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Spectrophotometric Measurements of the Kinetics of Ca^{2+} and Mn^{2+} Accumulation in Mitochondria*

Leena Mela and Britton Chance

ABSTRACT: The cation-sensitive indicator murexide was used to measure Ca^{2+} and Mn^{2+} accumulation by rat liver mitochondria. The absorbance change of murexide caused by Ca^{2+} or Mn^{2+} was measured in a dual-wavelength spectrophotometer at 540–510 mμ. Close to 100% of the indicator was found to be in the medium outside the mitochondria and thus murexide gives a direct measure of the extramitochondrial Ca^{2+} concentration. It is shown that the half-time of the accumulation of 380 μM Ca^{2+} in the absence of permeant anions is about 15 sec. Mn^{2+} accumulation is three to four times slower than Ca^{2+} accumulation. However, 25 μM Ca^{2+} added to the mitochondrial suspension together with 380 μM Mn^{2+} accelerates the Mn^{2+} uptake considerably. The permeant anion acetate facilitates the accumulation of the divalent cations. The kinetics of the Ca^{2+} accumulation are compared with the kinetics of the concomitant intramitochondrial pH change. The murexide technique as a kinetic method for measuring divalent cation accumulation in mitochondria is discussed.

The respiratory carriers respond to divalent cation accumulation very rapidly. The half-time of the oxidation of cytochrome *b* due to Ca^{2+}-activated electron transport is about 30–50 msec (Mela, 1968a). The Ca^{2+}-induced change of the intramitochondrial pH measured by bromothymol blue (Chance and Mela, 1966a) is rather slow with a half-time of approximately 20 sec. It has been difficult to correlate these changes to the actual cation accumulation by the mitochondria, because of the lack of adequate techniques for measuring the kinetics of the ion movements. Cation-sensitive electrodes are not ideal due to their slow response time (2–10 sec) (Chance and Yoshioka, 1966). Atomic absorption and radioactive ^{45}Ca measurements require 15–30

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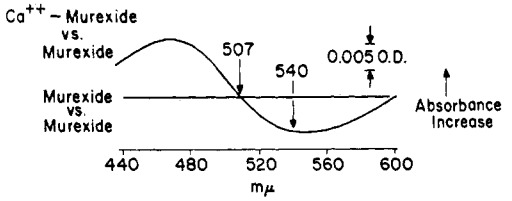

FIGURE 1: A difference spectrum of the Ca^{2+}–murexide complex *vs.* the free murexide, showing the absorbance trough at 540 mμ and the isosbestic point at 507 mμ. The spectrum was obtained by adding 370 μM $CaCl_2$ to the measuring cuvet, which contained 6.7 μM murexide in 20 mM Tris-Cl buffer (pH 7.4). The reference cuvet contained 6.7 μM murexide, but no $CaCl_2$.

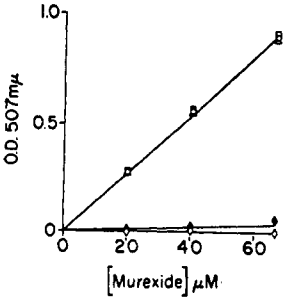

FIGURE 2: A plot of the absorbance at 507 mμ *vs.* added murexide concentration, showing the fractions of the added murexide found in the supernatant (10,000 rpm for 10 min) and pellet of the mitochondrial suspension as compared with the control curve obtained in the absence of mitochondria. The mitochondrial protein was 3.3 mg/ml. [Ca^{2+}], when added, was 330 μM. Final volume of the samples was 3 ml. (△) Control, (○) supernatant ($-Ca^{2+}$), (□) supernatant ($+330$ μM Ca^{2+}), (♦) mitochondrial pellet ($+330$ μM Ca^{2+}), and (◇) mitochondrial pellet ($-Ca^{2+}$).

sec for sampling and separation of the mitochondria from the surrounding medium. These difficulties encouraged us to examine the possibility of using a divalent cation-sensitive indicator, murexide, to monitor spectrophotometrically the accumulation of calcium and manganese by mitochondria.

Murexide has been used previously in biological systems to measure calcium binding both *in vivo* and *in vitro*. Ohnishi and Ebashi (1963) introduced the method to measure calcium binding in the particulate fraction from sarcoplasmic reticulum and Jöbsis and O'Connor (1966) used murexide *in vivo* to measure calcium movements in intact muscles. A detailed study of the kinetics of reactions of murexide with divalent and trivalent cations has been reported by Geier (1968).

