# Calcium and Manganese Interactions in Mitochondrial Ion Accumulation*

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ABSTRACT: The energy-linked accumulation of manganese by rat liver mitochondria is shown to produce only a small degree of membrane alkalinization during manganese accumulation in the absence of a permeant anion. If, however, a small concentration of calcium is present, proton ejection is accelerated, large alkalinization of the membrane occurs, and the extent of manganese accumulation is more than doubled. The phenomenon is investigated using the glass electrode as an indicator of extramitochondrial acidification, bromothymol blue as an indicator of intramitochondrial alkalinization, and electron paramagnetic resonance as a measure of free manganese.

It is concluded that calcium can have an activating effect on the uptake of manganese under our experimental conditions.

The employment of an intramitochondrial pH indicator has led to a more sensitive and effective evaluation of cation reactions with mitochondrial membrane (Mela, 1966; Chance, 1966; Chance and Mela, 1966a,b) than can be obtained with external pH indication, for example, by the glass electrode. While the energy-linked reaction of calcium with mitochondria elicits a very rapid respiration at low concentrations of $Ca^{2+}$ (Chance, 1965), the reaction with manganese requires much higher concentrations (Chappell *et al.*, 1963). This paper describes a novel phenomenon in which the addition of a small amount of calcium activates the reaction of the mitochondria with a much larger amount of manganese. The paper also presents observations of the effect of the accumulation of manganese upon the membrane alkalinization as measured by bromothymol blue (BTB).[1] In addition, electron paramagnetic resonance (epr) is employed to determine the extent of manganese accumulation.

## Experimental Procedure

BTB is added to rat liver mitochondria (Chance and Mela, 1966b) at a concentration of approximately 0.5 mμmoles/mg of protein. The reaction mixture has a low buffer capacity (20 mM sodium succinate) which facilitates the recording of extramitochondrial pH changes with the glass electrode. The components of the reaction medium do not effectively compete with the reactions under study; sodium is not rapidly accumulated, and succinate does not serve as an effective permeant anion (Chance and Yoshioka, 1965). The osmotic properties are maintained by 0.225 M mannitol and 0.075 M sucrose; pH changes are recorded by Leeds and Northrup electrodes connected to a Radiometer pH meter (Model 22) with a suitable output amplifier. The absorbance changes due to BTB are measured at 618 mμ with 700 mμ as a reference wavelength. These wavelengths largely compensate for the absorbance changes resulting from oxidation–reduction changes of the cytochromes. The wavelengths are obtained either with monochromators or with interference filters.[2] The sensitivity of the recording is sufficient to follow changes of intramitochrondrial pH of less than 0.02 (Chance and Mela, 1966a). Small amounts of calcium are also detected; for example, 30 mμmoles of $Ca^{2+}$/mg of protein gives an absorbance change of 0.03 $cm^{-1}$, and under our particular experimental conditions this absorbance change can be read to considerably better than 0.001. The addition of a solution of $CaCl_2$ at pH 7.4 to a reaction mixture containing all the constituents except the mitochondria causes no change of indicator absorption. If mitochondria are present at protein concentrations ranging from 0.4 to 6 mg/ml, a characteristic increased absorption of the indicator at 618 mμ is observed upon $Ca^{2+}$ addition. This is attributed to an alkalinization of the membrane space (cristae) of the mitochondria (Chance and Mela, 1966a,b).

In order to assay the $Mn^{2+}$ concentration external to the mitochondria, we have taken advantage of the finding of Chappell *et al.* (1963) that the epr signal due to $Mn^{2+}$ greatly broadens when the $Mn^{2+}$ is accumulated by the mitochondria. This method avoids uncontrolled errors in separation methods due to gains or losses of cations during filtration or centrifugation. Manganese concentrations were measured in the Varian E-3 epr spectrometer.[3] The epr experiments

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[1] Abbreviations used: BTB, bromothymol blue (3,3-dibromothymolsulfonphthalein); epr, electron paramagnetic resonance.

