Margolis, S. A., Lenaz, G., and Baum, H. (1967), *Arch. Biochem. Biophys. 118*, 224.
Mitchell, P. (1966), *Biol. Rev. 41*, 445.
Mitchell, P. (1961), *Nature 191*, 144.
Moore, C. and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562,
Nishimura, M., Ito, T., and Chance, B. (1962), *Biochim. Biophys. Acta 59*, 177.
Packer, L., Marchant, R. H., and Mukohata, Y. (1963), *in* Energy-Linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 51.
Sanadi, D. R. (1968), *Arch. Biochem. Biophys. 128*, 280.
Saris, N. E. (1963), *Soc. Sci. Fennica Commentationes Phys.-Math.*, 38.
Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.
Slater, E. C. (1953), *Nature 172*, 975.
Slater, E. C., and Colpa-Boonstra, J. P. (1961), *in* Haematin Enzymes, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., New York, N. Y., Pergamon, p 575.
Slater, E. C., and Tager, J. M. (1963), *in* Energy-Linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic, p 97.
van Dam, K., and Slater, E. C. (1967), *Proc. Natl. Acad. Sci. 58*, 2015.
van Gelder, B. F. (1966), *Biochim. Biophys. Acta 118*, 36.
Williamson, R. L., and Metcalf, R. L., (1967), *Science 158*, 1694.
Wilson, D. F. (1967), *Biochim. Biophys. Acta 131*, 431.
Wilson, D. F., and Azzi, A. (1968), *Arch. Biochem. Biophys. 126*, 724.
Wilson, D. F., and Chance, B. (1967), *Biochim. Biophys. Acta 131*, 421.
Wilson, D. F., and Gilmour, M. V. (1967), *Biochim. Biophys. Acta 143*, 52.
Wilson, D. F., and Merz, R. D. (1967), *Arch. Biochem. Biophys. 119*, 470.

Inhibition and Activation of Calcium Transport in Mitochondria. Effect of Lanthanides and Local Anesthetic Drugs*

Leena Mela

ABSTRACT: Using murexide to measure the Ca^{2+} accumulation rates and bromothymol blue to measure the rates of the intramitochondrial pH changes, and by measuring the oxidation–reduction changes of the respiratory chain carriers during Ca^{2+} accumulation, it was found that lanthanides at a concentration of about 0.05–0.07 mμmole/mg of protein specifically inhibit the reactions of Ca^{2+} with the mitochondrial membranes and the accumulation of Ca^{2+} into the mitochondria. No other functions of the mitochondria were found to be inhibited. It was also found that local anesthetics like butacaine enhance the accumulation of Ca^{2+}. Maximum activation was obtained at about 40 mμmoles/mg of protein. On the basis of these findings the nature of the mechanism of the mitochondrial divalent cation accumulation is discussed. It is suggested that in the mitochondria there exists a divalent cation carrier which can be identified and blocked by the lanthanides. This carrier is functionally specific and acts only as a divalent cation carrier. The functionally unspecific Ca^{2+} binding sites can be titrated with butacaine. By blocking the unspecific sites the Ca^{2+} accumulation can be enhanced.

Energy-dependent calcium accumulation in mitochondria has been found to be strongly inhibited by trivalent cations, lanthanum and praseodymium, which belong to the group of lanthanides (Mela, 1968a). The specific inhibition of calcium and manganese accumulation by lanthanides was shown by the diminished response of the respiratory carriers and the elimination of the membrane pH gradient induced by calcium accumulation. Recently the energy-independent binding of calcium to the mitochondrial membranes (Scarpa and Azzi, 1968) also has been shown to be inhibited by lanthanum ions (Rossi *et al.*, 1968).

It has also been shown that butacaine and a few other local anesthetic and antihistaminic drugs increase the intramitochondrial pH change which is concomitant with the calcium accumulation (Mela, 1968a; Chance *et al.*, 1968). Also Scarpa and Azzi (1968) have studied the effect of local anesthetics on the energy-independent calcium binding in submitochondrial particles and found an inhibition of this binding at higher concentrations of local anesthetics.

