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AN ELECTRON PARAMAGNETIC RESONANCE STUDY OF Mn^{2+} UPTAKE BY THE CHICK CHORIOALLANTOIC MEMBRANE

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SUMMARY

Mn^{2+} uptake in the chick chorioallantoic membrane, an embryonic epithelial tissue which transports Ca^{2+} in vivo was studied using electron paramagnetic resonance (EPR). Mn^{2+} was used as a paramagnetic analog for Ca^{2+}, since there is evidence that Mn^{2+} is accumulated by the Ca^{2+} transport mechanism.

After 1.5 h of uptake the EPR spectrum of the Mn^{2+} in the membrane indicated that 89 % of the Mn^{2+} was in a spin-exchange form, indicating close packing of Mn^{2+}. The Mn^{2+} spacing was estimated from the line width to be about 4.7 Å. The remaining Mn^{2+} was very likely Mn^{2+} hexahydrate.

At pH 7.4 the spin-exchange spectrum tended to broaden when uptake was inhibited, while at pH 5.0 the spin-exchange spectrum was completely abolished in the presence of inhibitors.

The EPR spectrum of Mn^{2+} in the chorioallantoic membrane had a broader line width than that of Mn^{2+} in isolated mitochondria, suggesting that in this tissue mitochondria are not directly involved in divalent cation transport. These EPR studies support the concept that divalent cations are sequestered in high concentrations from the rest of the cell contents during transcellular active transport.

INTRODUCTION

Electron paramagnetic resonance (EPR) spectroscopy is a technique which can give information on the physical environment of divalent cations in biological systems. Ca^{2+}-transporting systems may be studied by using Mn^{2+} as a paramagnetic analog for Ca^{2+}, which is not paramagnetic. EPR spectra of Mn^{2+} in the Ca^{2+} transport system are then analyzed to obtain information about the transport process. This approach has been used to study Ca^{2+} uptake by isolated mitochondria [1], which transport Mn^{2+} as an analog to Ca^{2+}. When Mn^{2+} is taken up by mitochondria under limited loading conditions, it gives a characteristic EPR spectrum

Abbreviation: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

indicating that most of the Mn^{2+} inside is closely packed. This spectrum disappears with the removal of substrates and ATP or upon the addition of transport inhibitors. Under massive loading conditions the EPR spectrum indicates that a manganese phosphate precipitate is formed. Roughly 3 % of the Mn^{2+} inside the mitochondria is Mn^{2+} hexahydrate in limited loading. Quantitation of the Mn^{2+} hexahydrate component has been used to estimate the internal concentration of the osmotically active Mn^{2+} [2] and to demonstrate a hydrogen ion gradient across the mitochondrial membrane [3].

The cellular mechanism by which epithelial membranes such as the intestine and the embryonic chick chorioallantoic membrane translocate large quantities of Ca^{2+} is not well understood. They may use existing cell mechanisms of Ca^{2+} homeostasis such as mitochondrial uptake, or they may use a separate, specialized mechanism. It has been suggested that in the chorioallantoic membrane Ca^{2+} is separated from the rest of the cell and held in special vesicles during transport [4]. Knowledge of the biochemical state of Ca^{2+} in the membrane transport system (whether it is hydrated, precipitated with anions, or bound to an organic substance) would be useful in evaluating proposed transport mechanisms and understanding epithelial Ca^{2+} transport at the molecular level.

In the previous paper [5] evidence was presented showing that Mn^{2+} is accumulated by the chorioallantoic membrane using the same energy-dependent system that transports Ca^{2+}. Mn^{2+} uptake is reduced significantly under conditions that reduce Ca^{2+} uptake, and more Mn^{2+} is taken up by the ectodermal side of the tissue than by the endoderm. Mn^{2+} uptake shows saturation kinetics and is competitively inhibited by Ca^{2+}. In addition, the electron probe distribution of Mn^{2+} in the tissue during uptake is similar to that of Ca^{2+}. These results indicate that Mn^{2+} may be a useful paramagnetic analog for Ca^{2+} and that information obtained from EPR studies of Mn^{2+} in the chorioallantoic membrane may give new insights into the mechanism of divalent cation transport in this tissue.

