Title

MANGANESE IN PHOTOSYNTHETIC OXYGEN EVOLUTION. I. ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE ENVIRONMENT OF Mn IN TRIS-WASHED CHLOROPLASTS

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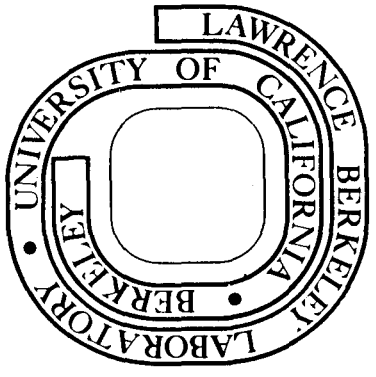

DISCLAIMER

MANGANESE IN PHOTOSYNTHETIC OXYGEN EVOLUTION. I. ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE ENVIRONMENT OF Mn IN TRIS-WASHED CHLOROPLASTS

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(Received)

Running Title: EPR Study of Mn in Chloroplasts

SUMMARY

A manganese EPR signal not present in untreated chloroplasts is observed in chloroplasts treated by washing in tris buffer (0.8 M, pH 8.0) for 15 min. Centrifugation indicates that the Mn responsible for the EPR signal is localized in the chloroplasts, not free in the supernatant liquid. Atomic absorption analysis demonstrates that less than 10% of total chloroplast Mn is lost upon tris treatment. Tris treatment converts 60% of the total chloroplast Mn pool to an EPR-detectable state.

Abbreviations: Chl, chlorophyll; tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol bis-(β aminoethylether)-N,N'-tetraacetic acid; G, gauss; ppm, parts per million (μg/g); SHN, 0.4 M sucrose, 0.05 M HEPES, pH 7.6, 0.01 M NaCl.

Sonication causes the Mn EPR signal to be divided proportionately between the chloroplast pellet and the supernatant liquid.

The EPR spectrum of Mn in tris-washed chloroplasts is identical with that of a divalent aqueous Mn spectrum in g factor, hyperfine splitting, and linewidth temperature dependence. It is concluded that upon tris treatment, Mn is released into the interior space of the thylakoid membrane.

Transport of Mn and anionic chelating agents across the thylakoid membrane was investigated using EPR. The rate of Mn diffusion through the thylakoid membrane is slow, with a $t_{1/2}$ of 2.5 h. The rate of transfer of chelating agents such as EDTA is much faster, with $t_{1/2}$ of 750 msec.

Tris-washing also destroys a weak Mn binding site on the exterior of the thylakoid membrane. It is suggested that perhaps the same mechanism is responsible for the change in environment upon tris treatment of the Mn involved in oxygen evolution.

INTRODUCTION

Manganese has long been associated with the oxygen evolving apparatus of photosynthesis[1]. Pirson[2], Kessler[3], Eyster et al.[4] and Spencer and Possingham[5] have reported that Mn deficient algae and spinach have a lowered rate of oxygen production. The current concept of two photosystems acting in series was first suggested in 1960 by Hill and Bendall[6], and soon gained wide acceptance. The work of Cheniae and Martin[7] assigned Mn a role in photosystem II, since Mn deficiency caused inhibition of photosystem II reactions while not affecting photosystem I reaction rates. Anderson et al.[8] found that 80% of the chloroplast Mn was found in a

system II enriched particle prepared by digitonin fractionation of chloroplasts.

Recent studies of Yamashita and Butler[9-11] on the site of action of Mn in the photosynthetic electron transport chain locate it on the oxidizing side of photosystem II. Their work relied on the use of various inhibitory treatments which mimic the effects of Mn deficiency. Some of these treatments involve mild heat[9], tris washing[10,11] and chaotropic agents[12]. DCMU-sensitive electron transport can be restored by adding various electron donors, showing that the photochemical apparatus is not destroyed by the treatments[10-12].

There has been a considerable controversy in the literature as to how much Mn these inhibitory procedures release. Tris treatment has been studied the most extensively. Homann[13] reported that tris treatment causes loss of 70% of tobacco chloroplast Mn. Cheniae and Martin[14] found that loss of Hill reaction activity was correlated with loss of 2/3 of the total spinach chloroplast Mn pool. Itoh et al.[15] found that tris treatment caused loss of 30% of the total Mn pool. Similar results were reported by Selman et al.[16] No explanation has been advanced to account for the large discrepancy in these results.

Yamashita et al.[17,18] reported a procedure for reactivating oxygen evolution in tris-treated chloroplasts. They measured Mn contents of untreated, tris-washed, and reactivated preparations and found that only about 10% of the total Mn pool is lost upon tris treatment, but the Mn content is lowered to about half its original value upon reactivation of oxygen evolution by rewashing in the presence of reducing agents.

Most models of O_2-evolving complexes advanced recently include Mn as an essential component[19-21]. In these schemes Mn is successively oxidized by the photoreaction, until four oxidizing equivalents are accumulated. Two water molecules then donate four electrons to the Mn complex, regenerating the initial state and producing O_2 and H^+. Mn is presumably included in these models because of its known association with photosynthetic oxygen evolution and its ability to assume a variety of oxidation states. This characteristic, coupled with the high redox potentials of many of the higher oxidation states of both free and complexed Mn, make it an attractive candidate for the catalyst in the oxygen-producing center.

Lozier, Baginski and Butler[12] showed that a Mn EPR signal appeared when chloroplasts are treated with tris or chaotropic agents. Their work prompted the experiments presented in this paper.

