Biochimica et Biophysica Acta, 291 (1973) 246–259

BBA 76183

CALCIUM TRANSPORT IN INTACT EHRLICH ASCITES TUMOR CELLS

A. CITTADINI*, A. SCARPA** AND B. CHANCE

Johnson Research Foundation, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104 (U.S.A.)

(Received June 6th, 1972)

SUMMARY

1. Ca^{2+} transport by both intact ascites tumor cells and ascites cell mitochondria was studied spectrophotometrically using murexide, a metallochromic indicator of Ca^{2+} concentrations.

2. Ascites tumor cells can accumulate *in vitro* up to 30 μmoles of Ca^{2+} per g dry wt within 3–4 min. The accumulation occurs when endogenous or added mitochondrial substrates provide for a coupled respiration. By contrast, glycolysis is unable to support Ca^{2+} uptake.

3. In the presence of succinate and rotenone, ascites tumor cells are able to accumulate and retain significant amounts of Ca^{2+}. In the presence of endogenous or added NADH-linked substrates, the uptake of Ca^{2+} was less and in a few minutes all the Ca^{2+} accumulated by the cells is released into the medium. This release is specific for Ca^{2+} and does not occur with Mr^{2+} and Sr^{2+}, which are accumulated and retained by the cells.

4. Respiration of ascites cells was stimulated by the addition of Ca^{2+}. In the presence of rotenone and succinate, the rate of respiration returns to basal levels after the Ca^{2+} has been transported into the cell. However, with other substrates, Ca^{2+} causes a stimulation of the respiration which continues even after the accumulation and subsequent release of Ca^{2+}.

5. Ca^{2+} uptake by ascites cells was only slightly affected by the variation of monovalent cations, pH or osmolarity of the medium. It was completely inhibited by the addition of 10 mM Mg^{2+} or 15 μM ruthenium red.

6. The results obtained indicate that ascites tumor cells possess a plasma membrane permeable to Ca^{2+} and that the Ca^{2+} uptake by mitochondria inside the cell accounts for the entire cellular Ca^{2+} accumulation. The use of ascites cells as a model for studying mitochondrial Ca^{2+} uptake *in vivo* and the metabolic aspects involved in Ca^{2+} transport are discussed.

Abbreviation: MOPS, morpholinopropane sulfonate; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethoxybenzimidazone.

* Present address: Universita' Cattolica Sacro Cuore, Istituto di Patologia Generale, Roma, Italy.

** To whom correspondence should be addressed.

INTRODUCTION

The role of Ca^{2+} in controlling properties and functions of mammalian cells is well established. Permeability, excitability, deformability, adhesiveness and multiplication exemplify cellular properties for which Ca^{2+} is considered as an important regulator[1-4]. Evidence is also accumulating which shows that metabolic functions of the cell such as rates of respiration, glycolysis and gluconeogenesis can be modified by the level and the compartmentation of the intracellular Ca^{2+} (ref. 5).

In recent years, the availability of advanced techniques for the separation and purification of subcellular fractions has generated much interest in the transport and the interaction of Ca^{2+} with isolated mitochondria, microsomes, plasma membranes. In these fractions the mechanism of Ca^{2+} transport, Ca^{2+} binding and the changes of cellular properties induced by Ca^{2+} have been investigated in great detail. In contrast, the transport of Ca^{2+} in whole cells has been studied with modesty of experimental design and some lack of sophistication. And there is little known about the mechanism of Ca^{2+} transport and its effect on the metabolic states of the cell.

The understanding of the above-mentioned problem is of even greater importance in the case of tumor cells because the presence of Ca^{2+} may be inherently related to the biological characteristics of these cells: particularly with respect to their uncontrolled and self-governing growth, cell adhesiveness and contact phenomena, and their specific metabolic regulation (*i.e.* high rate of glycolysis, Crabtree effect).

The purpose of the present work is to elucidate the mechanisms of transport and/or interaction of Ca^{2+} with tumor cells. Ehrlich ascites tumor cells were chosen for this study because they live suspended in the ascites fluid from which they can be separated in large amounts without significant contaminants and with a minimum of damage to their plasma membranes. Ca^{2+} exchange or Ca^{2+} transport has already been studied in ascites tumor cells by Thomason and Schoeffield[6], Bygrave[7,8] and Levinson and Blumenson[9]. The general conclusion was that ascites tumor cells are unable to accumulate Ca^{2+} both *in vivo* and *in vitro*. All these studies, however, were carried out with $^{45}Ca^{2+}$ and with long-term observations which were inadequate to follow the relatively rapid movement of Ca^{2+} under specific metabolic conditions. For this reason, we have chosen a method which allows the kinetic detection of Ca^{2+} transport under short-term metabolic states. This condition was obtained by the spectrophotometric detection of changes in absorbance of the dye murexide. This metallochromic indicator is sensitive to Ca^{2+} concentration in the medium and its use for the detection of Ca^{2+} transients in the presence of biological systems has been well established[10-13]. In the case of ascites tumor cells, the exclusion of murexide from the intracellular space has already been demonstrated and its use for kinetic measurement of Ca^{2+} movement successfully employed[13,14].

The results obtained show that intact ascites tumor cells are able to take up significant amounts of Ca^{2+} from the medium under some metabolic conditions. The mitochondria are the most probable candidates for such transport and evidence has shown that the Ca^{2+} accumulated exerts a regulatory function on the mitochondrial respiratory chain. In addition, we would like to introduce ascites tumor

cells as a simple system in which mitochondria can be studied for their Ca^{2+} transport ability in their physiological environment, the cytosol.

MATERIALS AND METHODS

The ascites tumor cells (Ehrlich-Lettre' hyperdiploid strain) were grown in albino mice (ICR–Swiss) by weekly intraperitoneal injection of 0.2 ml of a 7-day ascites fluid. The cells were harvested 6–8 days after inoculation, then washed and resuspended at a concentration of about 70–90 mg dry wt/ml in either of the following media: (1) 154 mM NaCl, 6.2 mM KCl, 10 mM morpholinopropane sulfonate (MOPS), pH 7.4; (2) 225 mM mannitol, 75 mM sucrose, 10 mM MOPS, pH 7.4. In saline medium, MOPS was used as buffer instead of the P_i buffer to avoid any possible interference of this anion with Ca^{2+}.

Mitochondria were prepared from isolated ascites cells according to the method of Kobayashi *et al.*[15] with slight modifications[16] and suspended at a concentration of about 10 mg protein/ml in the mannitol–sucrose–MOPS medium. Mitochondrial protein was measured by the biuret method.

