QUANTITATIVE MAGNETIC RESONANCE STUDIES OF MANGANESE UPTAKE BY MITOCHONDRIA

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ABSTRACT The uptake of the paramagnetic ion manganese by rat liver mitochondria is studied by electron paramagnetic resonance (EPR) spectroscopy. Emphasis is placed on: (*a*) obtaining accurate EPR quantitation of intramitochondrial manganese fractions previously described (Gunter, T. E., and J. S. Puskin, 1972, *Biophys. J.* **12**:625), (*b*) establishing competition for intramitochondrial binding between one of these these fractions and calcium, (*c*) demonstrating the effects of substrate and ATP concentrations on each fraction observed through EPR, and (*d*) demonstrating the effect of inorganic phosphate (P_i) concentration and pH on each fraction.

INTRODUCTION

Mitochondria represent one of the simplest biological systems which actively transport calcium and similar divalent cations, such as manganese. Mitochondria are found in the cells of all higher animals and plants and are thought (2) to play a role in intracellular calcium regulation. Therefore, an understanding of the mechanism by which mitochondria sequester divalent cations such as calcium and manganese and a knowledge of the manner in which these divalent ions are held inside mitochondria after uptake may provide important steps toward understanding intracellular calcium regulation and transcellular divalent cation transport.

The paramagnetic ion Mn^{2+}, because of its charge, charge symmetry and size, provides a likely analog to Ca^{2+}, Mg^{2+}, or both. Mn^{2+}, unlike Ca^{2+} and Mg^{2+}, can be studied by the electron paramagnetic resonance (EPR) technique.

Uptake of calcium, strontium, and manganese by the calcium transport system of mitochondria, and binding of these divalent cations to the mitochondrial membrane system has been studied by a number of techniques (3–12), including EPR (1, 7, 13–15). The disappearance of the signal of manganese hexahydrate ("free manganese") in a mitochondrial suspension was observed in the early studies with the unseen fraction being interpreted as having been taken up by the mitochondria (7, 13). Later it was found that treatment of the mitochondrial suspension with a slight excess of EDTA after Mn^{2+} uptake, effectively removed the external free manganese signal and allowed direct observations to be made on the state of intramitochondrial manganese (1). When manganous ion is taken up by mitochondria through the energy dependent transport process, two spectrally distinct fractions of manganese inside the mitochon-

dria may be observed through EPR. One of these fractions, hereafter denoted as E, shows the spectral characteristics of spin exchange (1). The other, hereafter denoted as S, is a hyperfine sextet similar to that of the manganese-water hexahydrate. Indeed, this fraction S can be identified under some conditions of manganese uptake by the mitochondria as being primarily composed of manganese hexahydrate, where the spectral lines have been broadened through the effects of intramitochondrial local viscosity (14).

The spectrum of E is generally narrower than the hyperfine envelope of the S spectrum. Furthermore, under conditions where no exogenous phosphate is added to the mitochondrial preparation, the line width of the E spectrum was observed to decrease with increasing mitochondrial uptake of manganese (1). It was concluded that the spectral lines of this manganese fraction are spin exchange narrowed and that this fraction is held in regions of high local concentration.

In some cases where uptake of manganese by mitochondria was large enough to give nearly Lorentzian line shape in spectra of the E fraction, the bulk of the manganese in the sample could be accounted for by EPR. In this paper a more satisfactory procedure is described for quantitating the manganese in the spin exchange fraction E, which is applicable to more general line shapes. Results obtained using this procedure are compared with atomic absorption measurements carried out on the same sample.

In these past studies, it was noted that parameters such as substrate concentration, ATP concentration, inorganic phosphate concentration, and pH greatly affect the characteristics of uptake; however, no detailed description of these effects has been given. Here we examine in more detail the effects of varying each of these parameters on the spectra of the E and S fractions.

EXPERIMENTAL PROCEDURE

As in references 1, 14, and 15 rat liver mitochondria were prepared by the usual modification of the Hogeboom-Schneider technique. EDTA was included in the medium at the time of tissue homogenization. Unless otherwise stated, the mitochondria were suspended in 0.25 M sucrose, 10 mM sodium succinate, 1 mg/ml BSA, 0.1 mM sodium ATP, 20 mM tris-chloride pH 7.4. Tris buffer was chosen because it does not interact strongly with manganese. Mitochondrial protein concentrations were determined by a modification of the biuret technique as discussed by Gornall et al. (16). Aliquots of a mitochondrial preparation, subject to the protocol of each day's experiment, were handled very much as in references 14 and 15.

Experiments on the kinetics of uptake by Chance and co-workers (7–9) serve to establish the characteristic times associated with uptake of manganese and calcium and, along with the work of Chappell et al. (13), the maximal amounts of uptake expected for calcium and manganese. Results of these past experiments have then helped to establish the conditions under which the present experiments were carried out.