MATERIALS AND METHODS

Chloroplast preparation

Spinach was grown as described by Sun and Sauer[22]. Fifteen g of leaves were picked at about the middle of the light cycle, rinsed with cold, distilled water, and deveined. One hundred ml of SHN (0.4 M sucrose, 0.05 M HEPES, pH 7.6, 0.01 M NaCl) was added, and the leaves were homogenized for 15 sec in a Waring Blendor. The homogenate was strained through 8 layers of cheesecloth, divided into 4 tubes, and centrifuged at 300 x g for 1 min to remove cell debris. The supernatant liquid was centrifuged at 5000 x g for 2 min to pellet the chloroplasts. The pellet was resuspended in 15 ml SHN per tube and centrifuged again at 5000 x g for 2 min. This procedure removed loosely bound Mn present in the intact

leaves, as evidenced by the disappearance of a small Mn EPR signal observed in whole leaves and unwashed chloroplasts. Chloroplasts prepared by this method were 95% broken, as determined by observation in a light microscope. Total chlorophyll was determined by the method of MacKinney[23].

Tris-washed chloroplasts were prepared by incubating a tube of chloroplasts in 15 ml 0.8 M Sigma Trizma tris buffer, pH 8.0, for 15 min in ambient room light. The chloroplasts were then pelleted by centrifugation at 5000 x g for 2 min, followed by resuspension in 0.5 ml of the supernatant liquid. The pH of the tris buffer was measured at 0° with Corning pH electrode #476024 and Corning calomel reference electrode #47002. These electrodes are free from errors that affect measurement of pH of tris[24].

SHN-washed chloroplasts were prepared the same way as tris-washed chloroplasts, except that SHN was substituted for the tris solution.

Sonicated chloroplasts were prepared using a sonifier cell disrupter, Heat Systems-Ultrasonics, Plainview, N. Y., Model #W185. Fifteen ml of chloroplasts (Chl content, 0.5 mg/ml) suspended in tris or SHN were subjected to 20 sec bursts of sonic power, separated by 20 sec cooling periods, for a total of 5 min sonication time. A fluted metal tube was used to contain the chloroplasts during sonication and served to facilitate heat transfer away from the chloroplast sample and into the surrounding ice bath.

The sonicated chloroplast sample was centrifuged for 3 min at 5000 x g to remove large thylakoid fragments not broken by sonication. The supernatant liquid was then centrifuged at 50,000 x g for 20 min to separate membrane fragments from supernatant liquid. The supernatant liquid was pale green and contained very little chlorophyll. EPR spectra

were taken of the pellet and of the supernatant liquid resulting from the 50,000 x g centrifugation.

Oxygen measurements

O_2 evolution was measured with a Beckman #39065 Clark-type electrode polarized at -0.7 volts *vs.* Ag/AgCl, and thermostated at 15°. The system was calibrated by bubbling with N_2, air and O_2.

White light from a 200-watt General Electric EJL projection lamp was passed through 9 cm of water and two Corning 1-69 infrared absorbing filters, and was focused onto the reaction chamber with condensing lenses. The electrode surface was mounted parallel to the light beam. No light-induced electrode responses were observed in the absence of chloroplasts. The light intensity was 1.7×10^6 erg cm^{-2} sec^{-1} as measured with a Hewlett-Packard 8334A radiant flux detector, and was of saturating intensity.

The reaction mixture for O_2 evolution contained 50 mM HEPES, pH 7.6, 20 mM NaCl, 10 mM NH_4Cl, and 5 mM $MgCl_2$. A mixture of 1.0 mM potassium ferricyanide and 1.0 mM potassium ferrocyanide was used as electron acceptor. Chl concentration was 30-50 µg/ml.

EPR measurements

EPR spectra were obtained using a Varian E-3 spectrometer. The cavity was continuously flushed with dry nitrogen. Instrument settings are given in figure captions. The 16 G modulation amplitude used in most experiments was chosen as optimum for best signal-to-noise without unduly broadening the spectrum. All spectra except those described in Fig. 4 were recorded at room temperature. For the experiment described in Fig. 4, a Varian E4557 variable temperature accessory was employed. It was calibrated using an iron-constantan thermocouple.

Chloroplast samples (Chl content 2.5-5.0 mg Chl/ml) were put in 1.0-mm inside diameter quartz tubes and positioned in the cavity using drilled teflon rods as guides. This system allowed a reproducibility of sample positioning such that the amplitude of the signal from a 3.6×10^{-5} M $MnSO_4$ solution varied by no more than 2% from sample to sample. All spectra were recorded after a 5-min dark incubation in the microwave cavity.

The stopped-flow experiments shown in Fig. 5 were performed by modifying an Aminco-Bowman stopped-flow device to work in conjunction with a Scanco S-804 continuous-flow mixing EPR cell, J. F. Scanlon Co., Costa Mesa, California. A 1024 channel Northern Scientific NS544 Digital Memory Oscilloscope was employed to combine the results of 16 successive cycles of the stopped-flow apparatus.

Mn analysis

Mn analyses were performed with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer. One ml of chloroplast suspension, containing between 2.5-5.0 mg Chl/ml, was digested with 2 ml 85% HNO_3 and 15% $HClO_4$. The solution was filtered and made up to a volume of 5.0 ml with distilled water. Determinations made using either the method of additions or externally prepared standards gave equivalent results. Blanks were run on all buffers and incubation solutions, which were found to be free of Mn. Mn absorption was linear over the entire range of concentration employed. In samples where standards were used instead of the method of additions, the working curve passed directly through the origin.

Standard Mn solutions for atomic absorption calibration and also for addition to EPR samples were obtained by dilution in the appropriate buffers of a 1000 ppm (1.82×10^{-2} M) standard $MnSO_4$ solution supplied by Bio Rad Laboratories, Richmond, California. Dilute standards were mixed freshly daily.