Experimental Methods

Rat liver mitochondria were prepared according to conventional methods paying special attention to the washing procedure in order to get mitochondria which are relatively free of endogenous calcium (Chance and Mela, 1966a). Calcium and manganese concentrations in the mitochondrial suspensions were measured with the cation-sensitive indicator murexide (ammonium purpurate) by monitoring the formation and disappearance of the Ca^{2+}–murexide complex in a dual-wavelength spectrophotometer (Chance, 1951) using 540 mμ as the measuring wavelength and 510 mμ as reference.

According to the Ca^{2+}–murexide *vs.* murexide difference spectrum, which is shown in Figure 1, decreasing absorbance at 540 mμ indicates an increase in the concentration of Ca^{2+}–murexide complex. The reference wavelength 510 mμ is near the isosbestic point. In the presence of 370 μM Ca^{2+} the change of extinction of murexide at 540–510 mμ is 1.0 mM^{-1} cm^{-1}. As can be seen from the spectrum the change of extinction would be three times as large, if 470 mμ were used as reference wavelength instead of 510 mμ. However, 510 mμ, which is a wavelength close to 540 mμ, was chosen to avoid large interference due to light-scattering changes (Jöbsis and O'Connor, 1966). When choosing proper wavelengths for measuring the murexide changes in mitochondria, control experiments were also done in the absence of the indicator to eliminate the possibilities of interference from the changes of the respiratory chain carriers, in this case particularly cytochrome *c*.

In some experiments the intramitochondrial pH changes as measured by the indicator bromothymol blue (Chance and Mela, 1966a) were monitored simultaneously with the measurements of the cation movements by murexide. The bromothymol blue absorbance changes were measured in a second dual-wavelength spectrophotometer at 625 (measuring wavelength) and 700 mμ (reference wavelength). The two spectrophotometers which were used to monitor simultaneously the absorbance changes of the two indicators in the same cuvet were both equipped with interference filters (540 and 510 mμ for murexide and 625 and 700 mμ for bromothymol blue). The two individual photomultipliers were guarded with Kodak Wratten gelatin filters (no. 65 and 26, respectively) to prevent interference from the other spectrophotometer (Mela, 1968b).

The rate-limiting factor in these instruments is the response time of the amplifier, which in our experiments was less than 0.1 sec. In such cases, a Tektronix oscilloscope was used to record the absorbance change. However, in cuvet experiments it is difficult to get mixing times less than 0.5–1 sec. Thus the first meaningful reading can be obtained within 1 sec.

The effect of the murexide on the respiratory control of the mitochondria was tested with an oxygen electrode. At the concentration used in these experiments the respiratory control ratio was not affected by murexide.

The reaction medium for the mitochondria in all these experiments consisted of 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-Cl (pH 7.4). Succinate was used as a substrate throughout. Murexide solution was prepared fresh daily. This appeared to be necessary, because murexide is unstable in solution, and there is a small but significant decrease in the concentration after 24 hr.

Results

Localization of Murexide. It was important to find out in which compartment of the mitochondrial suspension the murexide was located. Experiments were done in which concentrations of murexide ranging from 7 to 60

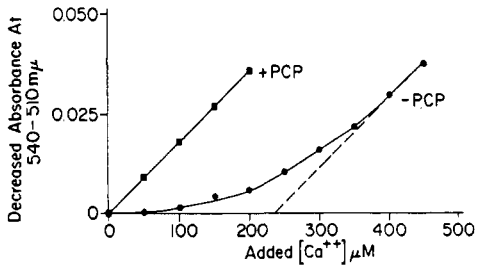

FIGURE 3: Titrations of the indicator, murexide, in a mitochondrial suspension in the presence (+PCP) and absence (−PCP) of 15 μM pentachlorophenol, with sequential additions of $CaCl_2$. The rat liver mitochondria were 4.9 mg of protein/ml in the presence of 4 mM succinate as substrate and 47 μM murexide as indicator.

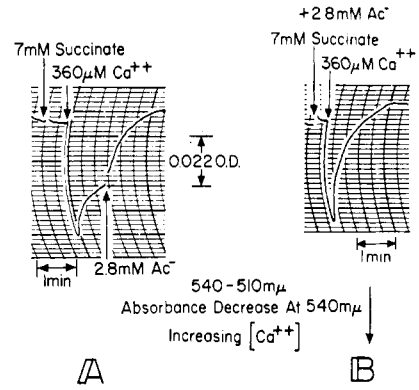

FIGURE 4: An experiment showing the kinetics of the Ca^{2+} accumulation using murexide at a concentration of 28 μM as an indicator in the absence (A) and presence (B) of 2.8 mM acetate. Rat liver mitochondria, 2.8 mg of protein/ml; 7 mM succinate was added as substrate.