EPR spectroscopy was carried out near 9.5 gHz on a Varian E-12 spectrometer

(Varian Associates, Palo Alto, Calif.). In quantitating the EPR spectra, dual cavity techniques were employed. The spectrum of a pitch standard was taken before and after the spectrum of each sample, allowing corrections to be made for changes in cavity Q. The spectra were analyzed into their component parts very much as described in reference 1. The Mn-EDTA spectrum was subtracted out by computer by maximizing the symmetry of the spectrum left after the Mn-EDTA spectrum was removed. Line width determinations were made by another subroutine of the same computer program.

The hyperfine sextet spectrum (S) was quantitated by comparing its line heights and widths to those of the Mn-hexahydrate spectrum produced by a diluted sample of Fisher certified atomic absorption standard manganese (Fisher Scientific Company, Pittsburgh, Pa.; lot 794867). The line height of each sample was compared with that of a standard having the same line width. The line width of a standard was controlled by adjusting the ratio of water to glycerol in making up the standard. Calculations of intramitochondrial free manganese concentration were made assuming an intramitochondrial volume of 0.4 μl/mg protein (14, 17).

The spin exchange spectrum of fraction E was quantitated by a computer program incorporating the following assumptions and operations:

(a) A straight line between a point 1,000 Oe below spectral center and a point 1,000 Oe above spectral center was taken as the initial base line for the derivative absorption spectrum of fraction E (See Fig. 1 a).

(b) Because the assumption used in a leaves out the tails of the experimental curve, a correction based on a derivative Lorentzian curve was added to the spectrum in a above in the range $H_e - 1{,}000 < H < H_e + 1{,}000$ based on the height and width of the spectrum of E, as indicated by the arrows in Fig. 1 b. The added spectrum is indicated by hatched area I in Fig. 1 c.

(c) The resulting spectrum shown in Fig. 1 c was integrated both from the low field side and from the high field side. These results were then interpolated to form an absorption spectrum. This approach is necessary because the best information relating to the height of the low (high) field absorption spectrum tail comes from integrating the low (high) field derivative absorption spectrum tail from the low (high) field side.

(d) A Lorentzian correction was made to the absorption spectrum to account for that part of the derivative absorption spectrum in the tails of the resonance ($H_e - 1{,}000 > H$ or $H_e + 1{,}000 < H$). This correction is indicated by hatched area II in Fig. 1 d.

(e) The absorption spectrum was integrated.

(f) Lorentzian corrections were made to the integral of the absorption spectrum to account for that part of the absorption spectrum in the tails of the resonance ($H_e - 1{,}000 > H > H_e + 1{,}000$). These corrections are equivalent to the areas of the hatched areas marked as III in Fig. 1 d.

(g) The area under the absorption spectrum was compared with the area under a corresponding absorption spectrum of a manganese hexahydrate standard (Fisher certified standard manganese).