RESULTS

Fig. 1 shows EPR spectra from chloroplasts washed with SHN and tris as described in Materials and Methods. The 6-line pattern observed in the tris-washed pellet is a typical signal from divalent, unbound Mn, resulting from the hyperfine interaction of the unpaired electrons with the 5/2 spin of the Mn^{55} nucleus[25]. The single line at g = 2.0 is the well known Signal II, associated with photosystem II[26]. The decreased magnitude of Signal II in the tris-washed sample results from an increased rate of decay of the radical responsible for signal II. This has been reported by Lozier and Butler[27].

The Mn signal is present only in the tris-washed pellet. The supernatant liquid has no observable signal. The SHN-washed sample has no signal in the supernatant liquid and only a very small one in the pellet. This latter signal most probably arises from denaturation effects during chloroplast isolation.

The fact that the Mn EPR signal sediments with the chloroplasts is surprising. It indicates that the Mn, although ostensibly free in solution on the basis of its symmetric EPR spectrum[25], is somehow associated with the chloroplasts themselves. The possibility of a weak binding site on the exterior of the chloroplasts sweeping the Mn down with the sedimenting chloroplasts was considered. This was ruled out when it was found

(experiments not shown) that Mn added during the tris washing showed no tendency to follow the chloroplasts upon centrifugation. Instead, the EPR signal was proportionately divided between pellet and supernatant liquid. It is evident that Mn has not been released into the suspending medium. Mn analysis supports this idea, as shown in Table I. Nearly all the Mn is retained in the tris-treated chloroplasts in the pellet, although their rate of O_2 evolution is low.

Fig. 2 shows the results of an experiment designed to measure the amount of Mn converted to an EPR-detectable state by tris treatment. A method similar to the method of additions used in atomic absorption was used to measure EPR-detectable Mn in tris-washed chloroplasts. The same samples were then analyzed for total Mn by atomic absorption. The horizontal-axis intercept in each case represents the amount of chloroplast Mn measured by each technique. The results indicate that 3.2 Mn/400 Chl was converted to an EPR-detectable state, while the total Mn content was 5.3 Mn/400 Chl. Tris treatment converted 60% of the chloroplast Mn to an EPR-detectable state. A possible ambiguity of this technique is that, unless the original Mn and the added Mn see the same set of weak binding sites, the amount of Mn^{+2} determined can be incorrect, because different fractions of the original Mn and added Mn will be removed from the solution by binding. The experiments presented in Fig. 7 argue against this interpretation, however. This possible error is considered in greater detail in the Discussion.

Two possibilities exist for Mn contained in the tris-washed chloroplasts to show an EPR spectrum such as that seen in Fig. 1. The first and simpler explanation is that the Mn has been released into an interior

space of the thylakoids upon tris treatment. In this view, Mn is completely uncomplexed and shows an EPR signal characteristic of the unbound ion. The second possibility is that the Mn is bound to the thylakoids in a very symmetric environment and, in an unprecedented fashion, shows a spectrum entirely unchanged from that of aqueous Mn. The following series of experiments is designed to answer the question of whether the Mn spectrum observed is truly that of unbound Mn.

Sonication of tris-washed chloroplasts

Tris-washed chloroplasts were sonicated to break the integrity of the thylakoid membranes. The results are shown in Fig. 3. The Mn giving rise to the EPR signal has been released into the supernatant liquid and now shows no preferential tendency to sediment with the chloroplasts. Control experiments using sonicated SHN-washed chloroplasts showed a small Mn EPR signal, but much less than the tris-washed chloroplasts. This observation is not surprising, as sonication is a rather drastic procedure which somewhat denatures the delicate oxygen evolving mechanism[28].

Linewidth temperature dependence

The temperature dependence of the peak-to-peak linewidth (ΔH_{pp}) of the g = 1.98 line of the Mn^{+2} EPR spectrum (fourth from the left in Mn spectra shown) gives an indication of the correlation time of the Mn ion, whether free or complexed[29-31]. To help in determining the environment of Mn in tris-washed chloroplasts, the temperature dependence of the EPR linewidth was studied over the range of 5-25°. The experiment was restricted to this limited temperature range because of the extreme sensitivity of chloroplasts to heat. The results are shown in Fig. 4. The chloroplast sample and an aqueous Mn control have virtually the same linewidth temperature dependence.

Transfer of Mn and anions across the photosynthetic membrane

The experiments presented so far strongly suggest that, upon tris treatment, Mn is initially released into an interior space of the thylakoids. Since the concentration of Mn outside the chloroplast is much lower than that inside, the Mn inside will tend to diffuse out. The rate of diffusion of Mn across the thylakoid membrane can be measured by observing the amount of Mn in the pellet versus the supernatant liquid as a function of time of incubation in tris. The kinetic behavior of this diffusion was investigated and the results are shown in Fig. 5. Chloroplasts were incubated in tris, and aliquots were taken at intervals. These aliquots were centrifuged as described in Materials and Methods, and EPR spectra were run on both the pellet and the supernatant liquid.

The signal of the pellet decreases with a half time of about 2.5 h. The signal in the supernatant liquid changes much less, owing to the tenfold larger volume compared to the pellet. The control chloroplasts, suspended in SHN instead of tris, show little Mn signal at all times.

It is somewhat more difficult to study the rate of transfer of anions across the photosynthetic membrane. An anion which complexes Mn^{+2} and destroys its EPR signal can be studied using the tris-treated chloroplast system, while an anion which has no effect on the EPR spectrum is impossible to follow using this method. A variety of Mn chelating and precipitation agents were surveyed for their ability to reduce or eliminate the Mn EPR signal, and six were chosen to study the accessibility of the Mn EPR signal in tris-washed chloroplasts. Table II shows the results of an experiment in which tris-washed chloroplasts were incubated with the various agents for 2 min, and then EPR spectra were taken. In all cases, except

for $K_4Fe(CN)_6$ addition, the added agents destroy or strongly decrease the Mn EPR signal in tris-washed chloroplasts. The behavior of $K_4Fe(CN)_6$ is not understood.