μM were added to mitochondrial suspensions of about 2–3 mg of protein/ml in the presence and absence of Ca^{2+}. After incubating the suspensions for 2 min, the mitochondria were separated from the medium by centrifuging at 10,000 rpm for 10 min at 0°. Both the supernatant fraction and the mitochondrial pellet were analyzed for murexide at 507 mμ. Results of an experiment of this type are given in Figure 2. The murexide appeared to be in the aqueous phase and there was no significant concentration of the murexide in the mitochondrial pellet. This experiment shows that murexide is an indicator for Ca^{2+} concentration in the solution external to the mitochondria.

Correlation of the Ca^{2+} Concentration with the Absorbance of Murexide. Figure 3 shows a titration of murexide with Ca^{2+} in mitochondrial suspensions in an uncoupled and coupled state of respiration. In the uncoupled state (+PCP) no Ca^{2+} uptake occurs. This titration gives the correspondence of the optical density change of murexide measured at 540–510 mμ to the added Ca^{2+} concentration, which in this case equals the concentration of Ca^{2+} external to the mitochondria. The $\Delta\epsilon$ 540–510 mμ in mitochondria extrapolated from the titration is 1.3 $mM^{-1}\ cm^{-1}$ for 370 μM Ca^{2+}. This curve serves as a calibration curve of the murexide change in mitochondrial suspension. The presence of mitochondria has only a slight effect on the murexide absorbance, and the titration in the presence of mitochondria gives a slightly higher extinction coefficient.

The second titration curve shown in Figure 3 (−PCP) is obtained by sequential additions of Ca^{2+} to coupled mitochondria. The difference from the calibration curve indicates the amount of Ca^{2+} which has been accumulated from the medium into the mitochondria, a space where it is not detectable by murexide. The extrapolation of the linear portion of this curve to the abscissa gives the total amount of Ca^{2+} taken up by the mitochondria. The absorbance change of murexide indicates the amount of Ca^{2+} which is free in the medium available for binding to murexide at the equilibrium state when maximal amount of Ca^{2+} has already been accumulated by the mitochondria.

Kinetics of the Ca^{2+} Accumulation. The main purpose of this investigation was to study the kinetics of the cation accumulation. Figure 4 shows an experiment of this kind. Rat liver mitochondria were suspended in the reaction medium at a concentration of 2.8 mg of protein/ml in the presence of 28 μM murexide. Following the addition of 7 mM succinate, 360 μM Ca^{2+} (129 mμmoles/mg of protein) was added. This causes a very rapid deflection toward decreased absorbance of murexide at 540 mμ, which is due to the formation of the Ca^{2+}–murexide complex in the medium (Geier, 1968). Following this change the absorbance increases (upward deflection) at a much slower rate. This deflection indicates the disappearance of Ca^{2+} from the medium into the mitochondria. The amount of Ca^{2+} accumulated is approximately 50% of the added Ca^{2+}, and this accumulation is complete in about 60 sec. Addition of 2.8 mM acetate, which acts as a permeant anion, however, facilitates the accumulation of Ca^{2+}, and about 2 min after the addition of Ca^{2+} or 1 min after the addition of acetate the murexide trace has returned to the starting level, indicating that all the added Ca^{2+} then has been accumulated by the mitochondria.

In Figure 4B, 2.8 mM acetate was added to the suspension before the addition of Ca^{2+}. In this case the Ca^{2+} accumulation is much faster than in the absence of acetate. All of the added Ca^{2+} is accumulated in about 60 sec.

According to this experiment, the kinetics of the overall reaction of Ca^{2+} uptake appears to be relatively slow compared with the very rapid response of the respiratory carriers. In agreement with previous reports the permeant anion acetate increases both the rate and the extent of Ca^{2+} accumulation in mitochondria (Chance and Yoshioka, 1965; Rasmussen *et al.*, 1965).