Ca^{2+}, Sr^{2+} and Mn^{2+} movements were followed in the above-mentioned media using murexide (ammonium purpurate) as indicator. Changes in absorbance of the dye were monitored by the dual-wavelength Aminco–Chance spectrophotometer at 540–507 nm as described before[13,14]. The same instrument was used for the measurement of cytochrome *c* content of intact cells and isolated mitochondria at 550–540 nm.

Oxygen consumption was measured polarographically with a Clark-type electrode in a stirred vessel and recorded.

All the experiments were performed at room temperature except where indicated. The 4,5,6,7-tetrachloro-2-trifluoromethoxybenzimidazone (TTFB) was a gift from Dr B. Beechey, Shell Research, Sittingbourne (England). Murexide and La^{3+} were purchased from K and K Chemicals, Plainview, N.Y., ruthenium red (tetraaminoruthenium hydroxychlorochloride) from Alfa Inorganics, Beverly, Mass., and was purified by recrystallization. 20 ppm of such material, dissolved in 0.1 M ammonium acetate (pH 6.9) gives absorbance of 1.47 at 533 nm (1-cm light path). All the other chemicals were of analytical grade.

RESULTS

Ca^{2+} uptake by ascites tumor cells

The addition of $CaCl_2$ to buffered saline solutions containing ascites tumor cells produced a rapid downward deflection of the trace due to the formation of Ca^{2+}–murexide complex which absorbs less light at 540 nm than does the free murexide (Fig. 1). When the cells were oxidizing endogenous substrate, the subsequent slow increase in absorbance indicates that Ca^{2+} is disappearing from the medium and therefore being taken up by the cells. This accumulation occurs during the first 2–3 min and is followed by the reappearance of the Ca^{2+} in the medium. After 6 min, all the Ca^{2+} previously accumulated by the cells is released. If the medium was supplemented with glutamate and malate the amount of Ca^{2+} taken up by the cells was twice as much and the following release was slower. In the presence of

rotenone and succinate a further enhancement of the Ca^{2+} accumulation was observed and there was no Ca^{2+} release during the first 10 min. However, if the cells were incubated in the presence of rotenone alone, the straight line after addition of Ca^{2+} indicates that none of the Ca^{2+} added has been taken up by the cells.

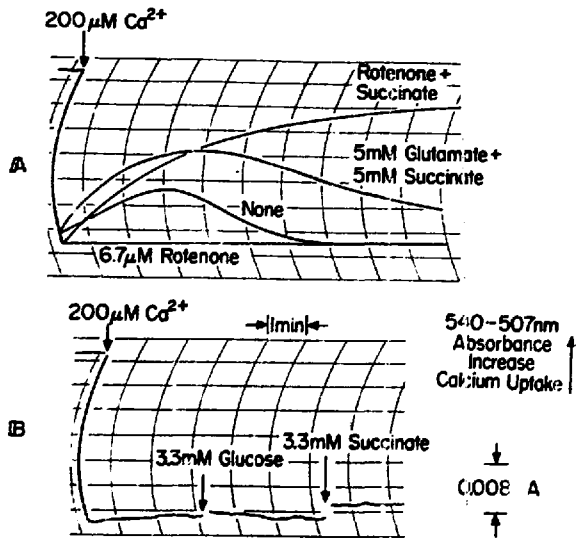

Fig. 1. A and B. Ca^{2+} uptake by intact ascites tumor cells under different metabolic conditions. The medium contained 154 mM NaCl, 6.2 mM KCl, 10 mM MOPS buffer, pH 7.4, 40 μM murexide, 15.6 mg dry wt/ml ascites tumor cells and the substrates and inhibitors indicated in the figure. In B, the experiment was carried out in the same medium saturated with nitrogen.

Similarly, Fig. 1B shows that in the absence of oxygen no measurable Ca^{2+} accumulation takes place. Under anaerobic conditions, glucose, which induces a fast and great production of ATP, in these cells[17] was unable to support the uptake of Ca^{2+}. This result clearly indicates that the ATP produced by glycolysis cannot be utilized by ascites tumor cells for the transport of Ca^{2+}. Moreover, Fig. 2 shows that the ATP produced by respiration is not necessary for the accumulation of Ca^{2+}. The percentage of Ca^{2+} accumulation induced by the addition of succinate and rotenone in tumor cells was plotted as a function of the amounts of oligomycin present in the medium. Oligomycin at an amount 3 times higher than that required to inhibit by 90 % the respiration of ascites tumor cells[17, 18, 19] did not inhibit the accumulation of Ca^{2+}.

Fig. 3A shows the results of an experiment in which the osmolarity of the medium was changed in order to achieve a different cell volume. Almost no differences in Ca^{2+} transport were observed between osmolarities of 0.08 and 0.35. Fig. 3B shows that the pH of the medium had only a limited effect on the Ca^{2+} transport, the rate of which was not proportional to the H^+ or OH^- concentrations. While in sucrose–mannitol media the rates of Ca^{2+} uptake by ascites cells was 50 % higher than that in NaCl, no effect was observed by substituting NaCl with either KCl or LiCl.

All these data indicate that experimental conditions acting extra-cellularly and leading to dramatic changes in the properties of the plasma membranes have very little effect on Ca^{2+} uptake by these cells.