The present paper presents data, on the one hand, on the inhibition of mitochondrial calcium accumulation by various lanthanides (Cotton and Wilkinson, 1967) and, on the other hand, on stimulation of the accumulation by local anesthetics, as measured with a cation sensitive indicator murexide. The application of the use of murexide in mitochondria has been reported elsewhere (Mela and Chance, 1968). This technique enables one to follow the kinetics of the calcium accumulation

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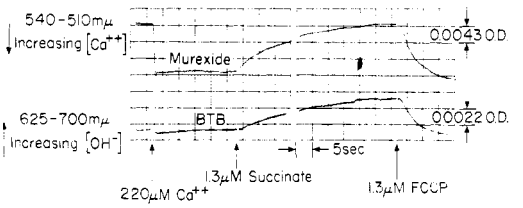

FIGURE 1: An experiment showing a simultaneous measurement of Ca^{2+} accumulation (murexide) and intramitochondrial pH change (bromothymol blue) in rat liver mitochondria, 2.5 mg of protein/ml suspended in standard medium in the presence of 3.3 μM rotenone to block endogenous respiration; 13 μM murexide and 1.7 μM bromothymol blue were present as indicators. The wavelengths used to measure the indicator changes and the additions are indicated on the figure.

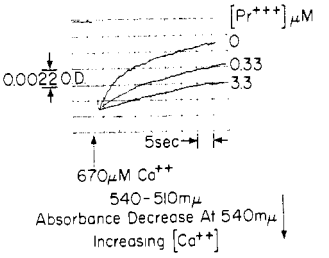

FIGURE 2: Oscilloscope traces of three separate experiments superimposed showing the effect of Pr^{3+} on the rate of Ca^{2+} accumulation in rat liver mitochondria, 4.8 mg of protein/ml; 10 μM murexide was used as Ca^{2+} indicator and 5 mM succinate as substrate. Pr^{3+} concentrations used are indicated on the diagram.

by the mitochondria, with a sensitivity of 5 μM Ca^{2+}. The inhibition and stimulation of the calcium movements are compared with the inhibition and stimulation of the other concomitant reactions, the response of the respiratory chain carriers and the development of the membrane pH gradient. The identification of the calcium carrier in mitochondria by means of the lanthanide inhibition is discussed.

A preliminary report of this work has been presented (Mela, 1968b).

Methods

Rat liver mitochondria were prepared by conventional methods in the presence of EDTA eliminating EDTA in two final washings (Chance and Mela, 1966a).

Dual-wavelength technique was used in all spectrophotometric measurements (Chance, 1951). The absorbance changes of the Ca^{2+} sensitive indicator murexide were measured at 540 mμ using 510 mμ as reference wavelength. The proper wavelengths were obtained in a filter double-beam spectrophotometer with narrow band interference filters. Murexide is not bound to the mitochondrial membranes, but remains free in the solution and so indicates the concentration of Ca^{2+} outside the mitochondrial membranes (Mela and Chance, 1968). Decreasing absorbance at 540 mμ indicates increasing concentration of Ca^{2+} in the suspending medium.

The intramitochondrial pH changes were measured with bromothymol blue[1] as a spectrophotometric indicator at a wavelength of 625 mμ with 700 mμ as a reference using narrow band interference filters (Chance and Mela, 1966a).

Murexide and bromothymol blue changes were also followed simultaneously with an arrangement of two dual-wavelength spectrophotometers measuring changes in one cuvete, as has been reported earlier (Mela, 1968a; Mela and Chance, 1968). To avoid interference of the 540- and 510-mμ light with the 625- and 700-mμ light, the individual photomultipliers were guarded with Wratten gelatin filters 65 and 29, respectively.

The same arrangement was also used to measure cytochrome *c* simultaneously with bromothymol blue. In this case the appropriate wavelengths for cytochrome *c* 550 and 540 mμ, were obtained with interference filters and a Wratten gelatin filter 65 was used as a guard filter on the photomultiplier tube.

In all experiments a Tektronix four-channel oscilloscope was used as a recorder. Thus the response time of the instruments was determined by the amplifier response, which in all experiments was less than 0.2 sec.

All the experiments were carried out in a standard reaction medium which consisted of 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-Cl (pH 7.4); 5mM succinate was used as substrate. Murexide, bromothymol blue, and Ca^{2+} concentrations used and other conditions are indicated on the figures or in the figure legends.