Comparison of the Kinetics of Murexide and Bromothymol Blue Changes. We have previously shown that the pH in the mitochondrial membrane rises when Ca^{2+} is accumulated in the absence of permeant anions (Chance and Mela, 1966a). Chance and Yoshioka (1966) have correlated this alkalinization to the amount of accumulated Ca^{2+} by using a Ca^{2+} -sensitive electrode to measure the Ca^{2+} concentrations. The rate of the Ca^{2+} uptake is compared with the rate of the intramitochondrial alkalinization in Figure 5. The top trace shows the measurement of Ca^{2+} movements as monitored by mu-

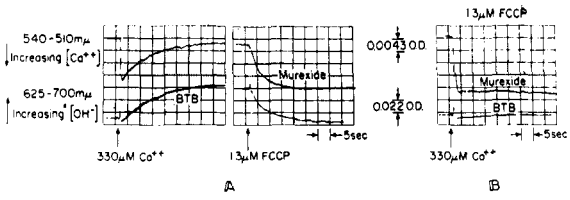

FIGURE 5: The kinetics of calcium accumulation and release as measured by murexide, compared with the kinetics of the intramitochondrial pH as measured by bromothymol blue, in the absence (A) and presence (B) of the uncoupler, FCCP. Rat liver mitochondria, 2.1 mg of protein/ml, in the presence of 6.7 mM succinate as substrate and 13 μM murexide and 1.7 μM bromothymol blue as indicators.

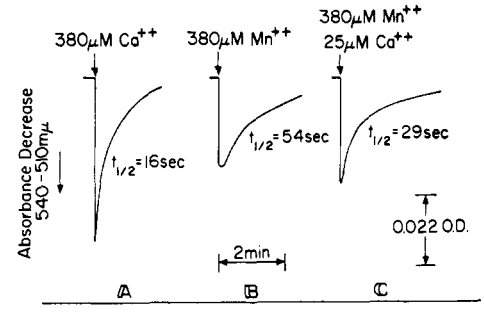

FIGURE 6: Comparison of the kinetics of uptake of 380 μM Ca^{2+} (A), 380 μM Mn^{2+} (B), and 380 μM Mn^{2+} in the presence of 25 μM Ca^{2+} (C). Rat liver mitochondria (6.3 mg of protein/ml) in the presence of 7.7 mM succinate as substrate and 46 μM murexide as indicator.

rexide and recorded simultaneously with the intramitochondrial pH measurement monitored by bromothymol blue (bottom trace). As shown in Figure 5A, the addition of 330 μM Ca^{2+} to a mitochondrial suspension is followed by accumulation of about 300 μM Ca^{2+} with a half-time of about 8 sec. The pH of the mitochondria rises with a slightly slower rate; the half-time of this reaction is 10 sec. This indicates that the intramitochondrial pH rises very nearly simultaneously with the Ca^{2+} accumulation, being, however, delayed about 1 sec after the Ca^{2+} movement. Thus the Ca^{2+} accumulation is the primary process preceding the concomitant pH change of the mitochondrial membrane. Upon addition of FCCP[1] to the Ca^{2+}-loaded mitochondria, the cation is discharged from the mitochondria with a half-time of 3.5 sec. The intramitochondrial alkalinity is neutralized with a slightly slower rate (half-time 4.5 sec).

Figure 5B shows a control experiment in which the uncoupling agent, FCCP, was added prior to the Ca^{2+} addition. The murexide trace shows no Ca^{2+} accumulation into the mitochondria. Furthermore, the bromothymol blue absorbance does not change. Thus, if Ca^{2+} is not accumulated by the mitochondria, the pH gradient across the membrane does not develop.

Accumulation of Manganese by Mitochondria. Murexide is also sensitive to manganese (Geier, 1968), and thus can be used to measure the accumulation of manganese by mitochondria. It is known that Mn^{2+}, like Ca^{2+}, is accumulated by mitochondria in an energy-dependent process, but more slowly than Ca^{2+} (Chappell *et al.*, 1963). We have also shown by bromothymol blue and electron paramagnetic resonance measurements that small amounts of Ca^{2+} facilitate the Mn^{2+} uptake (Chance and Mela, 1966b).

Figure 6 shows the comparison of the kinetics of Ca^{2+} and Mn^{2+} accumulation by mitochondria. As shown in Figure 6A,B, the absorbance change of the murexide at 540–510 mμ caused by 380 μM Mn^{2+} is about 50% of that caused by the same concentration of Ca^{2+} ($\Delta\epsilon$ for Mn^{2+} is 0.6 mM^{-1} cm^{-1}).

On each of the traces A–C, the upward deflection indicates the accumulation of the cation by the mitochondria. Trace A shows the uptake of 380 μM Ca^{2+}, trace B the uptake of 380 μM Mn^{2+}, and trace C the uptake of 380 μM Mn^{2+} in the presence of 25 μM Ca^{2+}. The half-time for the Ca^{2+} accumulation under these conditions (succinate as the substrate in the absence of permeant anions) is 16 sec, while the half-time for the Mn^{2+} uptake is 54 sec. However, in the presence of the small concentration of Ca^{2+}, the $t_{1/2}$ of the Mn^{2+} uptake is decreased to 29 sec. This evidence supports our earlier findings about the cooperative interaction of Ca^{2+} and Mn^{2+} ions in rat liver mitochondria (Chance and Mela, 1966b).