MATERIALS AND METHODS

Membrane preparation for EPR studies

The chorioallantoic membranes used in these EPR studies were taken from the air space region of 17-day-old White Leghorn eggs (Spafas Inc., Norwich, Conn.). The chorioallantoic membrane and attached inner shell membrane were mounted in an Ussing chamber, which has been previously described [6]. The tissue was prewashed twice for 2.5 min with the standard buffer solution before the solutions containing divalent cations were added. For the oligomycin, 2,4-dinitrophenol and *p*-chloromercuribenzenesulfonate (*p*Cl-HgBzs) inhibitor experiments, there were two 7.5 min prewashes with the inhibitor present, and there was also inhibitor in the ectodermal solution during incubation. In the standard experiment 0.75 mM Mn^{2+} and 0.1 mM Ca^{2+} were present on the ectodermal side and 0.75 mM Ca^{2+} on the endoderm. Using this procedure, only one side of the tissue was exposed to Mn^{2+} at a time and the transmembrane flux of Mn^{2+} in each direction was similar to that of Ca^{2+} [5]. Typically, the tissue was incubated in the Ussing chamber for 1.5 h at 37 °C, and the solutions were bubbled with 100 % O_2.

After the incubation, the tissue was cut from the chamber, washed twice in

buffer solution for 1 min, and then washed for 5 min in buffer solution containing 1 mM Ca-EDTA (ethylenediaminetetraacetic acid). The EDTA binds external Mn^{2+} and broadens its EPR spectrum so that effectively only the Mn^{2+} inside the chorioallantoic membrane is seen in the EPR spectrum [1]. The tissue was then placed in a quartz tissue sample holder, mounted in the sample cavity of a Varian E-12 EPR Spectrometer (Varian Associates, Palo Alto, Calif.), and examined at X-band frequency. About 15 min elapsed between the removal of the membrane from the Ussing chamber and the recording of the EPR spectrum.

Total Mn^{2+} content of each sample was determined by atomic absorption spectroscopy after the EPR spectrum was recorded. The tissue was placed in a platinum crucible, ashed overnight at 600 °C, and the Mn^{2+} recovered with 2 ml of hot 3 M HCl. This was brought to 10 ml with distilled water so that the final Mn^{2+} sample was in 0.6 M HCl. An Instrumentation Laboratory Model 153 Atomic Absorption Spectrophotometer (Lexington, Mass.) was used for analyzing samples, and solutions of 0.05-0.20 μg/ml Mn^{2+} in 0.6 M HCl were used as standards.

The studies of Mn^{2+} uptake in normal 2,4-dinitrophenol and *p*Cl-HgBzs-treated chorioallantoic membranes as a function of pH utilized the Dubnoff shaker uptake procedure described in the previous paper [5].

EPR quantitation

For quantitative estimation of the amount of Mn^{2+} represented by the EPR spectra, a TE 104 mode "dual" cavity system was used [7]. This allowed for correction of most of the errors introduced by variation in sample mounting. The amount of Mn^{2+} present is proportional to the area under the EPR absorption spectrum, and this was obtained by numerical double integration of the first-derivative spectrum of the sample to be quantitated. A Lorentzian line shape was assumed for the tails of the spectrum, which were difficult to integrate numerically. To obtain the absolute amount of Mn^{2+} present, the area of the sample was compared to the area resulting from the integration of a spectrum of a Mn^{2+} hexahydrate standard of known concentration run under similar conditions. The quantitation of EPR spectra and the possible sources of error have been discussed in greater detail elsewhere [8].

Solutions

The standard buffer solution was 140 mM Na^+, 5 mM K^+, 0.5 mM Mg^{2+}, 25 mM $Tris^+$, 170 mM Cl^- and 0.5 mM SO_4^{2-} adjusted to pH 7.4 at 37 °C. All salts were of reagent grade. For experiments run from pH 6.1 to 7.4, 25 mM PIPES (piperazine-*N*-*N'*-bis(2-ethanesulfonic acid)) was used as the buffer. In the range of pH 5.0–6.1, 5 mM sodium cacodylate was used. Control studies of Mn^{2+} uptake in different buffer solutions at the same pH showed no effect of these buffers on uptake. In addition, little interaction between these buffers and Mn^{2+} was observed. Interaction could be detected as a decrease in the amplitude of the Mn^{2+} hexahydrate EPR spectrum when the buffer was added to a solution of Mn^{2+} in distilled water.

