since neither ATP nor rotenone is present in normal extracellular fluid, and succinate is present in only very low concentrations, the effects produced by these agents are unlikely to represent the normal physiological activity of these cells in Ca^{2+} transport. However, a possibly specific relationship between succinate oxidation and Ca^{2+} uptake by liver slices was pointed out by van Rossum (1969).

Although the observations reported here are in general agreement with the current view that the mitochondria of animal cells contain a significant pool of cell Ca^{2+}, which has distinctive rates of Ca^{2+} influx and efflux and which shows characteristic responses to mitochondrial inhibitors (Borle, 1973; Rose & Loewenstein, 1975), the special conditions under which we observed maximal rates and amounts of Ca^{2+} uptake have raised a number of significant questions about the nature of the Ca^{2+}-uptake process in isolated cell preparations and the factors that are important in Ca^{2+} homoeostasis in cells.

Integrity of the cell membrane as a factor in Ca^{2+} transport studies

A central concern throughout this investigation has been the degree of intactness of the isolated Ehrlich-ascites-cell preparations. Although there have been many reports of active transport by various types of cancer cells or by isolated hepatocytes, nearly all such studies have involved study of inward transport across the cell membrane against a concentration gradient, for example the inward transport of glucose, amino acids or K^+. Under these conditions the presence of a few per cent of cells with damaged, i.e. leaky, membranes permitting unmediated passage of a metabolite or mineral ion, underestimates only slightly the rate of inward transport. However, in Ca^{2+} transport by whole-cell preparations, permeability damage to the cell envelope can introduce a much more serious error, because of the facts that the cytosol Ca^{2+} concentration is very low ($<1\ \mu M$) compared with the extracellular Ca^{2+} concentration, and that two distinct membrane-transport systems are involved, but in opposite directions. It is widely accepted that Ca^{2+} is pumped out of cells by an ATP-dependent process taking place in the cell membrane. Entry of Ca^{2+} into intact cells from the extracellular fluid, in which the free Ca^{2+} concentration is $800–1000\ \mu M$, is presumed to occur via a relatively slow passive process. Since the mitochondria of animal cells are extremely active in accumulating Ca^{2+} during respiration, damage to the plasma membrane could alow Ca^{2+} to enter the cytosol freely, thence to be pumped at a high rate into the mitochondrial matrix compartment. Thus a small fraction of cells with damaged plasma membranes but intact mitochondria could cause a very large over-estimation of the rate of influx of Ca^{2+} into the cells. This factor has been given little or no consideration in earlier studies of the compartmentation of Ca^{2+} transport by whole-cell preparations (Levinson & Blumenson, 1970; Borle, 1973; Cittadini *et al.*, 1973). Although

Ehrlich ascites cells are rugged and may be easily isolated in free form without blending, homogenization or enzymic treatment, our observations suggest that differences in the manner of isolation or treatment may yield significant differences in the access of extracellular Ca^{2+}, as well as extracellular succinate, to the mitochondria within the cells, as is illustrated by the differences in the behaviour of the Ehrlich-cell preparations reported here and those studied by Cittadini *et al.* (1973). Moreover, exposure of Ehrlich cells to a hypo-osmotic NaCl medium can evoke a large increase in the permeability of the cell membrane to both succinate and Ca^{2+} (Fig. 1). Our observations that two different Ehrlich-cell strains, as well as three other types of cancer cells, L1210, Novikoff hepatoma and Walborg HS30D, consistently showed little or no respiratory response to either succinate or Ca^{2+} in the absence of phosphate and rotenone, suggests that they were somewhat more 'intact' than the preparations studied by Cittadini *et al.* (1973).