Murexide was also tested for sensitivity to other cations of importance in mitochondria, such as K^+ and Mg^{2+}. At concentrations as high as 10 mM addition of these cations to mitochondrial suspensions has no effect upon the absorbance of murexide. However, the sensitivity of murexide to Ca^{2+} is changed by these cations. Thus, under conditions where K^+ or Mg^{2+} was present at millimolar concentrations, a recalibration of murexide sensitivity was required. The light absorbance of murexide is also pH sensitive. However, at buffer concentrations used in these experiments (20 mM Tris-Cl, pH 7.4), the pH change caused by the extrusion of H^+ ions during Ca^{2+} and Mn^{2+} accumulation is so small that it has a negligible effect on the murexide absorbance.

Discussion

The uptake of divalent cations, Ca^{2+} and Mn^{2+}, in mitochondria has been measured previously by means of cation-sensitive electrodes, atomic absorption, or radioactive isotope measurements. None of these methods is suitable for directly reading out the kinetics of cation accumulation.

The spectrophotometric method of measuring absorbance changes of murexide during Ca^{2+} and Mn^{2+} accumulation described in this communication has been found to be ideal for kinetic measurements, the rate-limiting factor in cuvet experiments being the mixing of the reagent with the mitochondrial suspension. To eliminate this factor, a rapid-flow apparatus with a mixing time of less than 1 msec can be employed to measure the initial kinetics of Ca^{2+} uptake in mitochondria, using murexide as the Ca^{2+} indicator. It is known from the work of Geier (1968) that the time constant for the re-

[1] FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

action of murexide with Ca^{2+} is less than 5 μsec, and for Mn^{2+} 10–12 μsec at 10° and pH 4.0. Thus, the murexide technique appears most appropriate for correlating the fast response of the mitochondrial respiratory chain enzymes with the cation movements themselves. It is also of importance to be able to study the kinetic relationship of the cation accumulation to the concomitant pH changes across the mitochondrial membranes.

The comparison of the response of murexide and bromothymol blue upon addition of Ca^{2+} to a mitochondrial suspension in the absence of permeant anions (see Figure 5) shows that the Ca^{2+} uptake starts within 0.5 sec, before the intramitochondrial pH begins to rise. Ca^{2+} accumulation and the bromothymol blue response follow the same exponential curve, but the bromothymol blue response is delayed approximately 1 sec after the Ca^{2+} accumulation. Deviation from the exponential curve occurs at about 20 sec after the addition of Ca^{2+}. In preliminary experiments with the rapid-flow apparatus, we have come to the conclusion that the initial steady-state changes of the respiratory chain enzymes have been completed (the half-time for cytochrome *b* being approximately 30–50 msec) (Mela, 1968a) by the time the detectable Ca^{2+} uptake starts (approximately 200 msec after the addition of the cation). This suggests that the primary step in the Ca^{2+} accumulation is the reaction with an energized carrier, which is closely connected to the respiratory carriers. A more detailed study of the fast kinetics of the cation accumulation will be reported elsewhere (L. Mela and B. Chance, in preparation).

In the presence of a permeant anion, such as acetate, the accumulation of Ca^{2+} is accelerated, due to the accumulation of the anion (Rasmussen *et al.*, 1965). According to Figure 4, the initial rate of Ca^{2+} accumulation in the presence of acetate is 2.3 times faster than in its absence. Our data also agree with the earlier findings of many investigators that more Ca^{2+} is accumulated by the mitochondria in the presence than in the absence of permeant anions.

With the murexide technique, we have been able to demonstrate the accelerative effect of a low concentration of Ca^{2+} ions upon the relatively slow Mn^{2+} accumulation.

The experimental evidence of this paper on Ca^{2+} and Mn^{2+} accumulation measured by murexide absorbance change is in good agreement with earlier studies of divalent cation accumulation. In well-controlled experiments from which unspecific changes, such as scattering and interference from other components (*e.g.*, cytochromes) have been eliminated, the Ca^{2+}- and Mn^{2+}-sensitive indicator, murexide, is an important tool for the study of cation accumulation in mitochondria. We stress its unique position as the only kinetic method now available for measurements of divalent cation uptake in mitochondria.

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