RESULTS

EPR spectrum of control membranes

When the chorioallantoic membrane was exposed to Mn^{2+} on the ectoderm and Ca^{2+} on the endoderm for 1.5 h in the Ussing chamber at pH 7.4, the Mn^{2+} in

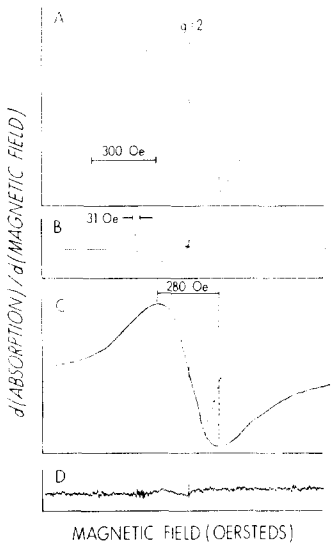

Fig. 1. Typical EPR spectrum of Mn^{2+} transported into the chorioallantoic membrane. (A) EPR spectrum of control chorioallantoic membrane exposed for 1.5 h to 0.75 mM Mn^{2+} at pH 7.4. (B) Spectrum of Mn^{2+} hexahydrate in 20 % glycerol/water mixture simulating hexahydrate component of A. (C) Mn^{2+} spin-exchange spectrum obtained when Mn^{2+} hexahydrate spectrum (B) is subtracted from total spectrum (A). (D) Background spectrum of standard buffer solution in tissue sample holder taken at 2.5 times the gain of spectrum A. All spectra are first-derivative spectra taken at X-band frequency. The peak to trough distances indicated are the linewidths of the absorption lines.

the tissue gave the first-derivative EPR spectrum seen in Fig. 1A. This spectrum is composed of two separate spectra, one spectrum of six narrow absorption lines superimposed on another spectrum consisting of a single broad line. These two spectra correspond to two different forms of Mn^{2+}, Mn^{2+} hexahydrate and an Mn^{2+} "spin-exchange" form respectively.

The Mn^{2+} hexahydrate spectrum, the six narrow absorption lines of Fig. 1A, is very similar to the spectrum of Mn^{2+} in distilled water. Mn^{2+} in water shows six narrow hyperfine lines which have widths of 24–26 Oe at room temperature [9]. Linewidth is defined as the peak to trough distance along the magnetic field axis of an absorption line. The Mn^{2+} hexahydrate spectrum in the tissue has linewidths of 28–34 Oe, indicating a decrease in the relaxation time of the complex. An Mn^{2+} hexahydrate spectrum with these linewidths can be simulated with Mn^{2+} in a 20 % glycerol/water mixture, and this spectrum is shown in Fig. 1B.

When the Mn^{2+} hexahydrate spectrum of Fig. 1B is subtracted from the total spectrum (Fig. 1A), the spectrum of Fig. 1C remains. The broad absorption line of this Mn^{2+} spin-exchange spectrum has a linewidth of about 280 Oe. This linewidth is much narrower than the width of the envelope of the six hyperfine lines, which is about 475 Oe [10]. This is an indication of a kind of averaging of the hyperfine lines. The averaging is produced by the exchange of electrons or "spins" between neighboring ions and is therefore called "spin-exchange". For this to take place, the ions must be closely packed, and the actual ionic spacing of the Mn^{2+} may be estimat-

ed from the linewidth. Comparison of this line width of 280 Oe with the line width of Mn^{2+} compounds of known ionic spacing [1] gives an apparent spacing of 4.7 ± 0.5 Å for the spin-exchange Mn^{2+} in the chorioallantoic membrane under these conditions. Fig. 1D shows a background spectrum of buffer in the tissue sample holder. A broad Mn-EDTA spectrum, which arises when EDTA complexes the Mn^{2+} external to the tissue, is also present in the total spectrum, but its contribution can be ignored in a first approximation.

Quantitation of EPR spectrum

The Mn^{2+} hexahydrate and spin-exchange spectra of many membranes could be quantitated separately to determine the amount of Mn^{2+} represented by each component of the spectrum. The Mn^{2+} hexahydrate was quantitated by comparison of its intensity with the intensity of a Mn^{2+} hexahydrate spectrum of a standard solution. The spin-exchange spectrum was quantitated by integration of the spectrum as described in the Methods section. The results of these quantitations were then compared to the total amount of Mn^{2+} in the same membranes as measured by atomic absorption spectroscopy. Membranes which were used in this quantitation analysis included normal control membranes, membranes treated with oligomycin and membranes incubated at pH 6.6. Membranes incubated at pH 5.0 could not be quantitated accurately because of the broadness of the spin-exchange spectrum.