[2] B. Chance and V. Legallais, in preparation.

[3] The kind assistance of Dr. Heinz Schleyer in operating the Varian E-3 instrument is gratefully acknowledged. In addition, Dr. Harold Bright kindly allowed us to use the instrument.

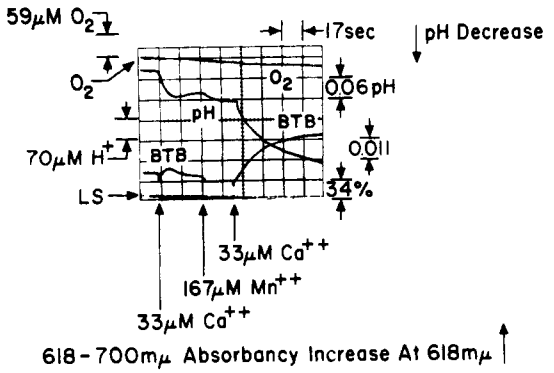


FIGURE 1: An experiment showing manganese accumulation in the presence of a low concentration of calcium. The traces indicate oxygen uptake, external pH change, change of BTB absorbance, and light scattering; rat liver mitochondria, 1.7 mg of protein/ml, 0.3 M mannitol–sucrose, 20 mM sodium succinate, pH 7.4, 3.3 μM BTB.

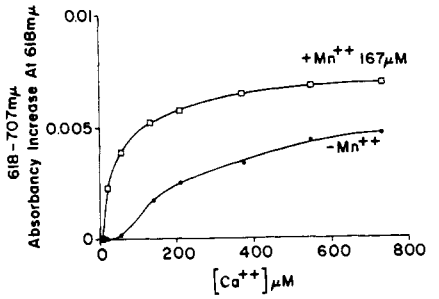


FIGURE 2: Interaction of 167 μM $Mn^{2+}$ on BTB changes caused by calcium titration in rat liver mitochondria; 2.2 mg of protein/ml, 0.3 M mannitol–sucrose, 20 mM sodium succinate, pH 7.4, 3.3 μM BTB.

were carried out in parallel with the optical experiments. The time for filling the capillary and adjusting the apparatus is about 75 sec; thus, the first reading of the epr was delayed that long after the addition of the last reagent. The concentration of manganese was evaluated from the amplitude of the first peak that was clearly resolved in the derivative trace. The spectra were scanned from low to high field. The sensitivity of the epr was calculated directly in terms of a known concentration of manganese.

## Experimental Results

The phenomenon which called our attention to the possibility that manganese accumulation was affected by the presence of calcium is illustrated in Figure 1. Here the techniques employed in previous studies (Chance and Mela, 1966a,b) show, on the top trace, oxygen utilization (59 μM $O_2$/division); on the second trace, extramitochondrial pH changes measured by the glass electrode (70 μM $H^+$/division); on the third trace, intramitochondrial pH changes measured by BTB (0.011 $cm^{-1}$/division); and, on the bottom trace, light-scattering changes (34%/division), on which time markers (5 sec/mark) are superimposed.