The time required for the apparent anion transport across the thylakoid membrane followed by chelation of Mn is less than 2 min. In order to study this reaction further, the stopped-flow apparatus was employed. This allowed a much finer time resolution than the manual mixing method used for Table II. The results of an experiment where EDTA and tris-washed chloroplasts were mixed and the Mn EPR signal was followed in time are shown in Fig. 6.

The chelation of free Mn^{+2} by EDTA in an aqueous control occurs within the 250-msec dead time of the measurement. The rate of chelation of Mn^{+2} observed in tris-washed chloroplasts is slower, with a $t_{1/2}$ of 750 msec. This most likely represents the time required for the EDTA to penetrate the thylakoid membranes. After diffusion through the membrane, the time required for chelation would be expected to be very short. An alternative explanation is considered in the Discussion. The curve in Fig. 6 exhibits first-order kinetic behavior.

Mn binding sites in untreated and tris-washed chloroplasts

The existence of a Mn binding site on chloroplasts has been demonstrated by Gross[32]. The results of an experiment designed to measure the strength and number of Mn binding sites in untreated and tris-washed chloroplasts are shown in fig. 7. The method used, first reported by Cohn and Townsend[33], utilizes the fact that Mn bound to proteins usually shows no EPR signal. Presenting the data in the form of a Hughes-Klotz[34] plot enables the measurement of the binding constant and the determination of the number of binding sites. The reciprocal of the horizontal-axis

intercept gives the dissociation constant, K_D, of the binding site. The reciprocal of the vertical-axis intercept gives the number of binding sites. Any deviation from a straight line indicates more than one class of binding sites[35].

The K_D of the binding sites for SHN-washed chloroplasts is calculated to be 1.2×10^{-4} M, and the number of binding sites is about 1 Mn binding site per 5.5 Chl. For tris-washed chloroplasts the total amount of binding is very low. The number of binding sites is reduced to one per 200 chlorophylls, while the K_D is essentially the same as for SHN-washed chloroplasts. No evidence of more than one class of binding sites is apparent with either chloroplast preparation.

DISCUSSION

The effect of tris washing on chloroplast Mn content and environment has been a controversial subject in the literature. The work of Yamashita and Butler localized the effects to the oxidizing side of photosystem II [9-11]. The experiments of Cheniae and Martin[14] indicated that Mn was removed from the chloroplasts upon tris treatment. However, Yamashita et al.[17] showed that reducing agents can prevent the loss of oxygen activity upon tris treatment and can also restore the O_2 evolution activity of chloroplasts which previously have been inhibited. Heat treatment inhibition of O_2 activity was not reversible by reducing agents. In a later paper, Yamashita et al.[18] showed that tris-treated chloroplasts actually showed very little loss of total Mn. The probable reasons for the discrepancy among various workers concerning amount of Mn lost by tris treatment will be the subject of a forthcoming paper.[36]

The results of Fig. 1, Table I, and Fig. 2, taken together, show that under our experimental conditions Mn is not lost from the chloroplasts upon tris treatment, but is merely altered in environment. Before tris treatment, no Mn^{+2} EPR signal is seen, and the rate of oxygen production is high. After tris treatment, an EPR signal characteristic of divalent Mn is observed and the rate of oxygen evolution is low. However, the total amount of chloroplast Mn is not decreased significantly. Furthermore, the results of Fig. 2 show that a significant fraction of the chloroplast Mn is converted to this EPR-detectable state.

The nature of the environment of the EPR-detectable Mn in tris-washed chloroplasts is a question requiring careful attention. The symmetric 6-line EPR spectrum is virtually diagnostic for hydrated Mn^{+2} free in solution[37,38]. Any chelation usually causes the spectrum to disappear entirely, or at least to become distorted[39]. In fact, the method used to determine Mn binding to chloroplasts assumes that any bound Mn has no observable EPR signal. Some spectra of bound Mn have been reported, but they are invariably distorted in some way[39-43]. The probable reasons for this behavior have been discussed by Reed and Ray[40], and by Reed et al.[39]

The question is then to determine how closely the spectrum observed in tris-washed chloroplasts resembles an aqueous Mn^{+2} spectrum. Any deviations from the aqueous signal could reflect the particular environment of the EPR-active Mn in tris-washed chloroplasts. The temperature dependence of the linewidth of the g = 1.98 line is an indication of the correlation time of the Mn^{+2} ion[29-31,44,45]. This analysis is possible only if there is no large static zero field splitting, which makes the

spectrum unobservable as in the case of the Mn-EDTA complex. The results of the experiments presented in Fig. 4 indicate that the Mn EPR signal in tris-washed chloroplasts shows a linewidth temperature dependence identical to that of an aqueous Mn control. The g value of the signal and the hyperfine splitting of the lines show no deviations from those of an aqueous Mn^{+2} signal.

The experiments presented indicate that the Mn EPR signal in tris-washed chloroplasts shows none of the distortion characteristic of bound Mn and indeed appears in every measurable way to be identical to a freely rotating, hexaquo divalent Mn ion.

Why then does the Mn EPR signal sediment as if it were bound into the chloroplast, as shown in Fig. 1? This is easily explained if one assumes that the Mn EPR signal seen in tris-washed chloroplasts is indeed that of free Mn, but Mn trapped in the interior space of the thylakoid.

This conclusion is strengthened by the sonication experiments presented in Fig. 3. If the Mn is indeed trapped in the interior of the thylakoid membranes, breaking these membranes open should release the Mn to the outside of the thylakoids where it will show no tendency to sediment with the chloroplasts. This is just the behavior seen.