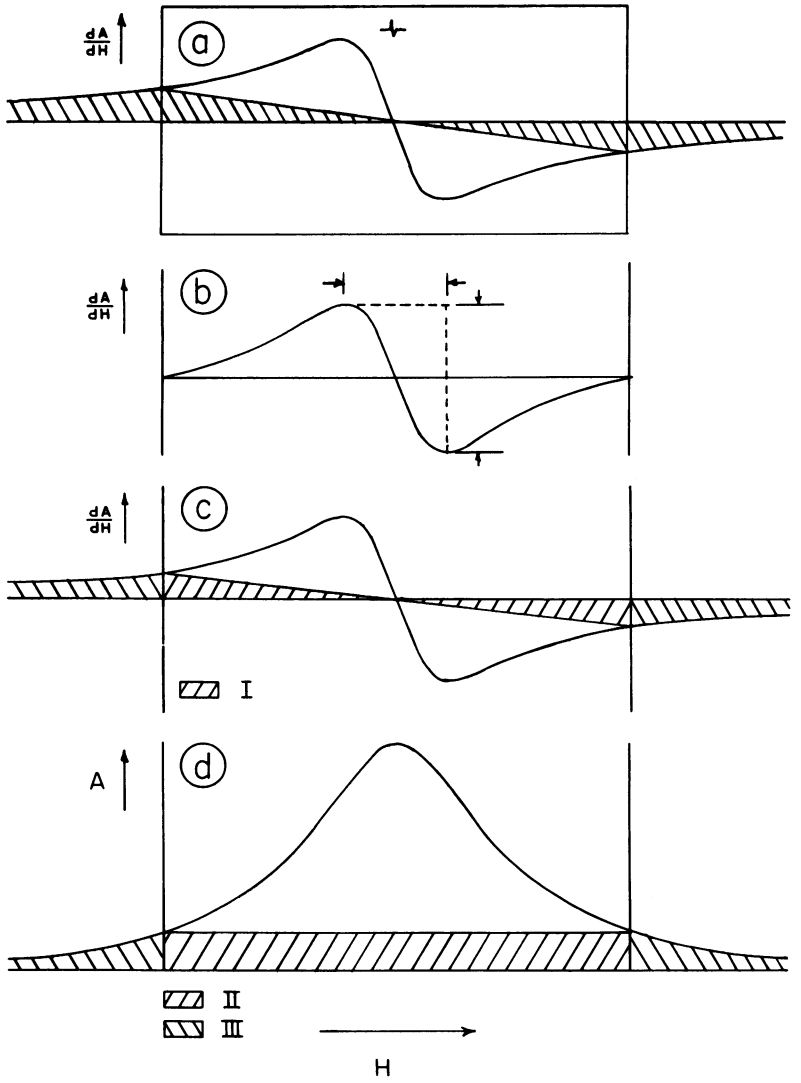


FIGURE 1 (*a*) Diagrammatic experimental spectrum drawn to represent a partially analyzed spectrum containing only spin exchange component (*E*) plus noise and spectrometer drift. The corrections are purposefully made large so as to be easily seen on the drawings. The part of the spectrum within the rectangle represents 2,000 Oe of field scan. Because of difficulty in determining the actual zero of the derivative absorption spectrum, the actual area shown hatched in the diagram cannot be accurately determined. Also the tails of the curve outside the ±1,000 Oe limits are undetermined. Hence, the computer program truncates the experimental spectrum removing the hatched area as in 1 *b*. Then in 1 *c* using the height and width of the spectrum it generates Lorentzian (or Gaussian) tails to replace the truncated experimental tails and uses the height of the generated Lorentzian (or Gaussian) at ±1,000 Oe from spectral center to approximate the lost hatched area (I). The integral of this curve is shown in 1 *d*. The integral of the tails from ± ∞up to the limits of the experimental spectrum generate the correction shown as hatched area II. In addition the area under the tails of the absorption curve (labeled III) must still be accounted for. This is done after the absorption spectrum is integrated.

(*h*) The process was gone through again from step *b* through step *g* using Gaussian corrections for the tails of the spectrum in place of Lorentzian. According to the theory of Anderson (18), the central region of an exchange narrowed line is "resonance" or Lorentzian in shape while the tails of the spectrum fall off more rapidly than Lorentzian. Hence, it might be expected that the best corrections to use for the tails of a particular spectrum are intermediate between Lorentzian corrections and Gaussian corrections.

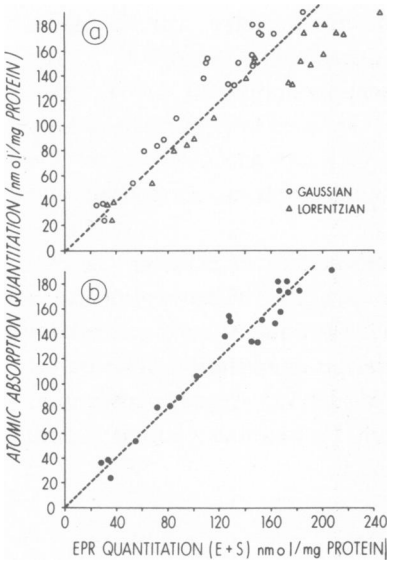
EPR quantitation, using both Lorentzian and Gaussian tail corrections, was carried out by a computer program which compared the area under the absorption curve for the standard with that of each mitochondrial sample. EPR quantitation carried out in the above manner was compared with atomic absorption quantitation done on an Instrumentation Laboratory, Inc. (Lexington, Mass.) Model 153 Atomic Absorption Spectrometer equipped with a Westinghouse (Pittsburgh, Pa.) Hollow Cathode Lamp designed for measurements of Mn^{2+} absorption.

RESULTS

Broad absorption lines are a common problem when one seeks to use transition metal ions as probes of biological systems. (When even the relatively slower relaxer Mn^{2+} is bound in a biological system to a protein molecule, for example, the EPR spectrum is often too broad to observe [19].) Consequently, it is important in magnetic resonance studies of Mn^{2+} in a biological system to discover how much of the manganese taken up by the system is detectable through EPR.

Fig. 2 compares mitochondrial manganese as measured by EPR to that measured by atomic absorption. Quantitation using both Lorentzian and Gaussian tail corrections is compared with atomic absorption quantitation in Fig. 2*a*. In Fig. 2*b*, a single point intermediate between the Gaussian and Lorentzian points is shown for the EPR quantitation of each sample. This point was obtained by minimizing the sum of the absolute magnitude of the variances between EPR quantitation and atomic absorption quantitation using a one parameter fit for the EPR data of the form: $\alpha[Mn^{2+}]_{Gaussian} + (1 - \alpha)\,[Mn^{2+}]_{Lorentzian}$. Atomic absorption determination of Mn^{2+} was chosen to compare with EPR since it does not suffer from the same quantitation problems as EPR, is several times more accurate than EPR quantitation, and can be carried out on the same sample as the EPR work, thus avoiding variation between samples.