The lanthanides praseodymium (Pr^{3+}), holmium (Ho^{3+}), and scandium (Sc^{3+}) and the local anesthetics were obtained from K & K Rare Chemicals Corp.; cerous chloride (Ce^{3+}) and ceric sulfate (Ce^{4+}) from C. F. Smith; and lanthanum (La^{3+}) from Fisher Chemical Corp.

Results

Murexide and Bromothymol Blue Measurements. Figure 1 shows a simultaneous measurement of murexide and bromothymol blue changes. Rat liver mitochondria were suspended in the standard medium at a concentration of 2.5 mg of protein/ml in the presence of 3.3 μM rotenone and in the absence of added substrate or permeant anions; 13 μM murexide and 1.7 μM bromothymol blue were added as indicators. In the upper trace the downward deflection indicates decreased absorbance of the indicator murexide. In the figure this is caused by an addition of 220 μM $CaCl_2$. After the first downward deflection neither murexide nor bromothymol blue absorbance changes, which indicates that both the Ca^{2+} concentration in the suspending medium and the intramitochondrial pH remain constant. There is no Ca^{2+} disappearance from the medium. Only after the addition of 1.3 mM succinate the Ca^{2+} accumulation starts. This is shown by a slow upward deflection of both murexide and bromothymol blue traces. Murexide shows the disappearance of Ca^{2+} from the extramitochondrial space (half-time about 10 sec) and the bromothymol blue trace shows a very nearly simultaneous increase in the intramitochondrial pH (half-time about 13 sec). Both traces reach a plateau after all added Ca^{2+} has been accumulated into the mitochondria. An addition of the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone discharges the accumulated Ca^{2+} as well as the membrane pH gradient.

Thus in the absence of energy there is no detectable dis-

[1] Bromothymol blue, 3,3′-dibromothymolsulfonphthalein; butacaine, 3-(*p*-aminobenzoxy)-1-di-*n*-butylaminopropane sulfate; murexide, ammonium purpurate.

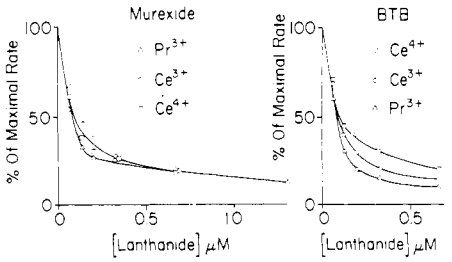

FIGURE 3: Titration of the Ca^{2+} accumulation rate and the rate of the bromothymol blue change with different lanthanides. Rat liver mitochondria at 3.0 mg of protein/ml were suspended in standard medium in the presence of 5 mM succinate, and 10 μM murexide and 1 μM bromothymol blue as indicators. The initial rates of both murexide and bromothymol blue changes after the addition of 330 μM Ca^{2+} were measured and plotted as percentage of control rate (−lanthanides) as a function of the lanthanide concentration. Each point corresponds to one experiment, where murexide and bromothymol blue were measured simultaneously.

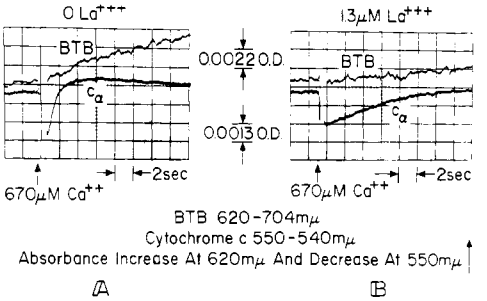

FIGURE 4: An experiment showing a simultaneous recording of bromothymol blue and cytochrome *c* response to an addition of 670 μM Ca^{2+} in the absence (A) and in the presence (B) of 1.3 μM La^{3+}. Rat liver mitochondria were suspended at a concentration of 4.2 mg of protein/ml in the presence of 0.67 μM bromothymol blue as pH indicator. An upward deflection on the bromothymol blue trace indicates alkalinization and on cytochrome *c* trace oxidation of cytochrome *c*.

appearance of Ca^{2+} from the extramitochondrial space into the mitochondria within 25 sec, neither is there any intramitochondrial pH change. Thus there is no energy independent Ca^{2+} binding to the mitochondria by murexide measurements. In many experiments which are not shown, the succinate addition was delayed for about 2–5 min after the addition of Ca^{2+}, and even then no Ca^{2+} disappearance was seen. Thus, we have not been able to demonstrate Ca^{2+} translocation supported by endogenously available high-energy source, which has been reported by Ernster *et al.* (1968). If Ca^{2+} binding under these conditions occurs, it either is complete within mixing time or the binding site is on the outside surface of the mitochondrion and is thus available for murexide as well since we know from our earlier studies that murexide itself stays outside the mitochondrial membranes (Mela and Chance, 1968).