Fig. 2 compares the Mn^{2+} content obtained from quantitation of the spin-exchange fraction only with the total amount of Mn^{2+} in the tissue of individual chorioallantoic membranes. A least squares straight line through these points has a slope of 0.89, indicating that on the average 89 % of the Mn^{2+} in the tissue is visible in the spin-exchange spectrum. A quantitation of the Mn^{2+} hexahydrate component of these same membranes indicated that about 3 % of the total Mn^{2+} in the tissue was

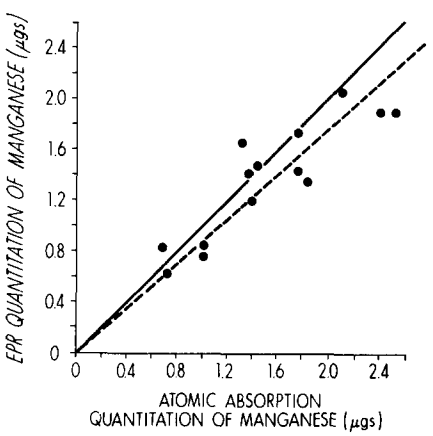

Fig. 2. Comparison of results of manganese quantitation by atomic absorption spectroscopy and EPR quantitation of the spin-exchange component alone. Membranes used were control membranes at pH 7.4 and 6.6 and membranes inhibited with oligomycin. The solid line represents one-to-one correspondence. The dashed line represents a least squares fit to the data and shows a slope of 0.89, 11 % less than the one-to-one correspondence line. The hyperfine sextet fraction was consistently found to be near 3 % of the total of the data that could be quantitated by EPR. This means that approximately 92 % of the manganese in the tissue can be accounted for by EPR.

in this fraction. The percentage of Mn^{2+} in each component was relatively constant in all the membranes quantitated, including those incubated with oligomycin and at pH 6.6. Thus, a total of 92 % of the total Mn^{2+} in the tissue was visible in either the spin-exchange or the hexahydrate spectrum. The remaining 8 % of the Mn^{2+} not accounted for may be Mn^{2+} in some form which gives a very broad spectrum that is not visible under these experimental conditions, or it may simply be the result of the uncertainty in quantitation of the EPR spectrum.

Mn^{2+} can be oxidized to Mn^{3+} or Mn^{4+} under the proper conditions, such as high O_2 concentration, high pH and high temperature. Normally, at pH 7 or below, Mn^{2+} can be kept in aqueous solution at room temperature for days with negligible loss due to oxidation. While Mn^{3+} and Mn^{4+} are paramagnetic, they would be expected to show rapid relaxation and hence be "invisible" to the EPR study. Thus the stability of the observed spectra with time may be taken as an indication that oxidation is not a problem in this system.

EPR studies of Mn^{2+} uptake at pH 7.4

Since the spin-exchange fraction accounted for almost all of the Mn^{2+} in the tissue, this fraction was investigated further. Table I shows the Mn^{2+} uptake by the tissue and the linewidth of the spin-exchange spectrum under different experimental conditions at pH 7.4. The uptake was measured by atomic absorption spectroscopy, and the linewidths were obtained by subtracting the Mn^{2+} hexahydrate component from the total EPR spectrum.

The first four lines of Table I show experiments with uninhibited membranes under different experimental conditions. The normal control chorioallantoic membranes were incubated for 1.5 h with 0.75 mM Mn^{2+} and had an average spin-exchange line width of 282 Oe. A typical EPR spectrum obtained under these conditions is shown in Fig. 3A. When the incubation time was reduced to 5 min, considerably less Mn^{2+} was taken up, but the linewidth was about the same as the 1.5 h control. Thus, the spin-exchange spectrum is associated with the initial movement of

TABLE I

MANGANESE UPTAKE AT pH 7.4

Average manganese uptake as measured by atomic absorption and spin exchange linewidth for both control and inhibited membranes. The results are expressed as the mean ±S. E. M. Uptake or linewidth values which are statistically different from the control are indicated by an asterisk.

Experiment	Mn^{2+} uptake (nmol/cm^2)	Spin-exchange linewidth (Oe)	Number of experiments
Control membrane	11.2±1.0	282±4	8
Control (5 min)	2.3±0.3*	289±2	4
Control (0.20 mM)	7.2±0.6*	297±4	4
Backflux membrane	4.2±0.5*	359±25*	5
Oligomycin (10 μg/ml)	5.4±0.5*	362±6*	6
2,4-dinitrophenol (0.2 mM)	8.4±1.0	305±10	4
10-day membrane	6.0±0.6*	278±10	5
Stripped membrane	7.5±0.6*	300±4*	4
*p*Cl-HgBzs (1 mM)	9.0±1.1	321±7*	5