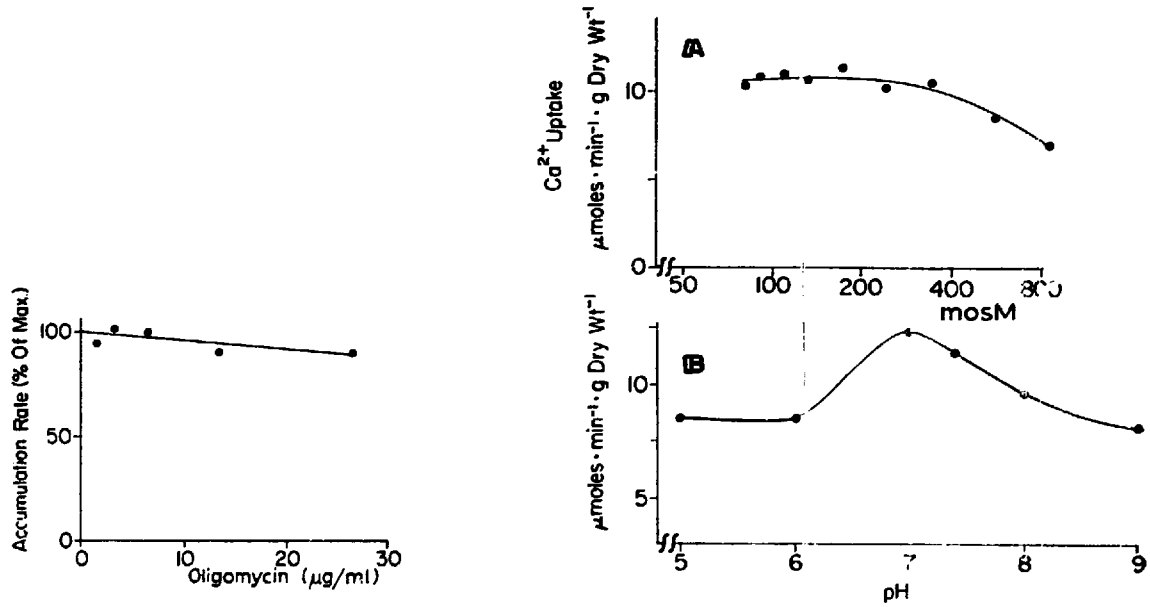

Fig. 2. Effect of oligomycin on the rate of Ca^{2+} uptake by ascites tumor cells. The medium contained 154 mM NaCl, 6.2 mM KCl, 10 mM MOPS buffer, pH 7.4, 40 μM murexide, 9.1 mg dry wt/ml ascites tumor cells, 6 μM rotenone and 5 mM sodium succinate. The amounts of oligomycin indicated in the figure were then added. The reaction was started by addition of 200 μM Ca^{2+} and the percent of initial rates of Ca^{2+} accumulation (with respect to the rate in the absence of oligomycin) were plotted *versus* oligomycin concentrations.

Fig. 3. A and B, the effect of osmolarity and pH of the medium on Ca^{2+} uptake by ascites cells. In A, the medium contained 10 mM MOPS buffer, pH 7.4, 40 μM murexide and a mixture of mannitol–sucrose (3:1, by vol.) to achieve the osmolarities reported in the figure. In B, the medium contained 225 mM mannitol, 75 mM sucrose, 40 μM murexide and 10 mM MOPS at the pH reported in the figure. In all the experiments, cells were incubated with 5 mM succinate and 5 μM rotenone and the reaction was initiated by 200 μM Ca^{2+}. The initial rate values of Ca^{2+} accumulation are reported. Cells were 9.8 mg dry wt/ml.

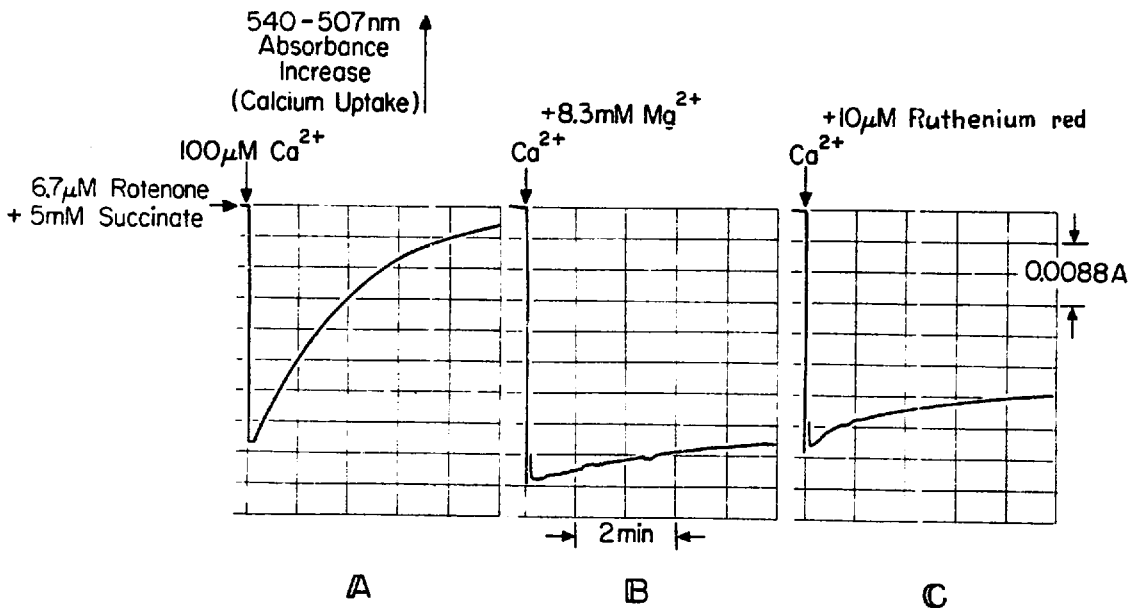

Fig. 4. A, B and C, Ca^{2+} uptake by ascites cells and the effect of Mg^{2+} and ruthenium red. Experimental conditions as in Fig. 2, except for the amount of cells which was 9.0 mg dry wt/ml.

Inhibitors of Ca^{2+} transport

The inhibitory effects of Mg^{2+} and ruthenium red on the Ca^{2+} transport in ascites tumor cells are shown in Fig. 4. Fig. 4B shows that $MgCl_2$ at a concentration of 8.3 mM is able to inhibit Ca^{2+} transport by more than 80 %. The same amount of Mg^{2+} is without effect on Ca^{2+} transport by mitochondria isolated from these cells (data not shown) or from other tissues[21,22].

Fig. 4C shows that ruthenium red, at a concentration of 10 μM is able to produce similar inhibition. Ruthenium red and La^{3+} have been widely used as markers in staining techniques for extracellular spaces in plasma membranes[23,24] and, in addition, their use has been successfully applied for the inhibition of the Ca^{2+} transport in isolated mitochondria[25,26].

Fig. 5, A and B, compares the inhibitions by ruthenium red and La^{3+} on the process of Ca^{2+} uptake by both whole ascites tumor cells and mitochondria isolated from these cells. Fig. 5A shows that nearly identical concentrations of ruthenium