Effect of Lanthanides on Ca^{2+} Accumulation. Using murexide to measure the extramitochondrial Ca^{2+} concentration, the kinetics of the disappearance of Ca^{2+} from the medium into the mitochondria were followed in the absence and presence of a lanthanide praseodymium, as is shown in Figure 2. In this figure oscilloscope traces of three separate experiments are superimposed using the time of the Ca^{2+} addition as the starting point. The rapid deflection of the murexide absorbance caused by the addition of 670 μM Ca^{2+} to a mitochondrial suspension of 4.8 mg of mitochondrial protein/ml is the same in each case. The difference in these three traces is seen in the Ca^{2+} accumulation rates. The upper trace gives the control rate of Ca^{2+} accumulation in rat liver mitochondria in the absence of lanthanides (0 Pr^{3+}) or added permeant anions. In this case the initial rate of Ca^{2+} accumulation is 10 mμmoles of Ca^{2+} accumulated per mg of protein per sec. After an uptake of about 190 μM Ca^{2+} (about 40 mμmoles/mg of protein), the uptake rate starts decreasing and appears to be exponential.

The second trace of Figure 2 shows the rate of Ca^{2+} accumulation in the presence of 0.33 μM Pr^{3+} (0.07 mμmole/mg of protein). This amount of Pr^{3+} has caused 75% inhibition in the rate, which now is only 2.5 mμmoles of Ca^{2+}/mg of protein per sec. In the presence of a ten times higher concentration of Pr^{3+} (0.7 mμmole/mg of protein) the inhibition is 85% and the initial rate of Ca^{2+} accumulation is only 1.5 mμmoles of Ca^{2+} accumulated per mg of protein per sec. The total amount of Ca^{2+} accumulated in all these cases is the same, only the time needed for it is longer in the presence of Pr^{3+} than in its absence.

In Figure 3 data from an experiment of the same kind as shown in Figure 2 are presented. Simultaneously with the measurements of the Ca^{2+} accumulation rates by murexide, also the rates of the intramitochondrial pH changes were measured by bromothymol blue. Experiments were done in the absence and in the presence of different concentrations of lanthanides, trivalent praseodymium, and both trivalent and tetravalent cerium. In Figure 3 the initial rates of these different cases as percentage of the control rates, which are the rates of the Ca^{2+} accumulation and the intramitochondrial pH change in the absence of the lanthanides, are plotted against the lanthanide concentration.

In diagram A the inhibition of the initial rates of the Ca^{2+} accumulation is shown as a function of the inhibitor concentration; 50% inhibition is achieved at less than 0.1 μM concentration of lanthanides which is about 0.03 mμmole/mg of protein. The extrapolated concentration for the 100% inhibition is 0.18 μM which equals 0.06 mμmole of lanthanide/mg of protein. However, the titration gives a biphasic titration curve, which may indicate that there are two binding sites. The one, which is more sensitive to lanthanides and takes care of most of the Ca^{2+} transport, exists at a concentration of about 0.06 mμmole/mg of protein.

The inhibition of the bromothymol blue change is shown in diagram B. Both 50 and 100% inhibition of the bromothymol blue response are achieved at the same concentration of lanthanides as in the murexide measurements. This indicates that the bromothymol blue response is closely related to the Ca^{2+} accumulation itself.

No significant differences between the different lanthanides were observed. Besides the ones shown on the figure, also holmium and lanthanum were tested. Titration with holmium is almost identical with the titration with praseodymium. A slightly higher concentration of lanthanum is needed for 50% inhibition of Ca^{2+} uptake. Trivalent cations scandium, which has a smaller ionic radius than the true lanthanides (Cotton and Wilkinson, 1967) and aluminum were also tested, but no inhibition of Ca^{2+} accumulation was observed in the presence of these cations.