All of the evidence presented is consistent with the interpretation stated earlier; namely, that tris treatment causes Mn to be released into the interior of the thylakoid membranes.

Gunter and Puskin[46] investigated an EPR spectrum of Mn accumulated inside mitochondria by the divalent ion pump active in such systems. They resolved the distorted signal observed into two main components: an aqueous Mn^{+2} signal with 52 G linewidth and a broad (300 G) single

line at g = 2.0, which they classified as an exchange-narrowed signal owing to the fact that it became narrower with increasing Mn concentration. Added EDTA had no effect on the Mn signal in their studies.

Assuming that the interpretation presented so far is correct, it is possible to study the kinetics of transfer of Mn out of the thylakoids, or kinetics of transfer of various anions into the thylakoid. These experiments are presented in Figs. 5 and 6. The results indicate that the rate of transfer of Mn^{+2} ions across the membrane of tris-washed thylakoids is slow, while the rate of transfer of anionic chelating agents is very much faster.

An alternative explanation for the behavior observed is that the various anions do not actually penetrate the membrane and complex the internal Mn, but cause a membrane conformational change which exposes a heretofore buried Mn binding site on the interior surface of the thylakoid. The wide variety of chemically unrelated species which cause the disappearance of the Mn EPR signal argues against this possibility, but it cannot be rigorously ruled out.

A schematic model which summarizes the data presented in this paper is shown in Fig. 8. Mn attached with two lines to the interior surface of the thylakoid membrane represents the 60% of the total chloroplast Mn which is EPR-active after tris treatment. Mn attached with four lines represents the more tightly bound 40% of the total Mn pool. This Mn is apparently unaffected by tris-washing. The depressions on the exterior surface of the thylakoid represent the weak Mn binding site measured in Fig. 7. The number of these sites per photosynthetic unit has been decreased for clarity in Fig. 8.

From the data presented in Fig. 7, it is apparent that tris-washing decreases the number of external Mn binding sites by about 35-fold, while their dissociation constant is essentially unchanged. This non-competitive (uncompetitive in Cleland's nomenclature[47]) inhibition is most easily explained by postulating a conformational change of the chloroplast membrane. The conformational change prevents the binding of the Mn ion[32].

Since the EPR signal of Mn^{+2} is very sensitive to its surroundings, one must observe appropriate precautions when comparing EPR signals from Mn in two different environments. In the experiments of Fig. 2 just such a situation exists. Mn ions are present inside the thylakoids due to the action of tris. Mn has also been added to the outside of the thylakoids. The presence of a weak Mn binding site on the outside surface which had no counterpart on the inside surface would cause an error in the measurement of the amount of EPR-detectable Mn present in the interior of the thylakoid.

Fig. 7 indicates that almost no Mn binds to the exterior of the tris-washed thylakoids. This means that the value measured for EPR-detectable Mn will not be too large.

The data of Figs. 1 and 2 indicate that tris treatment releases a major fraction of interior chloroplast Mn. It is possible that the same mechanism which is operative in changing the Mn binding site on the exterior of the thylakoid membrane also affects the internal Mn in the membrane responsible for oxygen evolution activity. The sites are very different in terms of strength of binding and number of sites per photosynthetic unit.

Recent experiments on proton evolution by Fowler[48] indicate that O_2 is produced in the interior of the thylakoid membrane.

No previous evidence exists as to the spatial distribution of Mn within the thylakoid. The results presented here indicate that Mn is released into an internal thylakoid space upon tris treatment. The simplest extrapolation from that finding is that before release the Mn resides on or near the interior surface of the membrane.

CONCLUSIONS

The results presented in this paper indicate that upon tris treatment 60% of the total amount of thylakoid Mn is released into the interior of the thylakoid but not immediately lost from the chloroplast. The procedure reported by Yamashita et al.[17,18] to reactivate oxygen activity in tris-washed chloroplasts apparently involves the return of the Mn to the configuration existing prior to the tris treatment. The assumption that Mn is essential for oxygen evolution (a statement that much indirect, but no direct, evidence supports) would suffer if Mn were irreversibly lost by tris treatment. If, however, Mn is still retained within the thylakoids after tris treatment, the reversibility of the procedure is easily visualized. This could involve another conformational change back to the original configuration. The Mn present inside the thylakoid could then rebind, and the original state would be restored.

Cheniae and Martin[14] reported two separate pools of Mn, one larger pool active in oxygen evolution and a smaller one not active with respect to oxygen evolution. The results presented here tend to confirm this result. Upon tris treatment, 60% of the total chloroplast Mn pool is

converted to an EPR-detectable state. The other 40% of the chloroplast Mn pool is apparently not similarly affected by tris-washing.

These experiments say nothing about the physical state of the chloro-p-at Mn prior to tris treatment. Unfortunately, no technique has been successfully used to study this Mn in its native state. No knowledge exists about the oxidation state of the Mn, or whether it changes upon illumination.

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Table I

TOTAL Mn CONTENT AND O_2 EVOLUTION RATE IN SHN-WASHED AND TRIS-WASHED CHLOROPLASTS.

Samples prepared as described in Materials and Methods, except 1 ml of supernatant was used to resuspend the chloroplasts. Total Mn measured by atomic absorption. O_2 evolution rates measured polarographically as described in Materials and Methods.