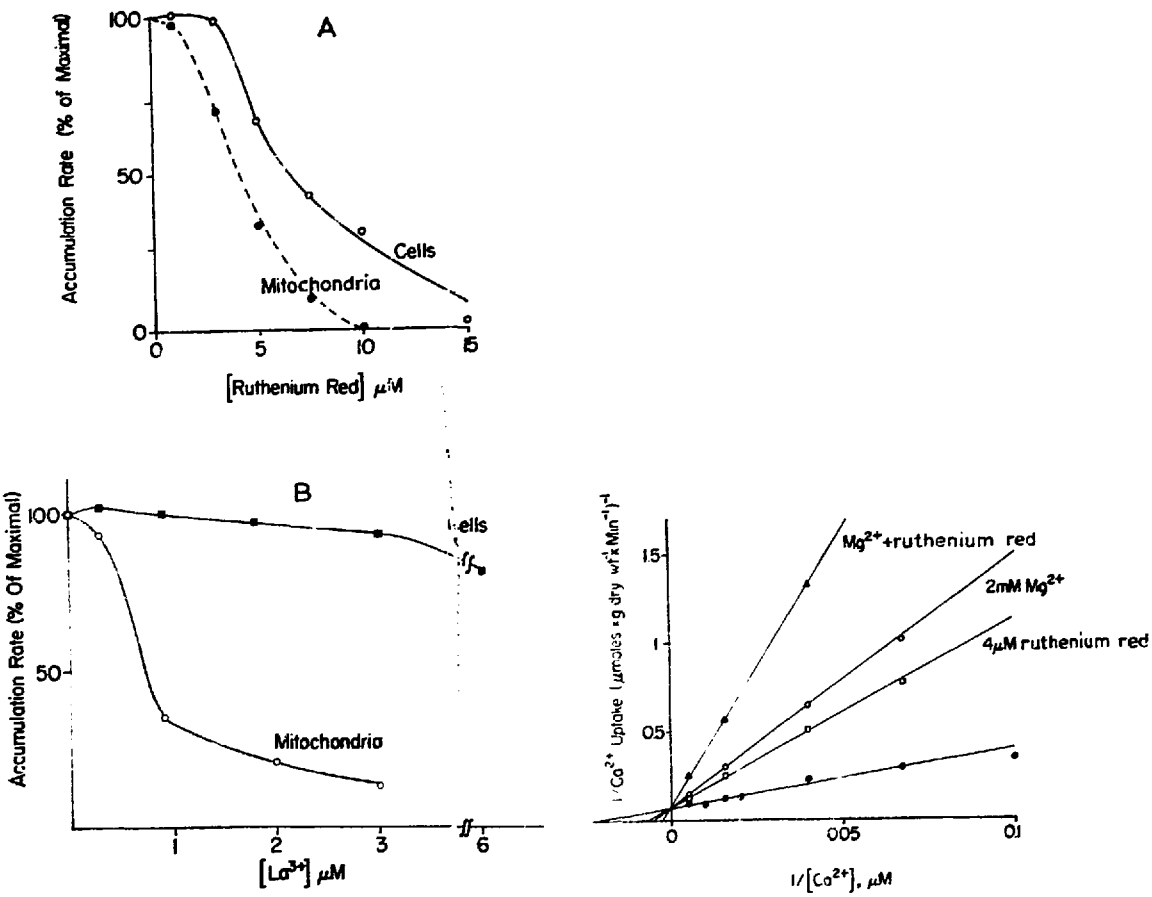

Fig. 5. A and B, the effect of ruthenium red and La^{3+} on Ca^{2+} uptake by ascites cells and by mitochondria from ascites cells. Experimental conditions as in Fig. 3B, except for the presence of ruthenium red or $LaCl_3$ when indicated. Percentages of initial rate of Ca^{2+} uptake with respect to the rate in the absence of inhibitor were plotted. Protein concentrations were 1.5 mg/ml and 9.8 mg dry wt/ml for mitochondria and ascites cells, respectively.

Fig. 6. Inhibition constants of Mg^{2+} and ruthenium red on Ca^{2+} transport by ascites tumor cells. The experiment was carried out in a medium identical to that of Fig. 3B, with the addition, when indicated, of 5 μM ruthenium red or 2 mM $MgCl_2$ or 2 mM $MgCl_2$ *plus* 4 μM ruthenium red. The reaction was started by the Ca^{2+} addition in varying amounts and the initial rates are reported. Cells were 10.1 mg dry wt/ml.

red are required to inhibit the energy-linked Ca^{2+} uptake by both intact cells and isolated mitochondria. In contrast (Fig. 5B), La^{3+}, while producing the known inhibitory effect on the isolated mitochondria, was unable to significantly impair the uptake by the whole cells even at large concentrations.

Of the two inhibitors, Mg^{2+} inhibits only Ca^{2+} transport by the whole cell, whereas ruthenium red inhibits transport, both of intact cells and of isolated mitochondria. Ruthenium red can therefore inhibit Ca^{2+} transport in the intact cell through two mechanisms: (a) either by diffusing into the cytosol and blocking the mitochondrial uptake inside the cell, or (b) by acting at both, the same site of Mg^{2+} in the case in intact cells and at the same site of La^{3+} in the case of isolated mitochondria.

The experiment shown in Fig. 6 was designed to investigate whether ruthenium red and Mg^{2+} possess the same site for inhibiting the transport of Ca^{2+} by ascites tumor cells. Based on the assumption that the velocity of Ca^{2+} uptake is proportional to the concentration of Ca^{2+} bound to binding sites of either fixed charges or carriers, the formula for competitive inhibition:

$$v = \frac{VN_T}{1 + \frac{K_{Ca^{2+}}}{[Ca^{2+}]} + \frac{K_{Ca^{2+}}[RR]}{[Ca^{2+}]K_{RR}} + \frac{K_{Ca^{2+}}[Mg^{2+}]}{[Ca^{2+}]K_{Mg^{2+}}}}$$

(where N_T = total number of sites) was used to calculate the inhibition constant for Mg^{2+} and ruthenium red (RR) from the intercepts on the abscissa. These values are 43.5, 360 and 0.93 μM for Ca^{2+}, Mg^{2+} and ruthenium red, respectively. Velocities were then measured at different Ca^{2+} concentrations in the presence of both inhibitors. The intercept on the abscissa, 0.0250, was compared to a value of 0.0212 calculated from the inhibition constant obtained independently. The mutual intercepts on the ordinate and the close agreement with the equation mean that the Ca^{2+} site must contain only one of the ions, either Ca^{2+}, Mg^{2+} or ruthenium red at any time and that Mg^{2+} and ruthenium red do not influence Ca^{2+} uptake except by binding at this site.

Transport of Sr^{2+} and Mn^{2+}

As previously observed (Fig. 1A), Fig. 7A shows that ascites tumor cells are able to accumulate only limited amounts of Ca^{2+} with endogenous substrates and when 3 min have elapsed the Ca^{2+} taken up is released into the medium. This effect was specific for Ca^{2+} and it could not be reproduced in the presence of other divalent cations. The uptake of Sr^{2+} and Mn^{2+} by ascites cells oxidizing endogenous substrates is shown in Fig. 7B and 7C, respectively. It is evident that both cations are accumulated by the cells in an energy-linked process. Sr^{2+} and Mn^{2+} were accumulated at a slower rate but to a larger extent than was Ca^{2+}. In addition, even after long periods of time, the cations accumulated were not released into the medium unless the uncoupler, TTBF, was added.