The characteristics of manganese uptake by mitochondria are like those of calcium rather than those of magnesium with regard to such variables as amount of uptake, effects on oxygen consumption, swelling, etc. Nevertheless, it is desirable to know more specifically whether the bound fraction of Mn^{2+} observed within mitochondria by EPR spectroscopy acts as an analog to calcium, an analog to magnesium or as an analog to neither. In Fig. 3 the results of a competition study are shown. In the experiments depicted here, a mitochondrial preparation was divided into a number of aliquots. Samples of the preparation were added to suspending medium and allowed to come to room temperature. Either calcium or magnesium ions were added in varying amounts. 2 min were allowed before addition of manganese. The amount of



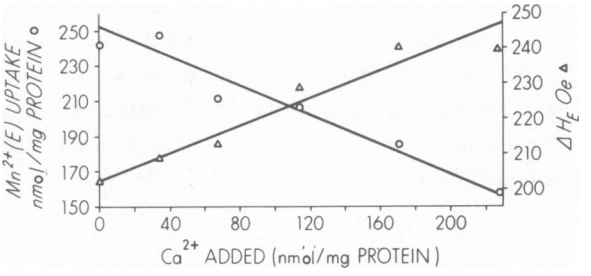


FIGURE 2

FIGURE 3

FIGURE 2 Total manganese as measured by atomic absorption vs. total manganese as measured by EPR. In 1 *a* points calculated from EPR data using both Gaussian and Lorentzian tail corrections for each spectrum analyzed are shown. In 1 *b* the total manganese uptake as measured by EPR is taken as the sum of (0.62) times the amount measured using Gaussian tail corrections plus (0.38) times the amount measured using Lorentzian tail corrections. These coefficients are determined by a fitting procedure as is explained in the text. Absolute error in the EPR quantitation is about 30% and relative error about 10%. Absolute error for the atomic absorption data is about 10%.

FIGURE 3 Manganese uptake in the *E* spectral fraction on the left ordinate and spin exchange line width on the right ordinate vs. calcium added. In the data shown here, calcium is added in varying amounts to each aliquot 2 min before 300 nmol/mg protein of manganese chloride is added.

manganese added was always the same (300 nmol/mg protein). The uptake of manganese and the spin exchange line widths were essentially unaffected by magnesium. For calcium, on the other hand, as shown in Fig. 3, the amount of manganese observed in the spin exchange form decreased and the spin exchange line width increased (representing a decrease in spin exchange averaging) with increasing $[Ca]^{2+}$. This is direct evidence for competition of manganese and calcium but not magnesium for the intramitochondrial spin exchange binding site.

From this data alone it is impossible to say whether the lack of evidence of competition in binding between manganese and magnesium is due to binding or to transport specificity. From previously published data, however, one might suspect transport effects (9). Although internal free manganese concentration can be approximately determined through EPR, it would be improper to infer relative binding affinity of manganese and calcium for this site from data like that of Fig. 3. One reason for this

is that binding affinity may be a function of intramitochondrial pH and, as will be discussed later, pH changes occur at the time of cation uptake.

Uptake of manganese increases when a substrate such as succinate is added to the mitochondrial preparation prior to manganese. The dependence of uptake on succinate concentration is illustrated in Fig. 4.

It is difficult to demonstrate the dependence of uptake on substrate (succinate) when either endogenous substrate or ATP is present in large concentration. The data shown in Fig. 4 were obtained after the mitochondria were subjected to two 20 min periods of room temperature incubation to deplete endogenous substrate. (The mitochondria were spun down and resuspended after each incubation period.) In order to achieve rapid depletion of endogenous substrate during the first incubation period, ADP and phosphate were added to the usual suspension medium containing sucrose, BSA and tris buffer; during the second period of incubation, only the suspension medium was used. Control aliquots to which both ATP and succinate were added subsequent to this treatment gave good uptake (greater than 200 nmol/mg protein).

When Mn^{2+} uptake is studied as a function of [ATP] a similar increase in uptake is seen. Adenine nucleotides enter the mitochondrion by the atractyloside inhibitable exchange mechanism (20) and the energy of ATP hydrolysis can be used to power ion transport (3). Furthermore, a divalent cation might enter directly as a $(M - ATP)^{-}$ complex (21). Since it is easier to demonstrate the dependence of uptake on [ATP] than that on succinate concentration, only one room temperature incubation (in suspension medium alone) was carried out prior to manganese uptake. It is interesting to note in Fig. 5 *a* that in the presence of sufficient substrate, ATP begins to increase total manganese uptake at a level almost a factor of 5 (in ATP concentration) lower than in the absence of substrate. One possible explanation is that in the absence of substrate, enough ATP must be present to provide energy for manganese transport; while if substrate is present in sufficient amounts, only enough ATP to complex intramitochondrial free manganese (never more than 10% of the total intramitochondrial manganese) is needed. Complexing the intramitochondrial free manganese would lower the intramitochondrial manganese activity, thus decreasing the free energy required for transport. It is difficult to obtain really definitive data by the method used here, and shown in Fig. 5 *a*, since there is still some endogenous substrate and ATP present. However, the data of Fig. 5 *a* is consistent with the above hypothesis.

