ION AND pH GRADIENTS ACROSS THE TRANSPORT MEMBRANE OF MITOCHONDRIA FOLLOWING Mn^{++} UPTAKE IN THE PRESENCE OF ACETATE

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Summary

The electron paramagnetic resonance (EPR) spectrum of Mn^{++} loaded mitochondria is affected by the presence of the permeant anion acetate (Ac^-) in the medium. The hyperfine sextet, shown earlier to have spectral characteristics like those expected of osmotically active Mn^{++} in the matrix space, grows in intensity with increasing [Ac^-]. From estimates of mitochondrial water, the free internal [Mn^{++}] can be calculated. The gradient of free [Mn^{++}] across the inner mitochondrial membrane is believed to be at least 500:1 under conditions of high [Ac^-]. Since Mn^{++} solubility is limited by [OH^-], it is possible to place an upper limit on the pH in the matrix space. The variation of free internal [Mn^{++}], as measured by EPR, with external pH indicates that the [H^+] gradient is 1-1.5 pH units in the absence of permeant anions and considerably less in the presence of 100 mM acetate.

Isolated rat liver mitochondria can accumulate Mn^{++} through the energy dependent Ca^{++} transport system in the absence of permeant anions (1). After treatment with EDTA to bind any non-transported Mn^{++}, the EPR spectrum of such Mn^{++}-loaded mitochondria can be resolved into a broad Mn-EDTA chelate spectrum plus two other components, corresponding to two distinct fractions of transported paramagnetic ions (2, 3):

a. a spin exchange narrowed line E, accounting for the bulk of the transported ions.

b. a hyperfine sextet S, which when uptake exceeds 70 nMoles/mg mitochondrial protein, appears to arise from a population of "free Mn^{++}", i.e. the mobile hexahydrate complex, in a region of relatively high local viscosity, presumably the mitochondrial matrix space (3). This identification was based on line width measurements of S over a range of temperatures and osmotic strengths.

Uptake of Ca^{++} by mitochondria can be enhanced by the addition of the

permeant anion acetate in the bathing solution. Concurrently, light scattering changes can be seen, indicating swelling of the mitochondria. A model consistent with these findings and the measured stoichiometry, provides for the active uptake of each additional Ca^{++} ion to be accompanied by the passive entry of one Ac^{-} (4). The Ca^{++} and Ac^{-} enter the matrix space, resulting in osmotic swelling. If this model and the interpretation above concerning the spectral component S are qualitatively correct and if Mn^{++} behaves analagously to Ca^{++} in this system, one would predict an enhancement of S when Ac^{-} is included in the medium during Mn^{++} uptake.

EXPERIMENTAL AND RESULTS

Mitochondrial samples were prepared as described earlier (2, 3) except that varying amounts of Ac^{-} were included. After addition of Mn^{++}, 15 minutes was allowed for uptake, EDTA was added and the EPR spectra taken.

Effects of acetate on EPR spectra.

Only minor changes in spectra were seen in the acetate concentration range 0 - 10 mM, consistent with the earlier report (3) that acetate had no marked effect on the spectra. However, further increase in acetate concentration led to a sharp increase in the intensity of S (Figure 1). At 100 mM Ac^{-} a slight broadening of E appeared. This may correspond to a decrease in bound Mn^{++} (2), but further work is necessary to elucidate this point.