Fig. 8 shows the uptake of Ca^{2+} (A), Sr^{2+} (B) and Mn^{2+} (C) by ascites tumor cells in the presence of succinate–rotenone (left) and glutamate–succinate (right). In the presence of succinate and rotenone the cells were able to take up and retain

large amounts of divalent cations: the extent of accumulation was similar for all the cations and some differences were seen on the rate of uptake ($Ca^{2+} > Sr^{2+} > Mn^{2+}$). However, in the absence of rotenone, a phenomenon similar to that in Fig. 7 was observed: Ca^{2+} was taken up and then released, whereas all the Sr^{2+} of Mn^{2+} added was taken up and retained. The results indicate that the release of Ca^{2+} with both endogenous of added substrates is not due to exhaustion of substrate but rather deals

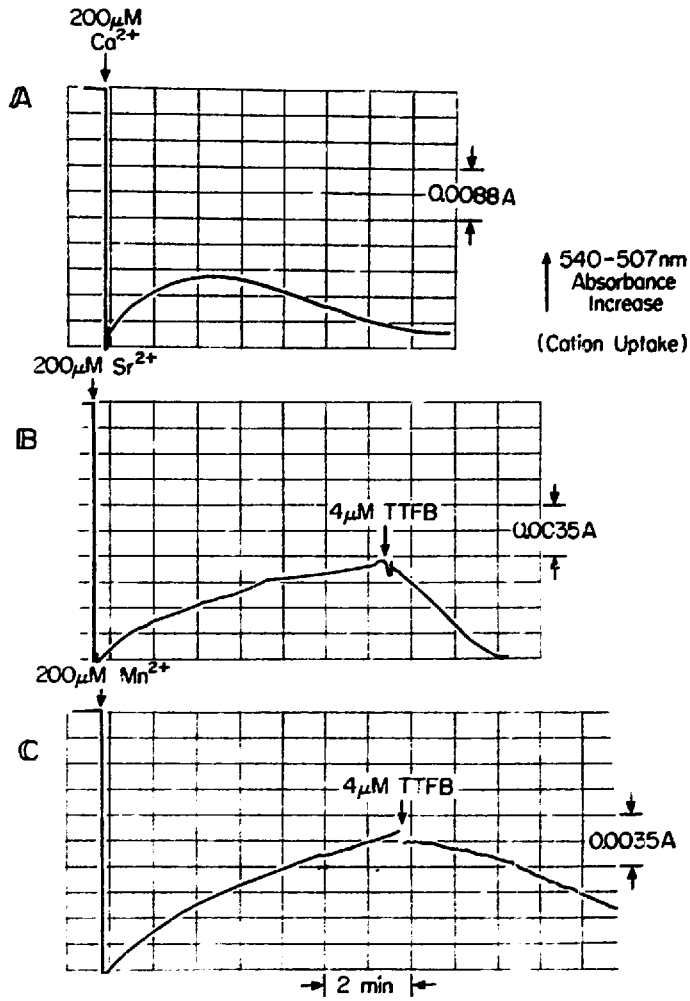

Fig. 7. A, B and C, the uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} by ascites cells oxidizing endogenous substrates. The medium contained 225 mM mannitol, 75 mM sucrose, 10 mM MOPS, pH 7.6, 40 μM-murexide and 9.8 mg dry wt/ml of ascites cells.

with specific irreversible interaction of Ca^{2+} at the level of the energy production of the cells. These findings are confirmed by the data reported in Fig. 9. Fig. 9A shows that the addition of Ca^{2+} to the cells is followed by the above described uptake and release of Ca^{2+}. When the Ca^{2+} was released, the cells were unable to accumulate added Mn^{2+}. By contrast, Fig. 9B shows that the cells maintained their ability to transport Ca^{2+} after they were preloaded with large amounts of Mn^{2+}. If any effect could be ascribed to the Mn^{2+} inside, then this would be an enhancement rather than inhibition of the Ca^{2+} transport.

Effect of Ca^{2+} on respiration

Fig. 10 shows the recording traces of the respiration of ascites tumor cells under different metabolic conditions. The left trace shows that the addition of Ca^{2+} produced an increase in the oxygen utilization which remained unchanged for more than 5 min (until anaerobiosis occurred). By contrast, the right trace shows that when the cells were incubated in the presence of rotenone, the increase in respiration by Ca^{2+} was followed by a decrease in respiration which could be further stimulated.