The BTB trace indicates alkalinization by an upward deflection following the addition of 33 μM calcium chloride. The increased alkalinity is, however, spontaneously neutralized and within 20 sec the trace has returned to the initial base line. This return of the trace is attributed to the buffer capacity of the mitochondrial membrane (Chance and Mela, 1966a,b). A marked proton ejection from the mitochondrial membrane is observed on the first addition of 33 μM $Ca^{2+}$; the $H^+/Ca^{2+}$ ratio is approximately 3, possibly due to calcium reaction with phospholipid components of the membrane (Chappell *et al.*, 1963). The second addition consists of 160 μM $Mn^{2+}$. The respiration trace shows some acceleration, the extramitochondrial pH trace shows an alkalinization to the extent of 20 μM $OH^-$, but the BTB trace shows no measurable change. At this point, 33 μM calcium is added. In a control experiment in which no manganese had been added, the response to the second addition of 33 μM calcium was almost identical with that shown here for the first addition of calcium (Chance and Mela, 1966a,b). In this experiment, however, the responses are different. There is a rapid ejection of protons from the mitochondria as indicated by the abrupt and large deflection of the pH trace, and at the same time the BTB trace shows a large alkalinization of the mitochondrial membrane. The deflection of the pH trace corresponds to an excess of 200 μM $H^+$. The BTB trace shows a degree of alkalinization that would have required approximately 200 μM $Ca^{2+}$ in the absence of $Mn^{2+}$. The respiration trace shows a slowing, characteristic of respiratory inhibition observed in state 6 (Chance, 1964). One interpretation of the result is that the entire amount of manganese plus calcium became available for proton ejection and alkalinization of the mitochondrial membrane.

*Calcium Concentration Required for Half-Maximal Effect.* Since it appears that calcium added after manganese is causing a much larger effect than that which would be expected for calcium alone, we have plotted in Figure 2 the response of the membrane to sequential additions of calcium in the presence and absence of manganese. In the absence of manganese, the usual titration curve is obtained where the initial additions of calcium caused no alkalinization of the membrane due to the endogenous phosphate and other sources of buffer capacity, and the titration starts when 23 mμmoles of $Ca^{2+}$/mg of protein has been added. In the presence of 167 μM $Mn^{2+}$, the titration curve is much more abrupt and a response is observed even at 6.7 μM $Ca^{2+}$. This response is very similar to that observed in mitochondria to which supplements of low concentrations of local anesthetics are made (E. J. Harris, B. Chance, and L. Mela, in preparation). The calcium concentration for half-maximal response is 40 μM. In the absence

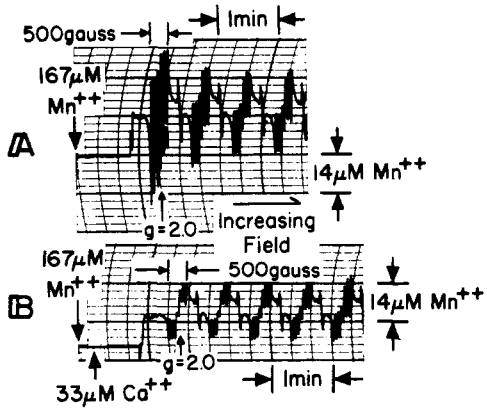


FIGURE 3: Epr study showing the disappearance of manganese signal in the absence (A) and presence (B) of 33 μM $Ca^{2+}$ in rat liver mitochondria; 4.7 mg of protein/ml; 0.3 M mannitol–sucrose, 20 mM sodium succinate, pH 7.4.

of manganese, this calcium concentration would have given no detectable response. Thus, we have evidence that both manganese and calcium are participating in the reaction described here.

*Response of Cytochrome b.* In order to verify that the over-all effects on hydrogen ion ejection and membrane alkalinization are reflected in the steady state of the respiratory carriers, we have, in a separate series of experiments, observed the response of cytochrome *b* under conditions similar to those of Figure 1. $Ca^{2+}$ addition causes a small cycle of oxidation and reduction of cytochrome *b*, which persists for a time equivalent to that of the BTB change. $Mn^{2+}$ then causes a negligible response of cytochrome *b*, and the second addition of $Ca^{2+}$ causes a large response of cytochrome *b* for a prolonged interval. In short, the responses of cytochrome *b* indicate that small amounts of $Ca^{2+}$ greatly activate the reaction of $Mn^{2+}$ with the mitochondrial electron transport and energy conservation systems. Addition of low concentrations of $Mn^{2+}$ to the $Ca^{2+}$-supplemented mitochondria causes no increased response of cytochrome *b* or of the indicator, BTB.