Calculation of internal $[Mn(H_2O)_6^{++}]$

The internal free Mn^{++}/mg mitochondrial protein was calculated from a comparison of the observed spectral component S with spectra obtained from standard solutions of $MnCl_2$ in water-glycerol mixtures (3). From published estimates of the free water volume in the matrix (.4µl/mg), corrected for the osmotic strength of the medium (3, 5, 6, 7), the internal free $[Mn^{++}]$ was calculated. A further correction (negligible except at the highest Ac^{-} concentrations) was made for swelling of the mitochondria in response

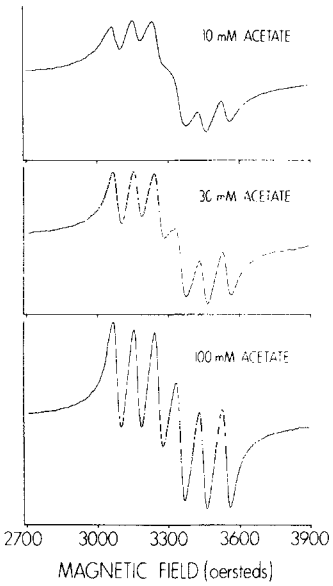

Figure 1. EPR spectra of Mn-loaded mitochondria as a function of sodium acetate concentration. To a suspension of mitochondria in a medium containing sucrose, this buffer, succinate, ATP and varying [NaAc], $MnCl_2$ (≃ 170 nMoles/mg protein) was added. After 15 minutes EDTA was added and the EPR spectrum taken.

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to MnAc accumulation, assuming that one Ac^- enters with each $Mn(H_2O)_6^{++}$ in accord with the model outlined above. The results of the calculation, as a function of increasing [Ac^-] are listed in Table 1. The errors inherent in the EPR technique are about ± 25% absolute and about ± 10% between samples; there may be a considerably greater error in the estimate of intramitochondrial water.

Manganese activity gradient.

From the intensity of the Mn-EDTA spectral components, it is possible to estimate the quantity of ions not transported. For each of the samples listed in Table 1, this was less than .1mM, which in turn is less than the initial [ATP] in the bathing solution and of the same order as the concentration of low affinity binding sites (8) on the outside of the mitochondria. It is possible to conclude then that the concentration of free Mn^{++} before addition of EDTA was reduced to .1 mM and probably considerably

TABLE 1

External $[Ac^-]$ (mM)	Internal Free Mn^{++} (nMoles/mg)	Internal $[Mn(H_2O)_6^{++}]$ (mM)
0	2.89	8.28
2.5	4.47	12.2
10	4.85	12.7
30	9.62	20.7
100	18.35	58.7

Table 1. Before addition of EDTA each sample (2ml total volume) contained 11.7 mg mitochondrial protein, 10mM Tris-Cl (pH 7.4), 10mM sodium succinate, .1mM ATP, .1% BSA, and 2 μMoles $MnCl_2$ and varying amounts of sucrose and sodium acetate. After allowing 15 minutes for uptake 2.5μMoles EDTA was added and the EPR spectrum taken. The external osmolarity was ≃ .28M except for the 100mM $[Ac^-]$ sample where it was .43M. Protein was determined by the Biuret Method.

lower. Combined with the results in Table 1, it appears that the mitochondria can maintain a concentration gradient of at least 500:1. A comment on activity coefficients is appropriate here. Because of the high concentration of phospholipids, small anions, and proteins, some of which bind Mn^{++}, the average activity coefficient for all the Mn^{++} transported is expected to be low. The narrow line-width (30-35 Oe) of S indicates that this spectral component arises from a population of ions, which during the characteristic spin state lifetime ($T_1 \simeq T_2$), are not immobilized or in strong association with negative groups. Each ion has a full complement of six waters of hydration in its first coordination shell and has no negative groups disturbing its second coordination shell except for a possible rapid exchange of singly charged anions (9, 10). The water molecules outside the first hydration sphere must be highly mobile (10, 11). The average activity coefficient for this fraction of Mn^{++} should be much higher (of order unity). Moreover, weakly bound fractions of Mn^{++} may exist that contribute to the activity but not to S. It is therefore conceivable that S could even under-

estimate the activity of $Mn(H_2O)_6^{++}$. This evidence then strengthens the contention that Mn^{++} is accumulated against a chemical activity gradient (3).