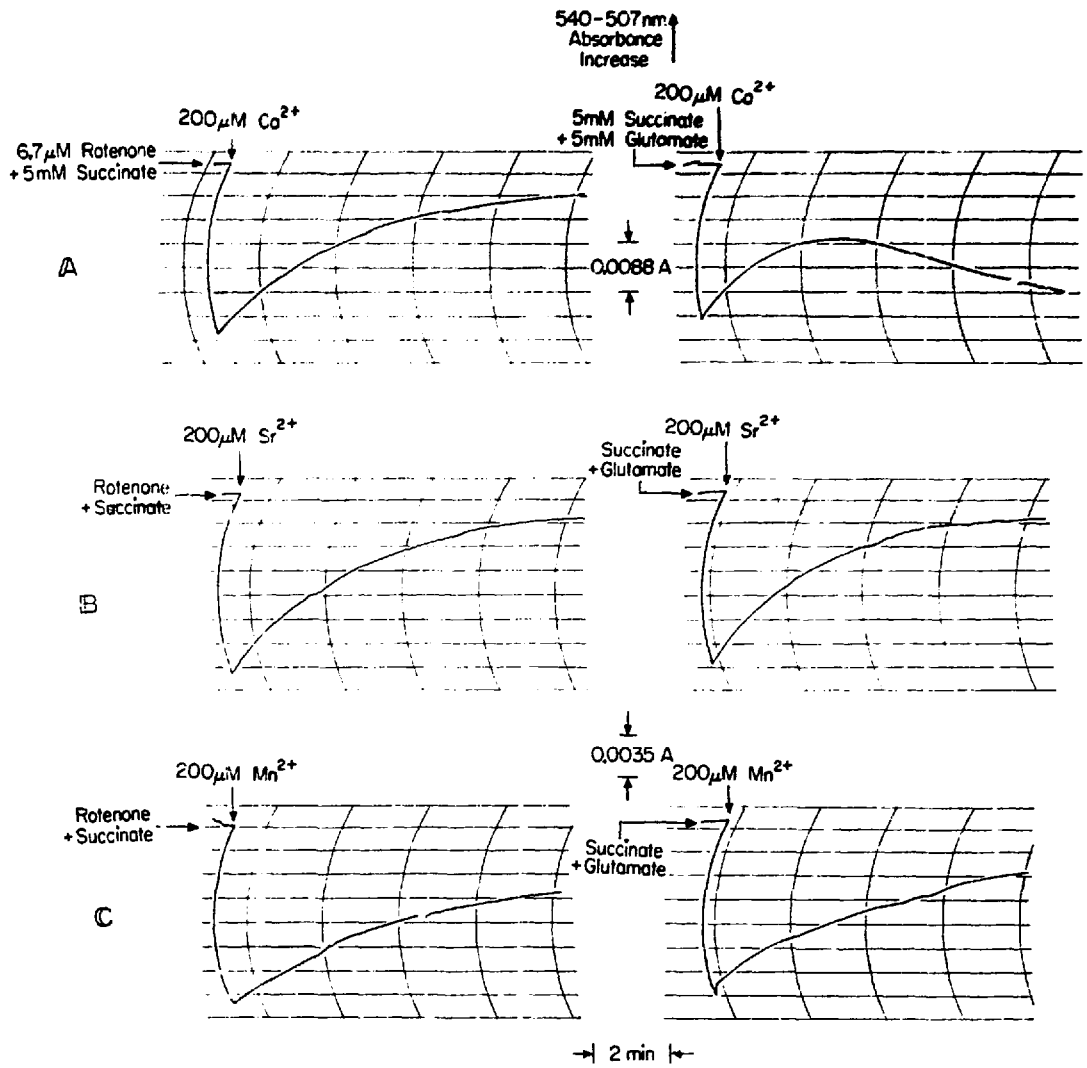

Fig. 8. A, B and C, the uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} by ascites cells in the presence of added substrates. Experimental conditions as in Fig. 7. Additions are reported in the figure.

Comparison of the traces of respiration and those of Ca^{2+} measurements shows that when Ca^{2+} is taken up by endogenous or added substrates, an increase of respiration occurred which was not depressed even when the Ca^{2+} was entirely released by the cells. In the presence of rotenone, however, the respiration rate was increased only for the time in which Ca^{2+} was taken up by the cells and then returned to steady-state levels after Ca^{2+} was accumulated.

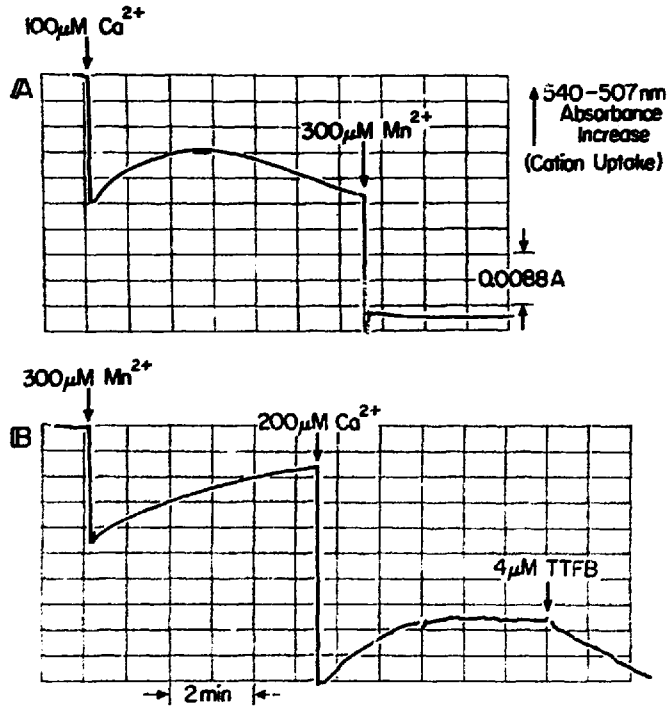

Fig. 9. A and B, the effect of Ca^{2+} on Mn^{2+} accumulation. Experimental conditions as in Fig. 7, except for the cell concentration which was 8.9 mg dry wt/ml.

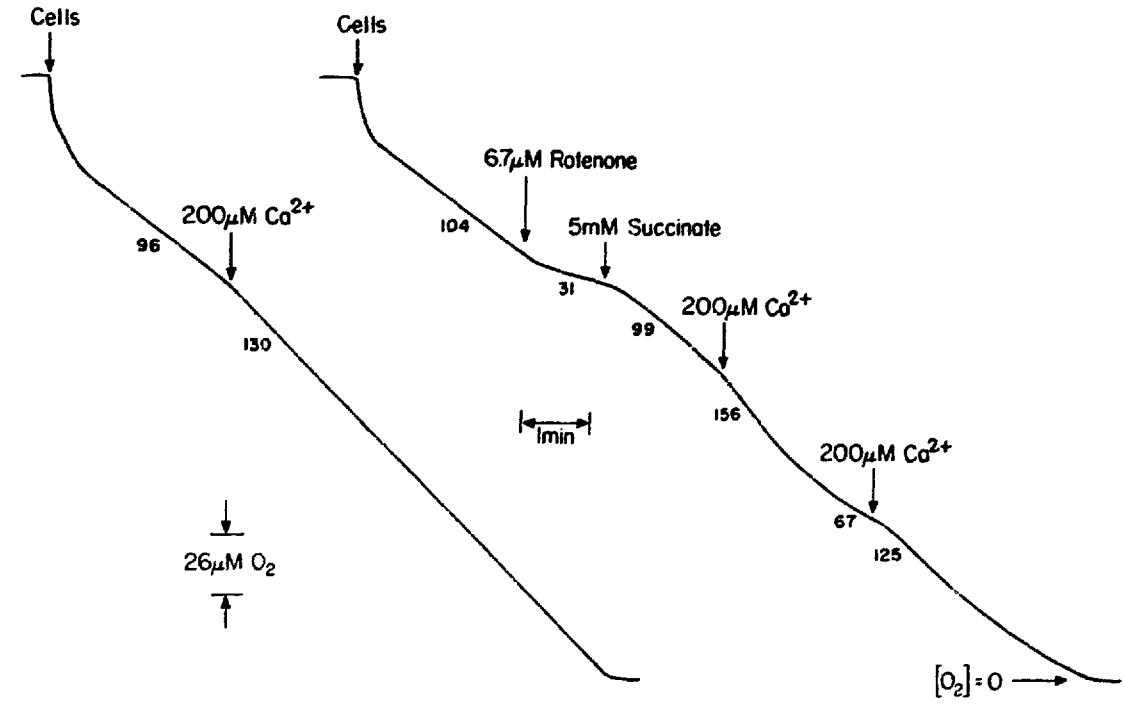

Fig. 10. The effect of Ca^{2+} on the rate of oxygen utilization by ascites cells. Experimental medium as in Fig. 1. All the additions are reported. The numbers in the figure are the value of oxygen utilization at the respective point (nmoles/min). Cells were 20.5 mg dry wt/ml.