*Fate of the Manganese.* In order to discover whether the manganese is accumulated during the interval in which protons are ejected and the membrane is made alkaline, we have measured in the epr apparatus the manganese concentration as a function of time (Figure 3). In each of the two portions of the figure, we plot repetitive scans of the hyperfine structure of manganese ion from low field to high field ($g = 2$ is indicated). The top trace shows that the amount of free manganese in the system has decreased considerably at the time of the first measurement (approximately 70 sec after the first addition of 167 μM $Mn^{2+}$). Each successive spectrum indicates a decrease of the amplitude of the hyperfine structure of the manganese signal; this decrease is characteristic of manganese binding by the mitochondrial membranes, presumably by its phospholipid components (Chappell *et al.*, 1963). Under these conditions the extramitochondrial manganese concentration is approximately 14 μM. If manganese and calcium are added as in Figure 1, at the first scan of the epr apparatus (approximately 80 sec after adding manganese), the residual signal is considerably lower than that observed in the upper trace and decreases no further with successive scans. This shows that the disappearance of manganese from a phase outside the mitochondria has accelerated and has proceeded to a greater degree following the addition of calcium.

## Discussion

These experiments point to a cooperative phenomenon in manganese uptake caused by the presence of low concentrations of calcium. Evidence for this conclusion is afforded most by the rates and the extents of the reaction as observed (a) by epr measurements of extramitochondrial manganese concentration, (b) by glass electrode measurements of the rate and extent of proton ejection by the mitochondrial membrane, and (c) by the much larger and more rapid degree of alkalinization of mitochondrial membranes as measured by BTB. It is noted experimentally that a prior addition of calcium will not activate the membrane toward the manganese; the order of addition is important. We attribute this to the fact that the completion of the accumulation of a prior addition of calcium involves its removal from the membrane or the carrier within the membrane to the interior of the mitochondria, and therefore calcium can only activate manganese accumulation if they are added together, or with manganese first. This result appears to be the first evidence of a cooperative interaction in mitochondrial ion accumulation.

Several explanations may be proposed for this interesting phenomenon. The range of explanations to be considered depends upon the nature of the epr and the BTB information. The former indicates that the manganese disappears from the aqueous phase, which suggests that the manganese is bound so that its paramagnetic resonance absorption no longer has hyperfine structure, but has changed to a much broader resonance not detectable under the particular conditions of field modulation employed. The combination of the glass electrode and BTB measurements indicates that proton movements to an extent commensurate with the manganese and not the calcium concentration have occurred. These proton movements not only affect the medium external to the mitochondria, but also cause a complementary change within the mitochondrial membranes. Similarity between these results and those obtained with higher concentrations of calcium seems sufficient to allow us to conclude that the manganese has been accumulated by the mitochondria in the same sense that calcium is accumulated. We must therefore seek an explanation of how a small amount of calcium added after manganese can accelerate the rate and

extent of manganese uptake. One simple mechanism based upon the postulate that ion transport involves a mobile carrier with specific binding sites for the ion transported (Davies, 1961) assumes that when one of the binding sites on this carrier is occupied by calcium, the carrier is more active toward manganese. Since the stoichiometric ratio of manganese to calcium is very large, it is necessary further to postulate that a portion of the calcium remains bound to the carrier until the manganese accumulation is complete.

Evidence in favor of this type of mechanism is provided by the data of Rasmussen and Ogata (1966) in which mitochondria internally labeled with calcium show external label upon a further addition of unlabeled calcium (Figure 15 of Rasmussen and Ogata, 1966). Thus circulation of calcium in and out of the membrane in the course of the reaction is already established.

A second mechanism does not involve an explicit carrier, but postulates that the pathway for manganese entry involves a series of steps through phospholipid membrane. When the phospholipid is altered by small amounts of calcium, its structure or reactivity is changed in such a manner that the manganese entry is much more rapid.

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