Experiment No.	Washing solution	Total Mn/400 Chl	O_2 rate $\frac{\mu moles\ O_2}{mg\ Chl\text{-}hr}$
1	SHN	5.53	156
	Tris	5.32	21
2	SHN	7.64	183
	Tris	6.54	53

Table II

LOSS OF Mn EPR SIGNAL UPON TREATMENT WITH CHELATING AND PRECIPITATION REAGENTS

Numbers refer to percent of original Mn^{+2} EPR signal remaining after addition. Concentrations of reagents refer to final concentrations. The first four reagents are chelating agents; the last two are precipitation reagents. The concentration of the inorganic $MnSO_4$ control (3.65×10^{-5} M) was chosen to give the same amplitude Mn EPR signal in the absence of additions as that of the tris-washed chloroplasts. Chlorophyll content 3.0 mg/ml.

Addition (final concentration)	% Mn EPR signal remaining	
	Tris-washed chloroplasts	3.65×10^{-5} M $MnSO_4$ in tris
1 mM EDTA	0	0
1 mM EGTA	0	0
5 mM 8-hydroxyquinoline	0	0
5 mM 8-hydroxyquinoline-5-sulfonic acid	0	0
1 mM $Na_4\ P_2O_7$	10	5.4
1 mM $K_4\ Fe^{II}(CN)_6$	91	11

FIGURE CAPTIONS

Fig. 1. Room temperature EPR spectra (1st derivative) of pellet and supernatant liquid from SHN-washed and tris-washed chloroplasts prepared as described in Materials and Methods. Instrumental conditions: microwave power 20 mW, modulation amplitude 16 G, time constant 0.3 sec, scan rate 250 G/min. Receiver gain was the same for all 4 spectra. Chlorophyll content 4.0 mg/ml in each of the pellets.

Fig. 2. Measurement of amount of EPR-detectable and total Mn in tris-washed chloroplasts by method of additions. The horizontal-axis intercept indicates the amount of chloroplast Mn measured by each technique. Points labeled o---o refer to total Mn as measured by atomic absorption. Points labeled •---• refer to EPR-detectable Mn. Chlorophyll content 2.88 mg/ml.

Fig. 3. Room temperature EPR spectra of sonicated tris-washed chloroplasts. Samples were prepared as described in Materials and Methods. Instrumental conditions: microwave power 80 mW, modulation amplitude 10 G, time constant 1.0 sec, scan rate 250 G/min.

Fig. 4. Linewidth (ΔH_{pp}) temperature dependence of the g = 1.98 EPR line of 3.6×10^{-5} M $MnSO_4$ in 0.8 M tris, pH 8.0 (o---o), and of tris-washed chloroplasts (Δ---Δ). To minimize denaturation effects, lower temperatures were recorded first. Instrumental conditions: Microwave power 100 mW, modulation amplitude 6.3 G. Chlorophyll content 2.80 mg/ml.

FIGURE CAPTIONS (Cont.)

Fig. 5. Decay kinetics of tris-induced Mn^{+2} EPR signal. Points represent: o---o, tris-washed pellet; ⊙---⊙, tris-washed supernatant liquid; Δ---Δ, SHN-washed pellet; ▲---▲, SHN-washed supernatant liquid. Large quantities of tris-washed and SHN-washed chloroplasts were prepared as described in Materials and Methods, but were not centrifuged. After different times, 10 ml aliquots were taken, centrifuged for 2 min at 5000 x g, and the pellet was resuspended in 0.5 ml supernatant liquid. EPR spectra were taken of resuspended chloroplast pellet and supernatant liquid.

Fig. 6. Kinetics of Mn^{+2} chelation by EDTA in tris-washed chloroplasts and in an aqueous solution of $MnSO_4$. The stopped-flow apparatus described in Materials and Methods was employed. Upper curve: tris-washed chloroplasts (2.2 mg Chl/ml) mixed with 10^{-4} M EDTA, 0.8 M tris, pH 8.0. Lower curve (control): 1.8×10^{-5} M $MnSO_4$, 0.8 M tris, pH 8.0, mixed with 10^{-4} M EDTA, 0.8 M tris, pH 8.0. The intensity of the Mn^{+2} EPR line at g = 2.12 (1st from left in Fig. 1 was monitored as a function of time. The baselines of the two curves have been artificially separated for clarity. Instrumental conditions: microwave power 100 mW, modulation amplitude 32 G, time constant 50 ms.

FIGURE CAPTIONS (Cont.)

Fig. 7. Measurement of external Mn^{+2} binding site in tris-washed and SHN-washed chloroplasts. Chlorophyll content 2.30 mg Chl/ml. A correction term was applied to each sample corresponding to the amount of Mn EPR signal present in the absence of any added Mn. This removes any contribution by interior Mn to the signal amplitude. Calibration curves were made for Mn^{+2} in both buffer systems; these were linear over the entire concentration range studied.

Fig. 8. Model for behavior of chloroplast Mn upon tris treatment. Mn attached with two lines represents loosely bound Mn released by tris-washing. Mn attached with four lines represents more tightly bound Mn not affected by tris-washing. Depressions in the exterior of the thylakoids represent weak Mn binding sites.