DISCUSSION

Ca^{2+} accumulation by intact tumor cells

The results presented in this paper clearly show that intact ascites tumor cells are able to take up significant amounts of Ca^{2+} from the surrounding medium. The accumulation of Ca^{2+} occurs when endogenous or added mitochondrial substrates support cellular respiration. The lack of Ca^{2+} accumulation in the presence of the uncoupler, TTFB, indicates that coupled respiration is required. The lack of Ca^{2+} transport in the presence of glucose, where ATP is synthesized, and the inability of oligomycin to block aerobic Ca^{2+} transport suggest that ATP as such is not involved in the uptake of Ca^{2+} by ascites tumor cells. We do not have an explanation of why ATP, while supporting Ca^{2+} uptake in isolated mitochondria by energization of a high energy intermediate[27], is unable to produce Ca^{2+} accumulation when present in the cytosol. This once again brings into question the specificity of the adenine nucleotide translocator or the existence of a compartmentation of ATP in ascites tumor cells[16,28,30]. On the other hand, glycolysis could induce changes in Ca^{2+} permeability of ascites cells and therefore further studies are required to clarify the problem.

Contrary to our observations, previous studies available in the literature conclude that Ca^{2+} is not accumulated by intact ascites tumor cells. This is the case of Bygrave[7,8] who states that ascites tumor cells are unable to take up Ca^{2+} *in vitro* and of Levinson and Blumenson[9] who describe different compartments for the exchangeable $^{45}Ca^{2+}$ and conclude that ascites tumor cells do not have an energy-dependent accumulation of Ca^{2+}. However, such a discrepancy can readily be explained by their experimental conditions. The longer times for incubation and sampling and the use of glycolytic substrates, easily accounts for the lack of accumulation observed by previous authors. Even under our conditions, no Ca^{2+} uptake was detectable in the presence of glucose. Moreover, our data, which show that Ca^{2+} is taken up and then released, are in line with the results of Levinson and Blumenson[9] who measured $^{45}Ca^{2+}$ distribution after 1 h and showed no Ca^{2+} uptake, only Ca^{2+} exchange.

The role of mitochondria in the Ca^{2+} uptake by ascites tumor cells

The requirement of a coupled respiration for Ca^{2+} transport by intact ascites tumor cells indicates that mitochondria are required for the process. The involvement of mitochondria can, however, be of two different types: the mitochondria can either supply the energy for an unidentified energy-dependent transport of Ca^{2+}, or they can accumulate Ca^{2+} themselves such that the observed cellular transport of Ca^{2+} can be confined to a simple mitochondrial uptake. All the evidence reported here suggests that the entire Ca^{2+} accumulation by ascites tumor cells can be accounted for by an uptake of Ca^{2+} by mitochondria inside the cells. This interpretation is based on the following considerations:

(1) Ca^{2+} uptake by ascites tumor cells presents characteristics similar to that of mitochondria isolated from the same cells or other sources: it is supported by oxidation of mitochondrial substrates, it is blocked by inhibition or uncoupling of respiration; and it is insensitive to oligomycin[27].

(2) As in the case of isolated mitochondria[31], the addition of Ca^{2+} to the cells

stimulates oxygen utilization. Even more specifically, the mitochondrial cytochrome *b* of ascites cells responds to changes of the extra-cellular Ca^{2+} concentrations with cyclic oxidation–reduction[14], in a way similar to that reported for isolated mitochondria[27].

(3) Ca^{2+}, Sr^{2+} and Mn^{2+} have been reported to be accumulated by isolated mitochondria at different rates[12,31,32]; a similar order for the initial rates of divalent cations accumulation has also been found in ascites tumor cells.

(4) If the mitochondria of ascites tumor cells are involved in cellular Ca^{2+} translocation simply for making the energy for the process available, then this energy cannot be in the form of ATP. This is demonstrated by the inability of ATP produced by glycolysis to support the uptake of Ca^{2+} and by the lack of effect of oligomycin on the cellular uptake of Ca^{2+} induced by mitochondrial substrates. Once again, cellular Ca^{2+} uptake resembles Ca^{2+} uptake by isolated mitochondria which, in contrast to that occurring in other membranes[33], is supported by a high energy intermediate which is not ATP[27].

Our conclusions therefore suggest that Ca^{2+} uptake by mitochondria inside the cells accounts entirely, or at least to a major extent, for the accumulation of Ca^{2+} by the whole cells. Two requirements must be satisfied to draw these conclusions: the capability of mitochondria to entirely accumulate the amounts of Ca^{2+} taken up by the cells, and a plasma membrane freely permeable to Ca^{2+}. The first point can be solved by comparing, on the basis of cytochrome *c* content, the rates and extents of Ca^{2+} uptake in ascites tumor cells and in mitochondria isolated from these cells. In intact cells Ca^{2+} uptake expressed per μmole of cytochrome *c* was 17.5 nmoles. In mitochondria isolated from ascites cells, it was 340 nmoles per μmole of cytochrome *c*. These results imply that mitochondria, when isolated, have the ability to accumulate an amount of Ca^{2+} which is about twenty times greater than that accumulated by whole cells containing the same amounts of mitochondria.

Plasma membranes and Ca^{2+} transport

Evidence for a plasma membrane permeable to Ca^{2+} can be drawn from previous results showing a metabolically independent compartmentation and exchangeability of Ca^{2+} in ascites tumor cells[9]. These findings are in agreement with our evidence which shows that changes in pH, monovalent cation and osmolarity of the medium slightly affect the process of Ca^{2+} translocation. The inhibition of the transport by Mg^{2+} and ruthenium red can be explained through their capacity of blocking the channels for Ca^{2+} or through combination with fixed charges, therefore inhibiting Ca^{2+} diffusion through the plasma membrane. The location of ruthenium red on the plasma membrane has been widely documented and ample evidence has been forwarded by Luft[24] that ruthenium red does not penetrate the plasma membranes of a variety of cells unless the membrane is broken.

An alternative argument is that plasma membranes of ascites tumor cells possess wide and aspecific permeability, either due to the intrinsic properties of the cells or induced by the damage of the plasma membrane during isolation. However, this possibility can be excluded by the minimum of manipulation of the cells during isolation, by the reported impermeability to other cations under the same conditions[34,35] and by the inhibition by Mg^{2+} on the cellular (and not the mitochondrial) Ca^{2+} transport.

In summary, our results indicate that plasma membranes of ascites tumor cells are permeable to Ca^{2+}. Externally added Ca^{2+} distributes at equilibrium between surrounding medium and cytosol. In the presence of coupled respiration, mitochondria accumulate cytosolic Ca^{2+} against a gradient and the uptake of Ca^{2+} by the cell is simply the result of the passage of Ca^{2+} through the plasma membrane to achieve a new equilibrium within the cytosol modified by the mitochondrial uptake.