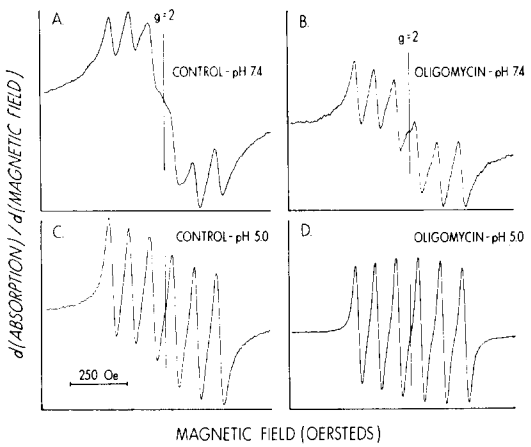

Fig. 3. EPR spectra of chorioallantoic membranes exposed to Mn^{2+} under various experimental conditions. A and B show spectra of control and oligomycin-treated membranes at pH 7.4 and C and D show spectra under the same conditions at pH 5.0. All membranes were exposed to 0.75 mM Mn^{2+} for 1.5 h. The relative gain of each spectrum was 3.1, 7.0, 2.9 and 1.0 respectively.

Mn^{2+} into the tissue. A spectrum of similar linewidth was also present when the membrane was exposed to 0.20 mM Mn^{2+} instead of 0.75 mM. Since less Mn^{2+} was taken up, this experiment shows that the linewidth does not depend directly on the amount of Mn^{2+} in the tissue. In the backflux experiment, where 0.75 mM Mn^{2+} was placed on the endodermal side of the chorioallantoic membrane for 1.5 h, there was significantly less Mn^{2+} in the tissue compared to the forward flux control. There was also a large increase in the spin-exchange linewidth from that of the forward flux control, and this represents an increase in the apparent interatomic spacing between Mn^{2+}.

The middle two lines of Table I show experiments with the metabolic inhibitors oligomycin and 2,4-dinitrophenol, which have been shown to inhibit divalent cation transport [5]. Oligomycin decreased Mn^{2+} uptake, and there was a large increase in linewidth from the control. Fig. 3B is a typical EPR spectrum of a membrane exposed to Mn^{2+} in the presence of oligomycin. With 2,4-dinitrophenol there was little shift in spin-exchange linewidth at this pH. 2,4-dinitrophenol also did not cause a large reduction in uptake in these experiments.

The last three experiments of Table I also involved chorioallantoic membranes which were not engaged in active transport. Membranes taken from 10-day-old eggs, membranes that have been stripped, which is a mechanical separation of the inner shell membrane from the chorioallantoic membrane, and membranes treated with the sulfhydryl-binding reagent *p*Cl-HgBzs have been shown to transport much less Ca^{2+} than normal membranes [11]. The EPR spectra of these membranes after incubation with Mn^{2+} all showed spin-exchange. The 10-day and stripped membranes showed little change in line width from the control while the *p*Cl-HgBzs-treated membranes were somewhat broader.

The uptake measurements in these experiments indicate that large amounts of Mn^{2+} may enter the tissue even though active transport of divalent cations has been blocked. In this respect the effects of *p*Cl-HgBzs on membrane permeability have been

extensively studied. Low concentrations of *p*Cl-HgBzs blocks active transport, but concentrations as high as 1 mM make tissues very permeable to divalent cations in addition to blocking transport [11]. Large amounts of Mn^{2+} are retained by the membrane under these conditions, and this binding is not inhibited by 2,4-dinitrophenol.

pH studies of Mn^{2+} uptake

One possible explanation for the spin-exchange spectrum seen with the 10-day, stripped and *p*Cl-HgBzs-treated membranes is that the Mn^{2+} which diffuses into the tissue under these conditions complexes with some anion such as phosphate. Calculations indicate that there is enough inorganic phosphate to precipitate with all the Mn^{2+} taken up by the tissue. Such Mn^{2+} precipitation may give spin-exchange linewidths similar to those observed.

If Mn^{2+} were precipitating with phosphate in the form of HPO_4^{2-}, the formation of this complex would be inhibited at low pH where most of the inorganic phosphate would be in the form of $H_2PO_4^-$. Since other experiments showed that the chorioallantoic membrane would actively transport Ca^{2+} down to pH 5.0, it was decided that Mn^{2+} uptake in this pH range should be studied in Dubnoff shaker experiments. Fig. 4 shows Mn^{2+} uptake as measured by radioactive tracer from pH 7.4 to pH 5.0. Mn^{2+} uptake by the control membranes (○---○) showed only a small decrease over this pH range, and Mn^{2+} uptake in the presence of 2,4-dinitrophenol (□- - -□) also decreased slightly at lower pH. At pH 5.0, over half the Mn^{2+} uptake was still dependent on metabolic energy since it was inhibited by 2,4-dinitrophenol. In contrast to the control and 2,4-dinitrophenol-treated membranes, membranes