Similar titrations were also done measuring the response of

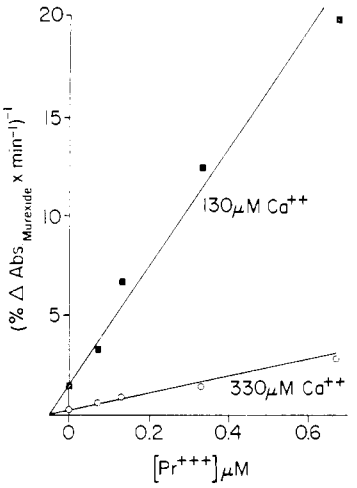

FIGURE 5: Dixon plot showing a noncompetitive inhibition of Ca^{2+} accumulation by Pr^{3+}. Each point corresponds to a separate experiment done in the presence of varying concentrations of Pr^{3+} and measuring the initial rate of the murexide absorbance trace after an addition of 130 or 330 μM Ca^{2+} to rat liver mitochondria at 1.8 mg of protein/ml and in the presence of 5 mM succinate as substrate and 10 μM murexide as the indicator. The reciprocal of the murexide rate is plotted as a function of Pr^{3+} concentration.

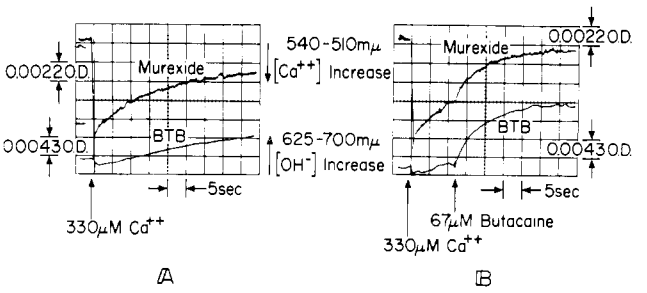

FIGURE 6: A simultaneous recording of murexide and bromothymol blue changes showing the effect of butacaine on Ca^{2+} accumulation and the concomitant pH change. Rat liver mitochondria were suspended at a concentration of 3.0 mg of protein/ml in the presence of 5 mM succinate and 10 μM murexide and 1 μM bromothymol blue.

the respiratory chain carriers during Ca^{2+} accumulation. With praseodymium 50% inhibition of cytochrome *b* response is obtained at about 0.05 mμmole of Pr^{3+}/mg of protein.

Figure 4 shows the effect of lanthanum on the response of cytochrome *c* measured simultaneously with bromothymol blue. Upon addition of Ca^{2+} to rat liver mitochondria cytochrome *c* changes rapidly to a more reduced steady state, which is transient and is followed by a slower change to a more oxidized steady state. The half-time of the oxidation reaction is less than 1 sec in the absence of La^{3+}; however, in the presence of La^{3+} it is about 6 sec. In the presence of 1.3 μM La^{3+} (0.31 mμmole/mg of protein) the initial rate of the oxidation of cytochrome *c* is about 90% inhibited. The initial rate of the bromothymol blue change measured simultaneously under the same conditions shows about 80% inhibition.

These experiments on the lanthanide inhibition show that all the different reactions induced by Ca^{2+} accumulation in mitochondria are inhibited by lanthanides to the same extent as the Ca^{2+} accumulation itself. This provides clear evidence that the changes of the respiratory chain carriers, membrane pH, and respiratory activity, which have been found to occur concomitant with the Ca^{2+} accumulation, are induced by the ion accumulation process.

Noncompetitive Inhibition by Lanthanides. The amount of lanthanides needed for the inhibition of Ca^{2+} accumulation is dependent on the concentration of the mitochondrial protein used. This suggests that the inhibition achieved at a certain concentration of lanthanides is dependent upon the total number of binding sites available.

Figure 5 shows the effect of the Ca^{2+} concentration on the inhibition by lanthanides, in this case praseodymium. The reciprocal of the initial rate of the Ca^{2+} accumulation measured by murexide absorbance change is plotted against Pr^{3+} concentration. Each point corresponds to an individual experiment where either 330 or 130 μM Ca^{2+} was added to a suspension of rat liver mitochondria at 1.8 mg of protein/ml in the presence of 5 mM succinate and 10 μM murexide. The murexide absorbance change was measured as in the other experiments at 540–510 mμ. The graph (Dixon and Webb, 1964) indicates that the inhibition by Pr^{3+} is not competitive with Ca^{2+}. It also shows that the K_i for Pr^{3+} is 0.05 μM.