Model for the study of mitochondrial Ca^{2+} uptake in vivo

Mitochondria, isolated *in vitro* from different sources, contain relatively large amounts of Ca^{2+} and are able to accumulate Ca^{2+} under various experimental circumstances. However, these findings do not allow extrapolation to *in vivo* conditions where the environment surrounding the mitochondria differs from any *in vitro* conditions. Indirect evidence has already indicated that mitochondria are able to accumulate Ca^{2+} *in vivo*. This is the effect of mitochondrial inhibitors and the function of cells in the presence and in the absence of Ca^{2+} (ref. 36), and the $^{45}Ca^{2+}$ distribution after separation of mitochondria from cells under different metabolic conditions[37]. However, these observations, which have been criticized on the basis of the experimental approach or on the uncertainty of the conclusion drawn[38], are very indirect and lack circumstantial evidence.

The ability of ascites tumor cells mitochondria *in situ* to accumulate amounts of Ca^{2+} in a way similar to that observed in the mitochondria isolated from these cells, could be used as a proof for a mitochondrial Ca^{2+} uptake *in vivo*. Furthermore, ascites tumor cells should prove a useful model for the study of Ca^{2+} accumulation by mitochondria and its effects in the whole cell. The above-mentioned characteristics (easy preparation, constant characteristics, well-studied metabolic properties and absence of contaminants) make the ascites tumor cell an ideal model for Ca^{2+} transport in which the uptake of Ca^{2+} by mitochondria *in situ* cannot only be demonstrated but also studied in great detail.

ACKNOWLEDGEMENTS

The present work was supported by Grants from the U.S. Public Health Service (GM 12202) and from the American Cancer Society. One of us (A.C.) acknowledges the financial support from Damon Runyon Fund for Cancer Research and Associazione Italiana Promozione Ricerche Cancro.

The authors are also grateful fo Mr Robert Hershberg for his help in preparing this manuscript.

REFERENCES

1 Manery, J. F. (1969) in *Mineral Metabolism* (Colmar, C. L. and Bronner, F., eds), Vol. 3, pp. 405–452, Academic Press, New York
2 Perris, A. D. (1971) in *Cellular Mechanisms for Calcium Transfer and Homeostasis* (Nichols, G. and Wasserman, R. H., eds), pp. 101–131, Academic Press, New York
3 Smyth, H. and Flaharan, E. (1969) *Life Sci.* 8-Part II, 1317–1322
4 Balk, S. D. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 271–275
5 Gevers, W. and Krebs, H. A. (1966) *Biochem. J.* 98, 720–735
6 Thomason, D. and Schoeffield, R. (1959) *Exp. Cell Res.* 16, 324–334
7 Bygrave, F. L. (1966) *Biochem. J.* 101, 480–487
8 Bygrave, F. L. (1967) *Nature* 214, 667–671

9 Levinson, C. and Blumenson, L. E. (1970) *J. Cell Physiol.* 75, 231–240
10 Ohnishi, T. and Ebashi, S. (1964) *J. Biochem. Tokyo* 55, 599–603
11 Jobsis, F. F. and O'Connor, H. G. (1966) *Biochem. Biophys. Res. Commun.* 25, 246–252
12 Mela, L. and Chance, B. (1968) *Biochemistry* 7, 4059–4063
13 Scarpa, A. (1972) in *Methods in Enzymology* (A. San Pietro, ed.), Vol. 24, pp. 343–351, Academic Press, New York
14 Cittadini, A., Scarpa, A. and Chance, B. (1971) *FEBS Lett.* 18, 98–102
15 Kobayashi, S., Hagighara, B., Mazumi, M. and Okunuki, K. (1966) *Biochim. Biophys. Acta* 113, 421–437
16 Maitra, P. K. and Chance, B. (1965) in *Control of Energy Metabolism* (Chance, B., Estabrook, R. W. and Williamson, J. R., eds), pp. 157–175, Academic Press, New York
17 Dallner, G. and Ernster, L. (1962) *Exp. Cell Res.* 27, 372–375
18 Dionisi, O., Cittadini, A., Gelmuzzi, G., Galeotti, T. and Terranova, T. (1970) *Biochim. Biophys. Acta* 216, 71–79
19 Slater, E. C. (1967) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 10, pp. 48–57, Academic Press, New York
20 Webb, J. L. (1966) *Enzyme and Metabolic Inhibitors*, Vol. 1, pp. 149–191, Academic Press, New York
21 Scarpa, A. and Azzi, A. (1968) *Biochim. Biophys. Acta* 150, 473–481
22 Scarpa, A. and Azzone, G. F. (1968) *J. Biol. Chem.* 243, 5132–5138
23 Hashimoto, K. (1971) *J. Ultrastruct. Res.* 36, 249–262
24 Luft, J. H. (1971) *Anat. Rec.* 171, 369–416
25 Mela, L. (1969) *Biochemistry* 8, 2481–2486
26 Moore, C. (1971) *Biochem. Biophys. Res. Commun.* 42, 298–305
27 Chance, B. (1965) *J. Biol. Chem.* 240, 2729–2748
28 Chance, B. (1964) *Acta Union Int. Contre Cancer*, 20, 1028–1032
29 Lee, I. Y. and Coe, E. L. (1967) *Biochim. Biophys. Acta* 131, 441–452
30 Ritter, C. and Thorell, B. (1971) *Exp. Cell Res.* 65, 233–239
31 Lehninger, A. L., Carafoli, E. and Rossi, S. C. (1967) in *Advances in Enzymology* (Nord, F. F., ed.), Vol. 29, pp. 259–320, Interscience, New York
32 Chappell, J. B., Cohn, M. and Greville, G. P. (1963) in *Energy-Linked Function of Mitochondria* (Chance, B., ed.), pp. 229–235, Academic Press, New York
33 Nichols Jr, G. and Wasserman, R. H. (1971) *Cellular Mechanisms for Calcium Transfer and Homeostasis*, Academic Press, New York
34 Wenner, C. E., Harris, E. J. and Pressmann, B. C. (1967) *J. Biol. Chem.* 242, 3654–3459
35 Poole, D. T., Butler, T. C. and Williams, M. E. (1971) *J. Membrane Biol.* 5, 261–276
36 Haugaard, N., Haugaard, E. S., Lee, N. H. and Horn, R. S. (1969) *Fed. Proc.* 24, 1657–1662
37 Carafoli, E. (1967) *J. Gen. Physiol.* 50, 1849–1864
38 Kübler, W. and Shinebourne, E. A. (1971) in *Calcium and the Heart* (Harris, P. and Opie, L. H., eds), pp. 93–123, Academic Press, New York