In the case of isolated hepatocytes, there is some evidence that permeability of the cell membrane to succinate correlates positively with Trypan Blue uptake (Mapes & Harris, 1975). However, even if it is assumed that the 5% of Ehrlich cells that are stained with Trypan Blue allow external succinate and Ca^{2+} to penetrate freely and that their mitochondria are completely intact, they cannot account for more than a small fraction of the Ca^{2+} uptake by the Ehrlich-cell preparations studied here, as shown by the data in Table 3. Moreover, there are other special characteristics of Ca^{2+} uptake by Ehrlich-cell suspensions that cannot be accounted for by the assumption that a large fraction of the observed Ca^{2+} uptake takes place in the 5% of permeability-damaged cells. Most striking is the observation that Ehrlich cells utilize succinate oxidation to support Ca^{2+} uptake, whereas they apparently cannot utilize oxidation of endogenous NAD-linked substrates or exogenous malate to support Ca^{2+} uptake. Extensive earlier work has shown that in isolated mitochondria from a number of animal tissues, the efficiency and stoicheiometry of all three energy-conserving sites in transporting Ca^{2+} is identical, yielding an uptake of 2 Ca^{2+} ions per 2 electrons per site (Rossi & Lehninger, 1963, 1964; Chance, 1965). Indeed, our data on whole-Ehrlich-cell preparations indicate that electron transfers through site 1 are inhibitory to Ca^{2+} uptake coupled to succinate oxidation, since rotenone, a specific inhibitor of electron flow through site 1, is required for maximal Ca^{2+} uptake coupled to succinate oxidation (Fig. 2).

Another significant difference between the behaviour of intact Ehrlich cells and isolated Ehrlich mitochondria is the rapid efflux of newly accumulated Ca^{2+} ions taking place in Ehrlich cells after about 4 min of incubation with succinate (Fig. 4), to be compared with the exceptionally high stability of newly accumulated Ca^{2+} in isolated Ehrlich-cell mitochondria, which retain Ca^{2+} tenaciously, in contrast with normal liver mitochondria (Thorne & Bygrave, 1973*a*,*b*, 1974*a*,*b*,*c*; McIntyre & Bygrave, 1974; A. C. Griffin & A. L. Lehninger, unpublished work). For these several reasons it must be concluded that the bulk of the Ca^{2+} uptake by the Ehrlich-ascites-cell preparations studied here is not primarily due to membrane leakiness of a small fraction of the cell population and the entry of Ca^{2+} into the mitochondria of the leaky cells.

Our observations on Ca^{2+} transport by isolated Ehrlich cells are in several respects similar to those on Ca^{2+} transport by isolated hepatocytes (Kleineke & Stratman, 1974). Ca^{2+}-stimulated oxygen consumption by hepatocytes was maximally supported by succinate and occurred at a rate and over time-spans comparable with those for Ehrlich cells. Moreover, respiratory inhibitors and Ruthenium Red inhibited Ca^{2+} uptake. L-Malate was less effective than succinate, and glucose failed to support Ca^{2+} uptake in the hepatocytes. Although free mitochondria could not be detected in the hepatocyte preparations used in that study, up to 15% of the cells in hepatocyte preparations can absorb Trypan Blue (Howard *et al.*, 1973). To this extent the observations on Ca^{2+} uptake in hepatocyte preparations are subject to some reservations, such as those expressed by Dubinsky & Cockrell (1975), because of the possibility that Ca^{2+} and succinate may penetrate rapidly only into those cells having a damaged plasma membrane (Mapes & Harris, 1975).

Although our observations on Ehrlich cells indicate that most of the Ca^{2+} uptake has taken place in intact cells with undamaged membranes, studies of Ca^{2+} transport in other types of cells may be compromised by significant inward leakage into damaged cells present in the preparations used.

Effect of phosphate

Another significant observation reported here is the requirement for phosphate in the medium for maximal uptake of Ca^{2+} by Ehrlich ascites cells (Fig. 5*a*). This is in agreement with the finding of Borle (1972) that increasing the concentration of phosphate in the medium surrounding cultured kidney cells can greatly expand (by up to 30-fold) the intracellular pool of Ca^{2+} that he has identified as mitochondrial. These observations are explained by the well-known fact that phosphate is the major physiological counter-anion for the respiration-dependent transport of Ca^{2+} into the mitochondrial matrix compartment (Lehninger *et al.*, 1967; Lehninger, 1974). Martin *et al.* (1975) have shown that phosphate accelerates $^{45}Ca^{2+}$ uptake by isolated fat-cells, and is taken up simultaneously.