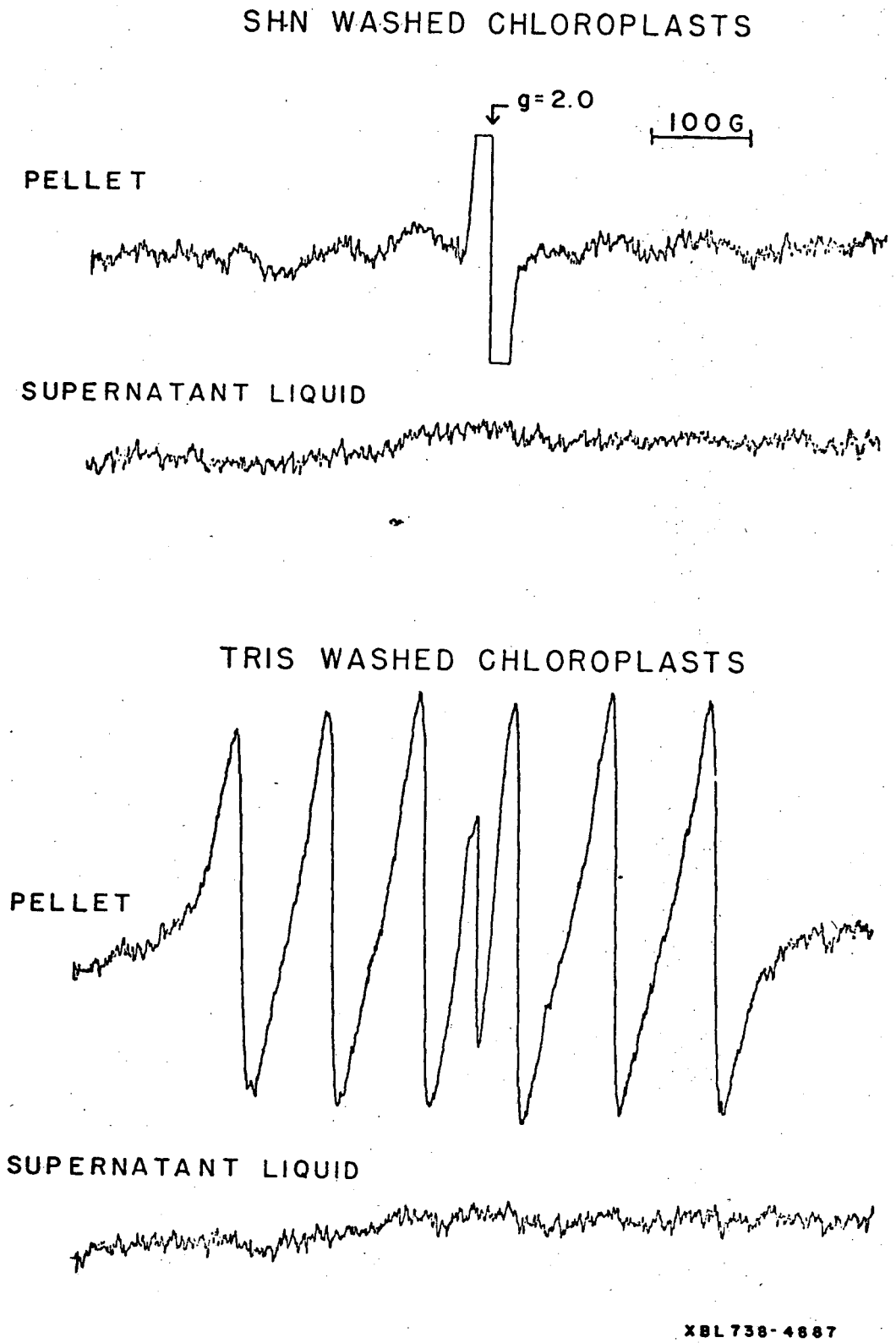
SHN WASHED CHLOROPLASTS
g=2.0
100G
PELLET
SUPERNATANT LIQUID
TRIS WASHED CHLOROPLASTS
PELLET
SUPERNATANT LIQUID
XBL738-4887

Fig. 1.

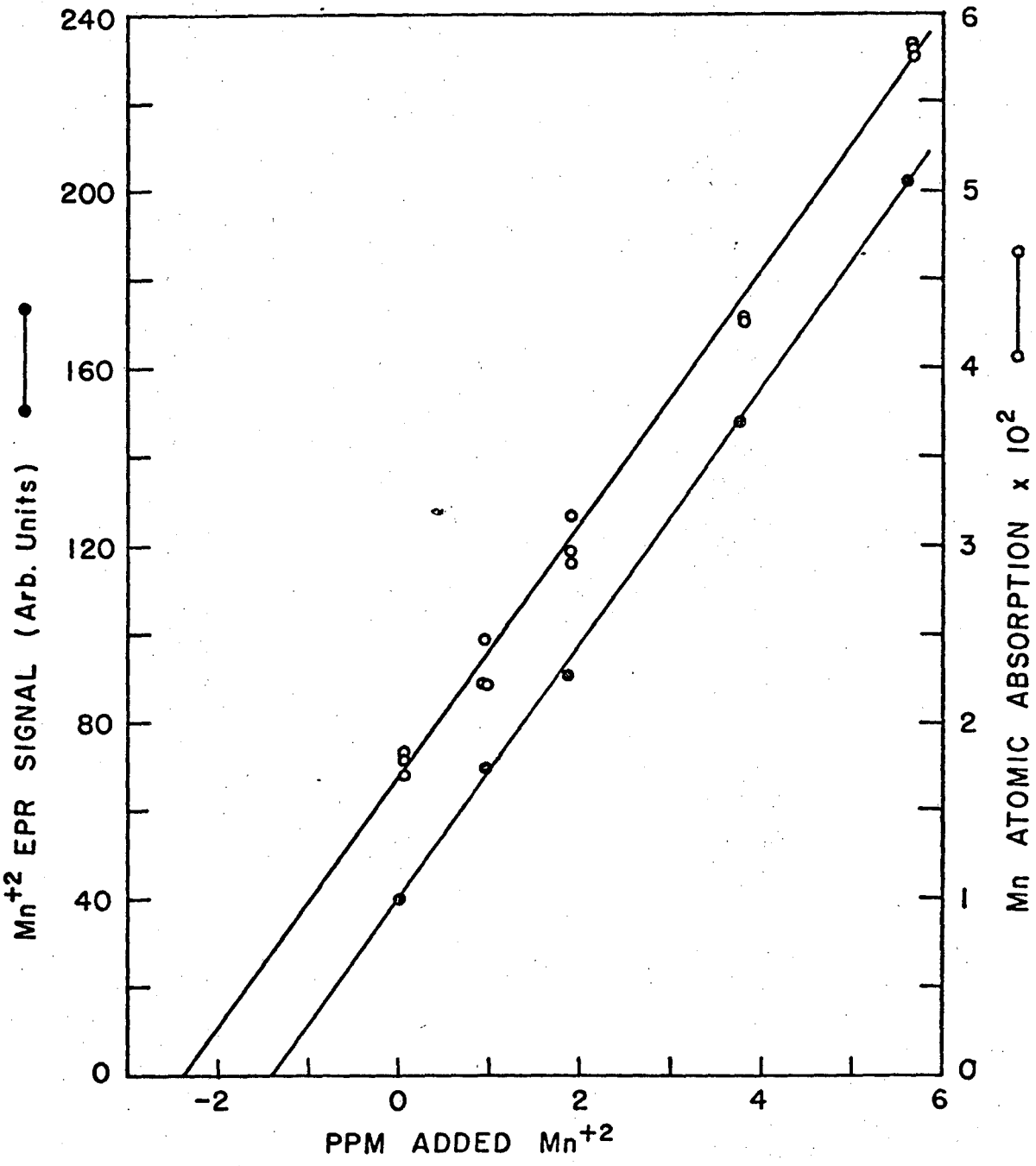
240
200
160
120
80
40
0
6
5
4
3
2
1
0
-2
0
2
4
6
Mn+2 EPR SIGNAL (Arb. Units)
Mn ATOMIC ABSORPTION x 10²
PPM ADDED Mn+2
XBL7311-4917