Effect of Butacaine on the Ca^{2+}-Uptake Rate. It has been reported earlier that butacaine and some other local anesthetic and antihistaminic drugs facilitate the intramitochondrial alkalinization which occurs during Ca^{2+} accumulation (Mela, 1968a; Chance *et al.*, 1968).

Figure 6 shows an experiment, in which the effect of butacaine on the Ca^{2+} accumulation as measured by murexide and on simultaneous intramitochondrial alkalinization as measured by bromothymol blue have been studied. Figure 6A shows the control experiment, where after the addition of 330 μM Ca^{2+} in the presence of succinate as substrate the indicators murexide and bromothymol blue respond typically to the Ca^{2+} addition. In Figure 6B after the addition of Ca^{2+}, 67 μM butacaine was added. At this point there is a rapid response of both indicators. The change in the rate of the murexide absorbance shows that Ca^{2+} is accumulated with much faster rate than in the absence of butacaine. Bromothymol blue changes rapidly, too, reaching a plateau value at a level about twice as high as in the control case.

A series of experiments were done where varying concentrations of butacaine were added to the mitochondrial suspension before the addition of Ca^{2+}. The initial kinetics of the Ca^{2+} accumulation and the bromothymol blue changes were compared under these different conditions. The results of these titrations are shown in Figure 7, where A shows the effect of butacaine on the initial rate of the murexide change and B shows its effect on the initial rate of the bromothymol blue change. The rates of the murexide change are given as millimicromoles of Ca^{2+} accumulated per milligram of protein per sec and the rates of the bromothymol blue change are given as percentage absorbance change per second. From these titrations it can be seen that the amount of butacaine needed for maximal stimulation of both these parameters is about 35–40 mμmoles of butacaine per mg of mitochondrial protein. This is of the same order of magnitude as the binding sites of butacaine in the mitochondrial membrane as reported by Scarpa and Azzi (1968). These titrations also illustrate that the rate of Ca^{2+} accumulation is stimulated by butacaine about 3-fold, however, the rate of the bromothymol blue change is stimulated as much as about 20-fold by butacaine. Qualitatively

similar results have been obtained with other local anesthetics (tetracaine, procaine), but the concentrations needed for half-maximal effect are different (for tetracaine, 500 μM and for procaine, 7 mM, as compared with about 100 μM for butacaine).

Discussion

These experiments shed some light on the molecular events that occur between the complexing of Ca^{2+} with the high-energy intermediate and the splitting of the complex (Chance, 1965) to transport Ca^{2+} to the interior of the mitochondria. The data presented suggest that the high-affinity binding sites for lanthanides, which according to the lanthanide titrations exist only at concentrations of about one-half of the respiratory chain carriers, are functionally very specific. It is suggested that these sites are divalent cation carriers, which in the presence of energy translocate Ca^{2+} and some other related divalent cations across the mitochondrial membranes. These carriers are phospholipid components of the membrane, and could be identical with the high energy intermediate X~I suggested by Chance (1965). If the carrier sites are blocked with the lanthanides, Ca^{2+} is not transported. Calculated from the extrapolated concentration of the lanthanides needed for 100% inhibition of Ca^{2+} accumulation (Figure 3) the concentration of these carriers in the membrane is less than 0.1 mμmole/mg of protein (0.05–0.07). Taking into account this concentration and the initial rate of Ca^{2+} accumulation, the turnover number of the carriers can be calculated to be about 140/sec (succinate as substrate).

In the absence of substrate and oxygen or ATP as energy source or in the presence of respiratory inhibitors or uncouplers Ca^{2+} accumulation does not occur. Thus the activation of the cation carrier is energy dependent, and needs for its function an interaction of a high-energy source.

The lanthanides were found to inhibit equally all the reactions involved in divalent cation accumulation, but they do not inhibit any other mitochondrial reactions, like oxidative phosphorylation or monovalent cation accumulation. Since the lanthanide binding sites do not seem to be involved in the transport of monovalent cations across the mitochondrial membranes, it is suggested that the two groups of cations, monovalent and divalent, must have a different specific transport mechanism in the mitochondrial membranes.