Failure of NAD-linked substrate oxidations to support Ca^{2+} uptake

A striking and unexpected observation made in the present study is the failure of added NAD-linked substrates, such as malate or β-hydroxybutyrate, to support Ca^{2+} uptake by the Ehrlich cells, whereas added flavin-linked substrates can support Ca^{2+}, with succinate giving maximal effects. These observations cannot be attributed to the failure of the NAD-linked substrates to penetrate into the cells, since the endogenous substrates of Ehrlich cells, consisting of fatty acids and amino acids, which are oxidized via NAD, also failed to support Ca^{2+} uptake. Especially significant is the observation that succinate oxidation by the ascites cells will support Ca^{2+} uptake maximally only when rotenone is also added to the cells; rotenone suppresses oxidation of endogenous NAD-linked substrates. Since much evidence exists (Rossi & Lehninger, 1963, 1964; Lehninger *et al.*, 1967) that in isolated mitochondria NAD-linked substrates are as effective as succinate in supporting Ca^{2+} uptake, it appears more likely that in intact Ehrlich cells, NAD-linked substrates may in some manner, possibly in co-operation with specific cytosol components, stimulate Ca^{2+} release from mitochondria. The latter possibility is supported by the observation of Chudapongse & Haugaard (1973) that NAD-linked substrates promote release of Ca^{2+} from isolated liver mitochondria by phosphoenolpyruvate, whereas succinate does not show this effect. Ehrlich cells have a high rate of glycolysis and therefore of phosphoenolpyruvate production.

Effect of external ATP

Another striking effect observed here is the action of added, i.e. extracellular, ATP in supporting Ca^{2+} uptake by Ehrlich cells (Figs. 5–7). Again, it might appear that ATP is effective only because it can gain direct access to the cytosol by leaking through the membrane of damaged cells, since ATP is not present in extracellular fluid and would not normally be available to tumour cells *in vivo*. However, extracellular ATP exerts some remarkable effects on 'intact' cell preparations. Dahlquist (1974) has described ATP-induced uptake of Ca^{2+} and Na^{+} by isolated mast cells, which respond by releasing histamine. He ascribed this effect to an alteration of membrane permeability by external ATP, which was also found to increase entry of Trypan Blue into the cells (Krüger *et al.*, 1974). Perdue (1971) found that Ca^{2+} uptake by cultured chick-embryo fibroblasts is also stimulated when ATP is added to the extracellular medium, an effect inhibited by oligomycin. As with the Ehrlich cells described here, both mast cells and fibroblasts showed specificity for ATP; other nucleoside 5′-triphosphates were inactive. Perdue (1971) postulated that external ATP interacts with microfilament components of the cell membrane, increasing its permeability to both Ca^{2+} and external ATP. Ronquist & Ågren (1975) described a Ca^{2+}- and Mg^{2+}-stimulated ATPase activity on the outer surface of Ehrlich ascites-tumour cells. They earlier showed that extracellular ATP can be generated by tumour cells (Ågren & Ronquist, 1969). The permeability-increasing effect of extracellular ATP was much more pronounced in mouse 3T3 cells after viral transformation (Rozengurt & Heppel, 1975).

Since the normal activity of the Ca^{2+}-transport systems of the plasma membrane is outward-directed and depends on intracellular ATP, the occurrence of an inward movement of Ca^{2+} supported by extracellular ATP represents a rather different process. In the experiments on Ehrlich cells reported in the present paper, extracellular ATP may actually have two effects: one to increase membrane permeability of the cell to both Ca^{2+} and ATP, and the other to supply energy during its oligomycin-sensitive hydrolysis in the mitochondria for the transport of cytosolic Ca^{2+} into the mitochondrial matrix.

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