Intramitochondrial pH

The activity of free Mn^{++} that can exist in solution above neutral pH is limited by the $Mn(OH)_2$ solubility product, log K_{sp} =-12.8, as depicted by the dashed line in Figure 2 (12). Conversely, the measurement of free Mn^{++} can provide an upper limit on pH. Using the intensity of S as an estimate of intramitochondrial free Mn^{++}, with the reservations discussed above, it is possible to put an upper limit on pH in the matrix space and therefore on the pH gradient across the transport membrane.

Mn^{++} uptake was studied as before but over a range of external pH. The free intramitochondrial Mn^{++} concentration, as determined from S, is plotted against the external pH in Figure 2. If the pH gradient across the membrane and the kinetics of transport do not vary rapidly with increasing external pH, one could predict that the intensity of S would remain relatively constant until an internal hydroxyl concentration was reached where the solubility product was exceeded and precipitation begun. This is the simplest interpretation of the results in Figure 2. Correspondingly, the

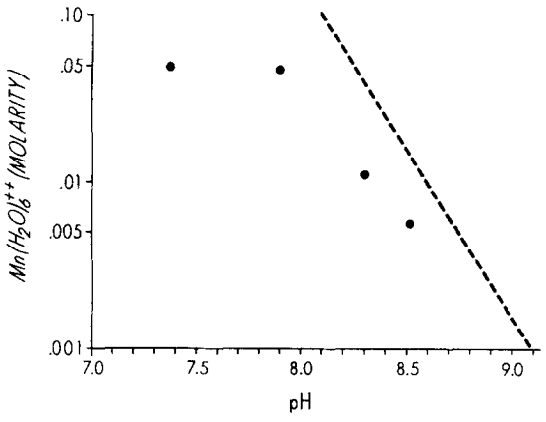

Figure 2. Each sample (2.55 ml) contains 10.7 mg mitochondrial protein, .16M sucrose, .1 M NaAc, 10 mM sodium succinate, .1mM ATP, 2.5 mg. BSA, 2 µMoles $MnCl_2$ and 20 mM tris-Cl buffer of varying pH. After 15 minutes, 27 µMoles EDTA was added and the EPR spectrum taken.

horizontal displacement of the experimental points from the dashed line in the region of the plot where the intramitochondrial $[Mn(H_2O)_6^{++}]$ falls off with increasing pH, should provide an estimate of the pH gradient across the membrane (in any case the displacement provides an approximate upper limit on the pH gradient). Under these conditions (100 mM Ac^- in the medium), the gradient is estimated to be .2 - .3 pH units. Assuming a possible error of a factor of 2 in activity coefficient or mitochondrial water (see above), the pH gradient is 0 - .5 pH units. In similar studies (13) carried out in the absence of permeant anion, there was an apparent $[H^+]$ gradient of 1 - 1.5 pH units.

DISCUSSION

The above observations are in good agreement with the bromthymol blue (BTB) measurements of Chance and Mela (14) indicating a pH gradient of about one unit following Ca^{++} uptake in the absence of permeant anion but little or no gradient in the presence of acetate. The results here complement those of Chance and Mela since the "pH indicator" in this case (Mn^{++} hexahydrate) is believed to be inside the matrix space rather than the intracristal space. The presence in the membrane of a high local $[Ca^{++}]$ and very few H^+ ions has led to criticism of the BTB work (15). Also consistent with these results are determinations of internal alkalinization made by titrating disrupted Ca^{++} -loaded mitochondria (15).

The magnitude of these pH gradients are such as to account for only a fraction of the $\simeq$ 240 mV proton motive force required by the chemiosmotic hypothesis for oxidative phosphorylation (7). However, they do not negate the possibility that sufficient membrane potential exists to make up the needed pmf. Moreover, such a membrane potential would allow for even the very large $Mn(H_2O)_6^{++}$ gradients observed here to be driven downhill energetically along an <u>electrochemical activity</u> gradient.

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