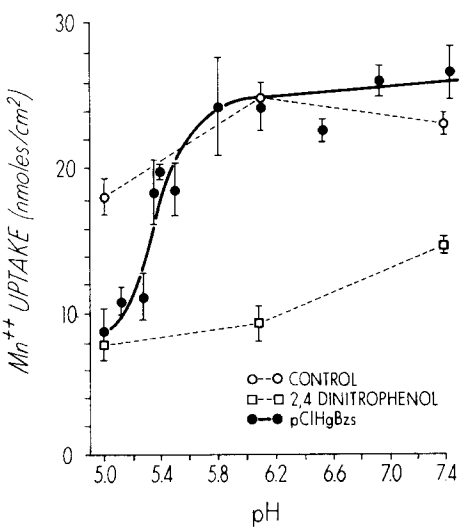

Fig. 4. Uptake of Mn^{2+} as a function of pH in Dubnoff Metabolic Shaker experiments. Membranes were placed in beakers containing radioactively labeled 0.75 mM Mn^{2+} buffer solution, shaken for 1.5 h at 37 °C, and then counted to measure uptake. Points represent the mean ±S.E.M. of four experiments for the *p*Cl-HgBzs curve and 10–18 experiments for the control and 2,4-dinitrophenol curves. The pH was measured at 37 °C at the end of the experiment.

exposed to *p*Cl-HgBzs showed a definite pH dependence (●–●). The concentration of *p*Cl-HgBzs used in these experiments made the tissue permeable so that all of the Mn^{2+} in the tissue presumably entered passively. From pH 7.4 to 6.0 membranes treated with *p*Cl-HgBzs retained as much Mn^{2+} as control membranes, but between pH 6.0 and pH 5.0 there was a sharp drop in tissue uptake to the levels seen with 2,4-dinitrophenol. This is consistent with a decrease in manganese phosphate formation in the tissue.

EPR studies of Mn^{2+} uptake at low pH

Since the chorioallantoic membrane accumulated normal amounts of Mn^{2+} down to pH 5.0, the EPR spectrum was examined at decreasing pH. The upper half of Table II shows Mn^{2+} uptake, which was measured by atomic absorption spectroscopy, and the spin-exchange linewidth as a function of pH. While the uptake was relatively unchanged, there was a progressive broadening of the spin-exchange linewidth from 282 to 394 Oe with decreasing pH. This linewidth shift can be seen by comparing Fig. 3C, the EPR spectrum of the control membrane at pH 5.0, with Fig. 3A, which is the control at pH 7.4. The broader absorption lines seen at pH 5.0 are more difficult to identify as arising from spin-exchange than the narrower lines at pH 7.4. In this case, however, the spectrum seen at pH 5.0 appears to be the result of a progressive broadening of the narrow spin-exchange seen at pH 7.4 and is, therefore, a true spin-exchange spectrum.

The lower half of Table II shows EPR studies of Mn^{2+} uptake in the presence and absence of transport inhibitors at pH 5.0, where manganese phosphate precipitation should no longer be a problem. The control tissues showed good Mn^{2+} uptake, and their spectra had an apparent linewidth of about 394 Oe. When the tissues were incubated with oligomycin or 2,4-dinitrophenol at pH 5.0, Mn^{2+} uptake was reduced. In these experiments oligomycin had less of an effect than 2,4-dinitrophenol. One reason for this may be that only 2 μg/ml of oligomycin were used, since it was more difficult to get into solution at this pH. However, both oligomycin and 2,4-dinitrophenol had a significant effect on the EPR spectrum. At this pH they completely abolished the broad Mn^{2+} spin-exchange spectrum seen in the control, leaving only

TABLE II

MANGANESE UPTAKE AT DECREASED pH

Average manganese uptake and spin-exchange linewidths are shown as a function of pH for control membranes and at pH 5.0 for inhibited membranes. The results are expressed as the mean ±S. E. M. Dashes indicate that no spin exchange was observed.