In the absence of added substrate, ATP hydrolysis must provide energy for uptake of manganese. Once inside the mitochondrion, however, the manganese ion finds itself in the presence of the products of ATP hydrolysis (ADP and inorganic phosphate, P_i). These products of ATP hydrolysis could lower the intramitochondrial manganese activity even more than ATP would. The data shown in Fig. 5 *b* is consistent with this picture. When ATP hydrolysis provides energy for manganese uptake, free manganese concentration is much smaller than when sufficient substrate is present at the same ATP concentration.

All this might be put together by saying, "In the presence of sufficient substrate, the

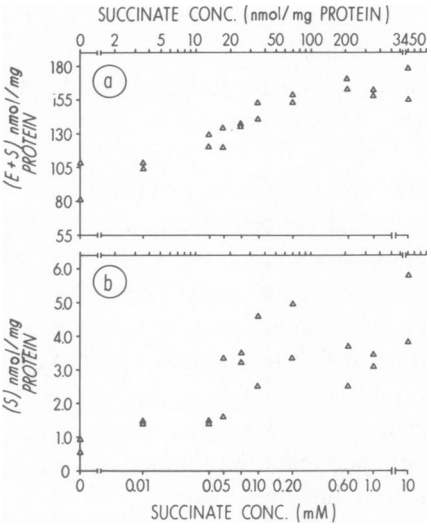


FIGURE 4

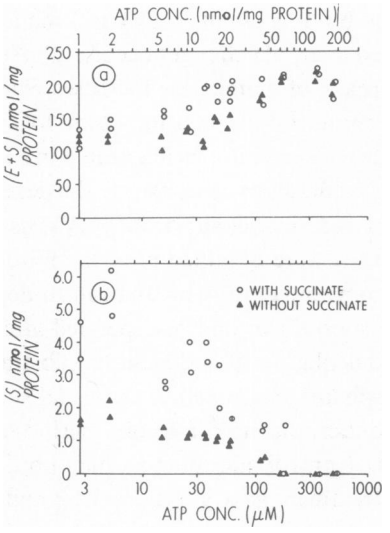


FIGURE 5

FIGURE 4 (*a*) Manganese uptake (*E* + *S*) vs. substrate (succinate) concentration. (*b*) Manganese uptake in the *S* fraction alone vs. substrate (succinate) concentration. No ATP is added to the mitochondrial aliquots studied here. The preparation involved two room temperature incubation periods of 20 min each as is described in the text. It is characteristic of *S* fraction data taken without ATP having been added that much more variance between identical aliquots is observed. Absolute error in quantitation is about 30% and relative error about 10%.

FIGURE 5 (*a*) Manganese uptake (*E* + *S*) vs. ATP concentration both with and without succinate. (*b*) Manganese uptake in the *S* fraction alone vs. ATP concentration both with and without succinate. The preparation involved one room temperature incubation period of 20 min as is described in the text. Absolute error in quantitation is about 30% and relative error is about 10%.

primary function of ATP is not to provide energy for manganese uptake but to lower intramitochondrial manganese activity; while in the absence of substrate, ATP provides energy for uptake and in so doing lowers intramitochondrial manganese activity even more."

This has been further tested by the following experiment: uptake of manganese into both the *E* and *S* intramitochondrial fractions has been studied under conditions where ATP alone, succinate alone, ATP plus succinate, and ATP plus succinate plus oligomycin are present in the suspending medium. The amount of manganese added to each aliquot has been varied so as to provide for each of the above cases a plot of [*S*] vs. total uptake. A linear, least squares fit has been made to each set of data in the uptake range 50–120 nmol/mg protein. The values of [*S*] are read from each of these least squares fit lines at a total uptake of 100 nmol/mg protein. The results are shown in Table I.

The results show that the intramitochondrial "free" manganese concentration is much higher when succinate oxidation provides energy for transport than when energy is provided by ATP hydrolysis. When ATP plus succinate are present the intra-

TABLE I

UPTAKE OF MANGANESE INTO *E* AND *S* INTRAMITOCHONDRIAL FRACTIONS

Case	Aliquot contains	Intramitochondrial $[Mn(H_2O)^{++}]$
		mM
1	ATP	2.2
2	Succinate	20.3
3	ATP + succinate	10.4
4	ATP + succinate + oligomycin	13.1

mitochondrial "free" manganese concentration is intermediate between the corresponding concentrations for the first two cases and reasonably close to the "free" manganese concentration of case 4. For case 4, since oligomycin is present, ATP cannot be used to provide energy for cation uptake; hence, it can only be used to lower intramitochondrial manganese activity. This data then supports the above hypothesis.