Other less specific sites are also involved in the mechanism of active Ca^{2+} transport in mitochondria. These sites can be titrated by butacaine or other local anesthetic drugs. The number of these sites according to the titration in Figure 7 is about 40 mμmoles/mg of protein. It has been shown by Scarpa and Azzi (1968) that the binding sites of local anesthetics in mitochondrial membranes are the phospholipids of the membrane. Figure 7 shows that if these sites are blocked by butacaine Ca^{2+} accumulation is enhanced. It is suggested that the enhancement of Ca^{2+} accumulation is achieved by blocking these unspecific Ca^{2+} binding sites by butacaine and thus leaving more Ca^{2+} available for the specific carrier sites. Also, it has been shown by A. Azzi and B. Chance (manuscript in preparation) by measuring the pH changes in the membrane (bromothymol blue) and the matrix (NAD/NADH) that butacaine inhibits the transport of the already accumulated Ca^{2+} from the mitochondrial membrane into the matrix space. This would explain the greater enhancement of the bromothymol blue change by butacaine compared with the activation of the Ca^{2+} accumulation itself (Figure 7).

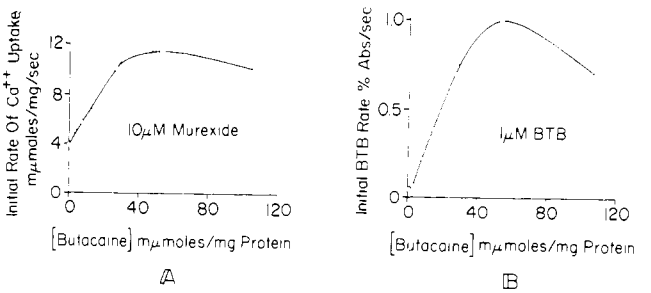

FIGURE 7: The dependence of the rates of the murexide and bromothymol blue changes on the added butacaine concentration. Each point corresponds to a separate experiment, where varying concentrations of butacaine were added before the addition of Ca^{2+}. Murexide and bromothymol blue changes were measured simultaneously after an addition of 330 μM Ca^{2+} in the presence of 5 mM succinate as substrate to a suspension of rat liver mitochondria at a concentration of 5.0 mg of protein/ml.

Since the carrier sites are very few and under normal conditions already turning over 140 times/sec, the enhancement of the Ca^{2+} transport by butacaine is limited, and is only about threefold. When Mn^{2+} is accumulated the carrier is turning over much slower than in the case of Ca^{2+} accumulation, and can be enhanced by butacaine up to fivefold. In the presence of low concentration of Ca^{2+}, the Mn^{2+} accumulation is also greatly enhanced (Chance and Mela, 1966b; Mela and Chance, 1968). This suggests that the divalent cation carrier can be activated much more effectively by Ca^{2+} than Mn^{2+}, and also that even in the presence of relatively high concentration of Mn^{2+} only small amounts of Ca^{2+} are needed for the activation of the carrier.

On the basis of the findings presented in this paper, we propose a mechanism of the accumulation of divalent cations in mitochondria. (1) In the mitochondrial membrane there exists a cation carrier, which is activated specifically by divalent cations like Ca^{2+} and Mn^{2+}, but not by monovalent cations. The activation of the carrier only occurs in the presence of an energy source. This carrier exists at a concentration of about 0.05–0.07 mμmole/mg of protein, which is about one half of the respiratory chain carriers. The carrier can be titrated and blocked completely by trivalent cations lanthanides. (2) Besides these carriers which provide the high affinity binding site of Ca^{2+}, there are some less specific divalent cation binding sites which in an energy-dependent process bind the amount of Ca^{2+} exceeding the binding capacity of the carrier sites. These sites then release the cation when the carrier site is free. These unspecific sites do not transport the cation across the membrane. For that process the carrier is necessary. Thus, our experimental evidence supports one of the two possible mechanisms for cation translocation, which were left to be established in a recent discussion of Scarpa and Azzone (1968) on the role of surface binding on the aerobic Ca^{2+} translocation in mitochondria. The unspecific binding sites can be titrated by butacaine and some other local anesthetics, and the titration gives a value of about 40 mμmoles per mg of protein. Blocking these sites enhances the activity of the cation carriers. (3) The release of Ca^{2+} from the mitochondria consequent to the loss of energy also occurs through the same car-

rier sites as the uptake, since it can be inhibited by lanthanides and enhanced by butacaine.