Experiment	pH	Mn^{2+} uptake (nmol/cm²)	Spin exchange (Oe)	Number of experiments
Control	7.4	11.2±1.0	282±4	8
Control	6.6	12.3±1.1	306±12	4
Control	6.1	10.2±0.5	362±8	4
Control	5.0	7.8±0.4	394±10	7
Oligomycin (2 μg/ml)	5.0	6.2±0.6	—	4
2,4-dinitrophenol (0.2 mM)	5.0	3.9±0.6	—	4
*p*Cl-HgBzs (1 mM)	5.0	2.2±0.5	—	4

an increased Mn^{2+} hexahydrate fraction. Fig. 3D shows the EPR spectrum of a membrane inhibited with oligomycin at pH 5.0. The oligomycin has removed the spin-exchange spectrum seen in the control at pH 5.0 (Fig. 3C). The spectrum of Fig. 3D also contrasts with the spectrum of tissue inhibited by oligomycin at pH 7.4 (Fig. 3B), which shows some spin-exchange. The last line of Table II shows that *p*Cl-HgBzs treatment at pH 5.0 greatly inhibited Mn^{2+} uptake and also abolished the spin-exchange spectrum. This is in sharp contrast to the results at pH 7.4, where little effect on uptake or on the EPR spectrum was seen (Table I).

DISCUSSION

The EPR spectra of chorioallantoic membranes which had accumulated Mn^{2+} indicated that 89 % of the Mn^{2+} was in a closely packed spin-exchange form with an ionic spacing of about 4.7 Å. The other 3 % had a spectrum very similar to that of Mn^{2+} in water. Since the spin-exchange spectrum accounts for the bulk of the Mn^{2+} in the tissue, this component is likely to have the most physiological significance. There are several lines of evidence indicating that, depending on the pH, most or all of the spin-exchange spectrum arises from Mn^{2+} in the Ca^{2+} transport system.

First, it is significant that the spin-exchange spectrum is present after only 5 min of exposure to Mn^{2+}. In the previous paper it was shown that Mn^{2+} and Ca^{2+} uptake during the first 2 min follow the same kinetics as seen after several hours of active transport [5]. This suggests that Mn^{2+} and Ca^{2+} rapidly enter the transport system after the tissue is exposed to them. Likewise, the spin-exchange spectrum arises during the first few minutes of Mn^{2+} uptake.

The experiments at pH 7.4 in which uptake was inhibited (Table I) also show that much of the spin-exchange spectrum is dependent on metabolic energy. However, there are indications that at this pH there may be two sources of closely packed Mn^{2+} that give rise to spin-exchange. One source is the Mn^{2+} in the Ca^{2+} active transport system of control membranes. This spectrum has an apparent linewidth of 282 Oe and is broadened in the backflux and oligomycin experiments. A second narrow spin-exchange form appears when energy-dependent uptake is inhibited, but at the same time membrane permeability is increased as in the case of the 10-day, stripped and *p*Cl-HgBzs-treated membranes. Precipitation of the Mn^{2+} which passively diffuses into the tissue could account for the spin-exchange spectrum seen under these conditions.

To minimize any spin-exchange due to precipitation, the pH was lowered so that the spin-exchange due to Mn^{2+} in the Ca^{2+} transport system could be studied more easily. The results of experiments at pH 5.0 (Table II) provide definite evidence that the broad spin-exchange spectrum seen in the control membranes at this pH is dependent on the presence of metabolic energy. Oligomycin, 2,4-dinitrophenol and *p*Cl-HgBzs all reduced Mn^{2+} uptake and abolished this spin-exchange spectrum, leaving only the Mn^{2+} hexahydrate spectrum. Since no residual spin-exchange was visible, it can be concluded that all the spin-exchange Mn^{2+} seen at pH 5.0 in the absence of inhibitors is Mn^{2+} in the divalent cation transport system. As can be seen from Fig. 4, the chorioallantoic membrane takes up Mn^{2+} at pH 5.0, and other experiments indicate that Ca^{2+} is transported at this pH. In addition, in experiments not shown, measurements were made of the potential difference across the tissue in an

Ussing chamber at pH 5.0 with Mn^{2+} present. These fell within the range of normal values measured at pH 7.4 [12].

The increase in spin-exchange linewidth seen with decreasing pH (Table II) gives some insight into the nature of the spin-exchange spectrum. A change in linewidth with pH is not the result which would be expected if the spin-exchange spectrum were due to just a manganese phosphate precipitate. Such an Mn^{2+} complex would be expected to have a constant linewidth, since the ionic spacing of Mn^{2+} would be constant. Its intensity would decrease with pH, but its linewidth would not be expected to change. The observed increase in linewidth could be due to the direct effect of increased hydrogen ion concentration on the spin-exchange component, such as competition between H^+ and spin-exchange Mn^{2+} for binding sites. On the other hand, it may be that the spin-exchange spectrum at pH 7.4 is composed of several spectra with different linewidths that superimpose to give one apparent spectrum. If one spin-exchange component, such as a manganese phosphate precipitate in the extracellular fluid, had a narrow linewidth and were pH sensitive, the linewidth of the total spectrum would appear to broaden with decreasing pH as the narrow linewidth spectrum of manganese phosphate diminished.