It has been reported (for example by Reed and Lardy [22]) that the inorganic phosphate (P_i) levels of mitochondria decrease with time upon incubation at elevated temperatures (30°C) for periods of 5–10 min. Experiments were carried out in this laboratory using the molybdate assay for inorganic phosphate described by Chen et al. (23). After 15 min incubation at 22°C, the intramitochondrial P_i level was found to be 5 or 10 nmol/mg protein, consistent with values given by Reed and Lardy.

On several different days samples were prepared by incubating for 15 min at 22°C in the usual medium less ATP. These samples were spun down and then resuspended in fresh medium. New protein assays and assays for P_i were carried out (yielding 5–10 nmol/mg protein). These preparations were then diluted into series of aliquots containing the usual suspension medium plus varying amounts of inorganic phosphate. Uptake increased monotonically with exogenous P_i. As can be seen in Fig. 6, the line width of *E* decreases to a minimum (individual samples show a minimum line width of ~206 Oe) and then increases again to a value of ~240 Oe, characteristic of manganese phosphate. (Note: The statement that manganese phosphate typically shows a line width of 280 Oe in reference 1 is an error not caught at the time of proofreading.) It is not necessary that all the exogenous P_i entered the mitochondria, and hence the $[P_i]$ scale should be viewed as merely indicative of increasing P_i. The results shown in Fig. 6 suggest that P_i plays a role in the spin exchange fraction observed in mitochondria even when no exogenous phosphate is added.

As was pointed out in reference 1, the line width of the spin exchange fraction under limited loading conditions can be narrower than that observed under massive loading (P_i added) conditions or that observed for amorphous manganese phosphate. This, and the fact that there was insufficient P_i in the mitochondria to account for a 3:2 Mn:P_i stoichiometry, led to the conclusion that the spin exchange fraction was not simply manganese phosphate.

The above observations lead to the hypothesis that the spin exchange fraction is a kind of plaque formed on the inner surface of the inner mitochondrial membrane.

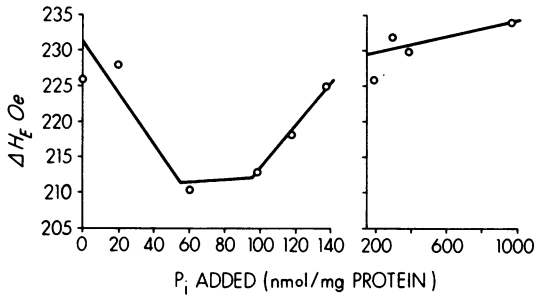


FIGURE 6

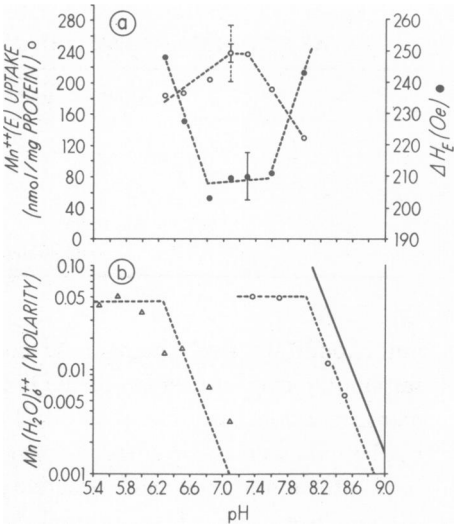


FIGURE 7

FIGURE 6 Spin exchange line width vs. the amount of inorganic phosphate added to each mitochondrial aliquot prior to adding manganese. The preparation involved one room temperature incubation period as is described in the text. The points shown here are plotted from averages of four aliquots (taken on different days) for each point shown. Error in line width determination is about 10 Oe.

FIGURE 7 (*a*) Uptake in the *E* fraction (left ordinate) and spin exchange line width (right ordinate) vs. extramitochondrial pH. (*b*) Uptake in the *S* fraction both with chloride as the counter ion (left) and with 100 mM acetate present (right) as a function of extramitochondrial pH. Absolute error in quantitation is about 30% and relative error is about 10%. Error in line width determination is about 10 Oe. The solid line in 7 *b* represents the solubility product of manganous hydroxide while the dashed lines are drawn through the experimental data points parallel to the solubility product curve.

Manganous ions might initially associate there with negative groups on proteins (particularly phosphoproteins) or phospholipids and then be stabilized by further association with soluble anions such as $HPO_4^{=}$ or OH^- so as to form a close-packed two-dimensional array. Under conditions of maximum uptake without P_i being added, such a plaque would cover something like 35–40% of the inner surface of the inner mitochondrial membrane. As P_i increases, more Mn^{2+} and P_i would be deposited and the plaque thickened. This picture is consistent with electron microscope observations of Ca^{2+} and Sr^{2+} uptake in the presence of exogenous phosphate (24, 25).