Acknowledgments

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References

Chance, B. (1951), *Rev. Sci. Instr. 22*, 634.
Chance, B. (1965), *J. Biol. Chem. 240*, 2729.
Chance, B., and Mela, L. (1966a), *J. Biol. Chem. 241*, 4588.
Chance, B., and Mela, L. (1966b), *Biochemistry 5*, 3220.
Chance, B., Mela, L., and Harris, E. J. (1968), *Federation Proc. 27*, 902.
Cotton, F. A., and Wilkinson, G. (1967), Advanced Inorganic Chemistry, New York, N. Y., Interscience, p 1052.
Dixon, M., and Webb, E. C. (1964), The Enzymes, New York, N. Y., Academic, p 315.
Ernster, L., Hollander, P., Nakazawa, T., and Nordenbrand, K. (1968), *in* Mitochondria-Structure and Function, Drahota, Z., and Ernster, L., Ed., London, Academic (in press).
Mela, L. (1968a), *Arch. Biochem. Biophys. 123*, 286.
Mela, L. (1968b), *Trans. N. Y. Acad. Sci.* (in press).
Mela, L., and Chance, B. (1968), *Biochemistry 7*, 4059.
Rossi, C., Carafoli, E., and Lehninger, A. L. (1968), *in* Mitochondria—Structure and Function, Drahota, Z., and Ernster, L., Ed., London, Academic (in press).
Scarpa, A., and Azzi, A. (1968), *Biochim. Biophys. Acta 150*, 473.
Scarpa, A., and Azzone, G. F. (1968), *J. Biol. Chem. 243*, 5132.

The Amino Acid Sequence of Ferredoxin from *Clostridium acidi-urici**

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ABSTRACT: The sequence of amino acids in *Clostridium acidi-urici* ferredoxin was determined using two derivatives, *S*-β-aminoethylated ferredoxin and oxidized ferredoxin. Peptides derived from tryptic, papain, and partial acid hydrolyses were used to construct the total sequence. The sequence of amino acids in the individual peptides was determined by the Edman degradation and the exopeptidases leucine aminopeptidase, carboxypeptidase A, and carboxypeptidase B. Like the other two clostridial ferredoxins previously sequenced, *C. acidi-urici* ferredoxin has an amino terminal alanine. Out of a total of 55, 37 amino acids are found in identical positions in all three species, including 8 cysteine and 3 proline residues.

Ferredoxin, an iron-containing polypeptide of low potential, has been found in plants and bacteria. The structure of the ferredoxin isolated from spinach contains five cysteinyl residues and seems distantly related in structure to the bacterial ferredoxins (Matsubara *et al.*, 1967). Structural studies on the bacterial ferredoxins from *Clostridium pasteurianum* (Tanaka *et al.*, 1966) and *Clostridium butyricum* (Benson *et al.*, 1966) have shown that these polypeptides are very closely related, each consisting of two nearly homologous halves. Within each half there are four cysteinyl residues, the spacing of which is probably significant in the binding of iron and inorganic sulfide (Malkin and Rabinowitz, 1966) as required for biological function. From the evolutionary standpoint, these similarities between plant and bacterial ferredoxin and between the two halves of an individual bacterial ferredoxin are currently of concern in comparative biochemistry (Eck and Dayhoff, 1966).

There are differences in the properties of ferredoxin from *Clostridium acidi-urici* on the one hand, and those from *C. pasteurianum* and *C. butyricum* on the other. Ferredoxin from *C. acidi-urici* crystallizes more readily and has a lower activity in the phosphoroclastic assay than the other two (Lovenberg *et al.*, 1963). As a step in correlating these variations in properties with structural differences, and also to aid in the comparative study, the amino acid sequence of ferredoxin from *C. acidi-urici* was determined.

Methods

Purification of Ferredoxin. Ferredoxin from *C. acidi-urici* was isolated and purified according to the method of Lovenberg *et al.* (1963), and was the generous gift of Dr. R. Malkin and Dr. J. C. Rabinowitz. The purified protein had an $A_{390}/A_{280} = 0.78$.

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