An 11 M concentration of free Mn^{2+} in solution would be necessary to produce a spin-exchange line width as narrow as that observed in the chorioallantoic membrane. Since this concentrations cannot be reached with the anions present in this membrane, spin-exchange Mn^{2+} must be in a bound form.

Some indirect evidence for the physiological importance of the spin-exchange spectrum is also given by electron probe X-ray microanalyzer studies. This technique shows Ca^{2+} [4] and Mn^{2+} [5] in high local concentrations in the ectoderm of the chorioallantoic membrane after exposure to these ions. It is interesting that both electron probe and EPR studies show that Mn^{2+} is concentrated in the tissue during uptake. It may be that the Mn^{2+} concentrations detected in the electron probe are the same concentrations responsible for the spin-exchange seen in the EPR spectra. The fact that the electron probe shows Mn^{2+} and Ca^{2+} in similar locations in the tissue is evidence that these Mn^{2+} concentrations are in the Ca^{2+} transport system.

In general, the EPR studies of the chorioallantoic membrane support the concept that a separate, specialized mechanism is responsible for divalent cation transport. It has been postulated that the chorioallantoic membrane transports Ca^{2+} by forming endocytotic vesicles that separate the Ca^{2+} from the rest of the cell cytoplasm [4]. The Ca^{2+}-filled vesicles are transported across the cell, and the Ca^{2+} is released on the other side by refusion of the vesicles with the cell membrane. The finding that almost all of the Mn^{2+} in the chorioallantoic membrane during energy-dependent uptake is closely packed and is not in the hexahydrate form supports the idea that the transport mechanism sequesters divalent cations in high concentrations.

At the molecular level it is not known what is complexing the Mn^{2+} so that the ions are near enough to each other to produce the characteristic spin-exchange spectrum. Manganese precipitation with anions can give spin-exchange linewidths similar to those observed in the control membranes. It may be that precipitation takes place inside the proposed transport vesicles as part of the transport mechanism. This would be different from the Mn^{2+} precipitation which may occur in the presence of *p*Cl-HgBzs and which is not related to divalent cation transport. On the other hand

it is possible that Mn^{2+} is bound to phospholipid or protein. Several proteins such as phosvitin and casein bind enough divalent cations to give spin-exchange (unpublished observations).

The EPR spectra of the chorioallantoic membrane may also be used to evaluate the role of mitochondria in Mn^{2+} uptake, since the EPR spectra of isolated mitochondria which have accumulated Mn^{2+} have been studied extensively [1–3]. Most of the ectodermal cells of the chorioallantoic membrane are not rich in mitochondria [13]. If the spin-exchange spectrum of the chorioallantoic membrane were due to Mn^{2+} inside of mitochondria, then calculations indicate that the mitochondria would have to be massively loaded to account for all the Mn^{2+}. However, the EPR spectrum of Mn^{2+} in the chorioallantoic membrane is significantly different from the spectrum of Mn^{2+} in massively loaded mitochondria. The typical spin-exchange linewidth for massively loaded mitochondria is 240 Oe (incorrectly reported as 280 Oe in ref. 1), while the narrowest linewidth seen in the chorioallantoic membrane is 282 Oe. The bulk of the spin-exchange spectrum in the chorioallantoic membrane arises from Mn^{2+} in some form other than that found in massively loaded mitochondria. This indicates that mitochondria probably do not have a major role as carriers in divalent cation transport in the chorioallantoic membrane. This conclusion is supported by the electron probe X-ray microanalyzer studies. The high local concentrations of Ca^{2+} that are seen are found in areas that contain few mitochondria, and they do not appear to be calcium phosphate in massively loaded mitochondria [4]. In addition, oxygen consumption studies indicate that the stimulation of oxygen consumption observed during Ca^{2+} transport is not due to the quasi-uncoupling effect of Ca^{2+} on mitochondria [14].

In addition to giving information about transport on the whole tissue level, the EPR spectrum of Mn^{2+} in the chorioallantoic membrane may be useful in isolating transport vesicles or proteins. The characteristic spin-exchange spectrum might possibly be used to follow the divalent cation-binding components through each stage of tissue fractionation. Once they were isolated, the EPR spectrum of Mn^{2+} bound to these transport components could be compared to the spectrum of Mn^{2+} in the whole tissue. Transport components such as calcium-binding proteins have been isolated from other epithelial tissues such as intestine [15]. The EPR studies described here for the chorioallantoic membrane may also be useful in studying Ca^{2+} transport in these other epithelia.

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