The pH dependence of the spin exchange line width shown in Fig. 7 also supports this view. As the pH of the suspending solution is increased from 5.5, the line widths of the *E* fraction spectra first decrease to a minimum of 210 Oe at pH ~7.4, and then increase as pH is increased further. External pH was measured in these experiments, while the spectral effects should be related to internal pH. It is assumed that internal pH increases monotonically with external pH.

In terms of the discussion as to the composition of the spin exchange fraction, the initial narrowing of the spin exchange *E* line width could be ascribed to decreasing competition between Mn^{2+} and H^+ for association with negative groups on the inside of the inner membrane. The increase in spin exchange line width above pH 7.4 could be due to increasing amounts of $Mn(OH)_{2s}$ formed at a higher pH. $Mn(OH)_{2s}$ precipitates at a pH of around 8.5, at a $[Mn \cdot (H_2O)_6^{2+}]$ roughly equivalent to that value calculated from the *S* fraction of Mn^{2+} within mitochondria assuming an intramitochondrial volume of 0.4 μl/mg protein (14, 17). This data on *E* fraction line width would imply that a pH gradient of perhaps 1 pH unit existed across the mitochondrial inner membrane under these conditions.

Another way to estimate the pH gradient across the inner mitochondrial membrane was suggested in reference 15 and is shown in Fig. 7 *b*.

The free intramitochondrial manganese level at physiological pH is generally much lower in the absence of a permeant anion like acetate which does not precipitate with manganese. At lower pH however, the free intramitochondrial manganese level may be high in the absence of such an anion. The effect of this lower pH on the mitochondria is unknown although uptake of manganese still occurs. Regardless of possible effects of low pH on mitochondria, the data can still be used to provide an upper limit on intramitochondrial pH. The data shown on the left side of Fig. 7 *b* was obtained with no permeable anion added to the mitochondrial samples while that shown on the right side of Fig. 7 *b* (as in the case shown in reference 15) was obtained with 100 mM NaAc present. While it is not known whether or not the separation in pH units between the dashed line on the left side of Fig. 7 *b* and the solid line on the right side actually represents a pH gradient across the inner mitochondrial membrane, it is certain that it sets an upper limit to the pH gradient possible under these experimental conditions, since under a higher pH gradient it would be impossible to obtain the observed levels of $Mn \cdot (H_2O)_6^{2+}$. The data presented in Fig. 7 *a* and *b* is consistent with a gradient of 1 to 2 pH units across the inner mitochondrial membrane after cation uptake in the absence of permeant anion but 0–0.5 pH units in the presence of a permeable counterion.

The data in Fig. 7 *a* support the view that cation binding in the *E* fraction increases with increasing pH to a maximum somewhere between an intramitochondrial pH of 8 and 9 and decreases above that pH.

DISCUSSION

Detectability and Competition

Two of the fundamental questions which must always be treated in studying the paramagnetic ion manganese as an analog to calcium are: (*a*) can all the manganese be accounted for by EPR?, and (*b*) is manganese really acting as a calcium analog in this system? The data shown in Fig. 2, show that all the manganese can be accounted for by EPR in a straightforward manner. It is still possible that a small fraction of the

manganese present in the system has lines too broad to be seen by EPR, but this fraction of manganese, if it exists at all, is small.

The data shown in Fig. 3 clearly indicate competition for binding between calcium and manganese. In the experiment from which these data were drawn, the system was treated with calcium two minutes before treatment with manganese. The results were similar when calcium and manganese were added together.

It may be argued that one cannot separate competition in binding from competition in transport. Calcium and manganese do not show simple competition kinetics with respect to transport. Manganese by itself is transported more slowly than calcium (7–9). The addition of calcium increases the rate of Mn uptake (9). It has been suggested that this may be an indication of binding to more than one site on a membrane carrier molecule, with the binding of calcium to one site increasing the affinity of another site for manganese (9). However, in studying possible competition between Ca^{2+} and Mn^{2+} for binding to energized mitochondria, it should be remembered that the internal activities of the two ions may bear little or no resemblance to initial uptake kinetics. If, e.g., the transport is driven solely by a membrane potential, the steady-state activity gradients for the two ions will be equal.

Such questions cannot be completely resolved by the data shown in Fig. 3. However, the data of Fig. 3 does provide some direct information on competition between calcium and manganese ions for intramitochondrial binding sites. In the absence of Ca^{2+}, the uptake of as little as 150 nmol/mg mitochondrial protein results in a spin exchange line width for *E* fraction of 210 Oe or slightly broader. In the presence of calcium, however, the spin exchange line width is much broader, indicating greater average separation between manganese ions while total uptake in the two cases is about the same. This indicates competition between Mn^{2+} and Ca^{2+} ions for spin exchange binding sites.

Effects of Substrate and ATP

Using the quantitation techniques described in the Experimental Procedures section and checked for consistency with atomic absorption quantitation as discussed above, it is possible to add further information to that in the literature on the effects of substrate and ATP on the free and bound fractions of intramitochondrial manganese. It has been known for some time that rat liver mitochondria, in the absence of added phosphate, sequester somewhat more calcium or manganese when ATP is present, than when substrate alone is present. The data shown in Figs. 4 and 5 and Table I support the hypothesis that when divalent cation is taken up by mitochondria having sufficient substrate and ATP, the energy for cation accumulation comes primarily from substrate oxidation. More cation is accumulated in the presence of ATP, however, because ATP lowers the activity of intramitochondrial manganese and thus lowers the energy required for uptake.

Another conclusion is suggested by this data. At the pH of the mitochondrial matrix space (≥ 7.5) the equilibrium constants for ADP^{3-}- or ATP^{4-}-Mn^{2+} complexes (26) are such as to imply no observable free internal Mn^{2+} signal unless all the adenine nu-

cleotide were saturated with divalent cations. The addition then of external ATP in the presence of oligomycin cannot provide additional internal binding sites for Mn^{2+} if the ATP were only to exchange for internal ADP or ATP. The observed decrease (Table I) in internal free Mn^{2+} under these conditions strongly suggests a net uptake of ATP concomitant with Mn^{2+} uptake. This result may be related to the observed stimulation of adenine nucleotide transport by mitochondria by divalent cations (20). It may also be related to the net accumulation of adenine nucleotides during massive loading of calcium phosphate by mitochondria (27).

The Role of P_i and pH

The data shown in Fig. 6 provides a strong indication that inorganic phosphate is involved in the formation of the *E* spectral fraction even in the case where no additional phosphate is added to the mitochondrial suspension medium. At the same time, it corroborates the early conclusion (1) that *E* is not a simple precipitate of manganese with inorganic phosphate in this case. While the role played by this phosphate in manganese binding is not actually known, it is possible to make at least two viable hypotheses. First, while some of the inorganic phosphate in the mitochondria seems to be easily extractable by room temperature incubation, it is possible that this phosphate, while inside the mitochondrion, is associated as labile esters with moieties such as serine or threonine. The affinity of manganese for phosphoproteins such as phosvitin or casein is high. At pH > 8.3 and high protein and manganese concentrations, it has been possible to see spin exchange in such systems, although not lines as narrow as 210 Oe. However, the concentrations of binding sites and manganese in mitochondrial experiments may be higher than those employed for technical reasons in our in vitro studies. Hence, labile phosphate groups on phosphoproteins may play a role in the formation of the spectral fraction designated as *E*.

Second, the inorganic phosphate may not bind covalently to any other molecule but, as was suggested above, it might simply associate with manganese held in a surface layer by nonlabile surface phosphate groups, sulfhydryl groups, etc. In this way P_i could help stabilize manganese in a surface layer of high local concentration. This is consistent with the electron microscopy data presented by Greenawalt et al. (24). Any combination of these two hypotheses is also possible.

Either case is consistent with surface binding providing high local concentration and with "plaque" formation as discussed above. These hypotheses are not contradictory to the data of Chappell et al. (13). For manganese taken up under similar conditions to those discussed here, they found values of enhancement, ϵ (the enhancement of water proton relaxation), of less than 0.5. The enhancement value for manganese in a manganese phosphate precipitate is much lower than 0.5; that for protein-bound manganese is usually greater than unity but for tightly bound manganese within the protein molecule an enhancement of 0.5 can be observed. Even if manganese is initially bound to the outside of a protein molecule, the formation of a plaque as described above would be expected to decrease the enhancement value.

While the estimates of intramitochondrial pH after cation transport obtained from

the data shown in Fig. 7 must be taken as upper limits, in the two cases shown, they are close to the values of pH obtained in corresponding cases by other investigators. Chance and Mela (7, 28), using the indicator bromothymol blue obtained a pH gradient of approximately 1 pH unit in the absence of a permeant anion while the data of Fig. 7 requires an upper limit of about 2 pH units in this case. The bromothymol blue experiments showed no effective pH gradient in the presence of permeant anion, while the data of Fig. 7 requires an upper limit of 0.25 pH units (less than 0.5 pH units even if a factor of two error in intramitochondrial manganese activity measurements is assumed). Through measurements on the pH of solubilized mitochondria, Gear et al. (29) arrived at a gradient of approximately 1.5 pH units following the accumulation of 80 nmol Ca/mg protein in absence of permeant anion.

It is interesting to note that Colonna et al. (30) have reported competition for binding within mitochondria between divalent cations and the safranines (cationic dyes) and have been able to estimate distances between anionic binding sites for both safranine and cyanine dyes of between 4 and 5 Å. This is in quite good agreement with spin exchange line width estimates of average manganese-manganese spacing of 4.0 ± 0.6 Å.

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