Fig. 2.

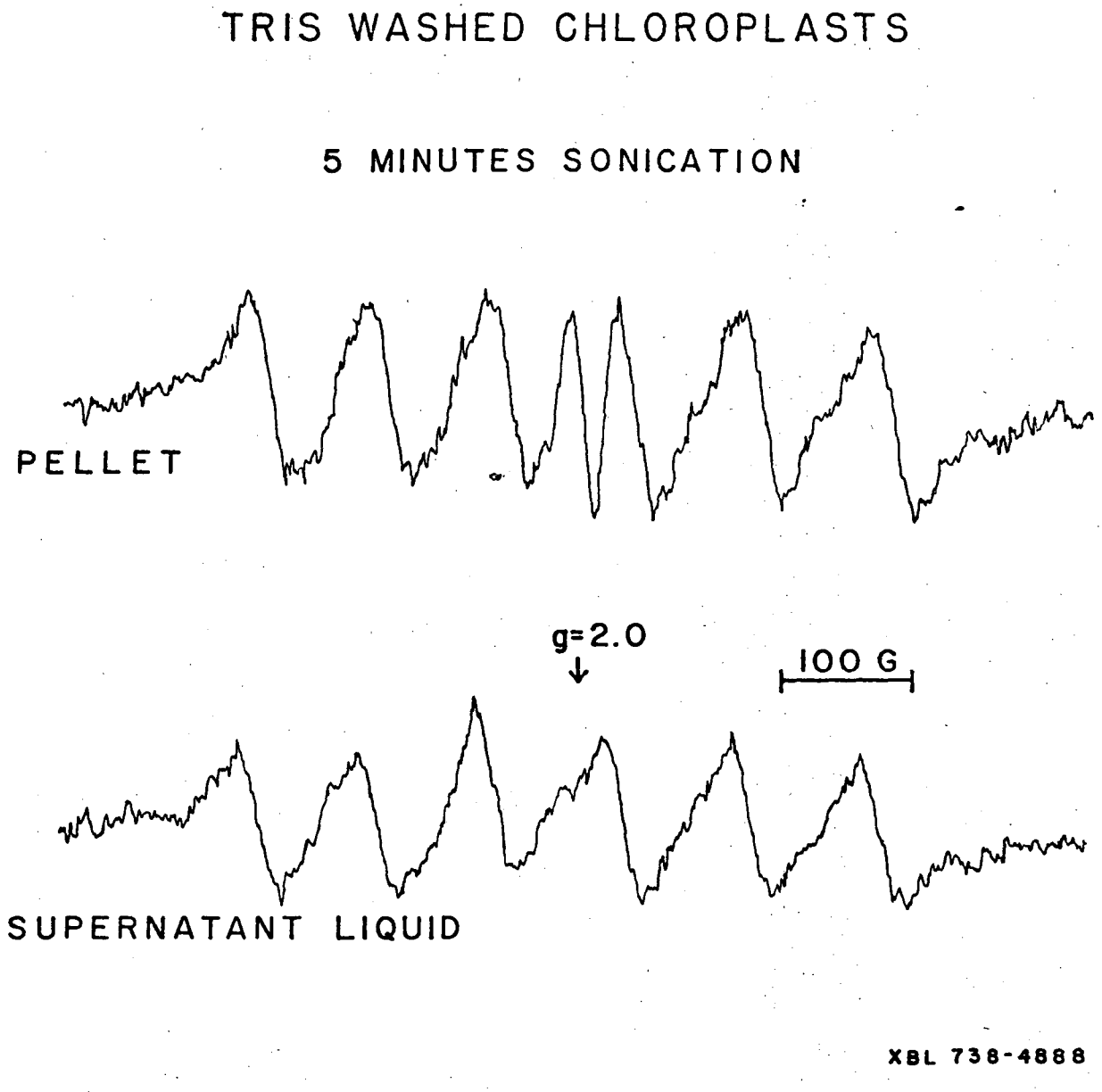
TRIS WASHED CHLOROPLASTS
5 MINUTES SONICATION
PELLET
g=2.0
100 G
SUPERNATANT LIQUID
XBL 738-4888

Fig. 3.

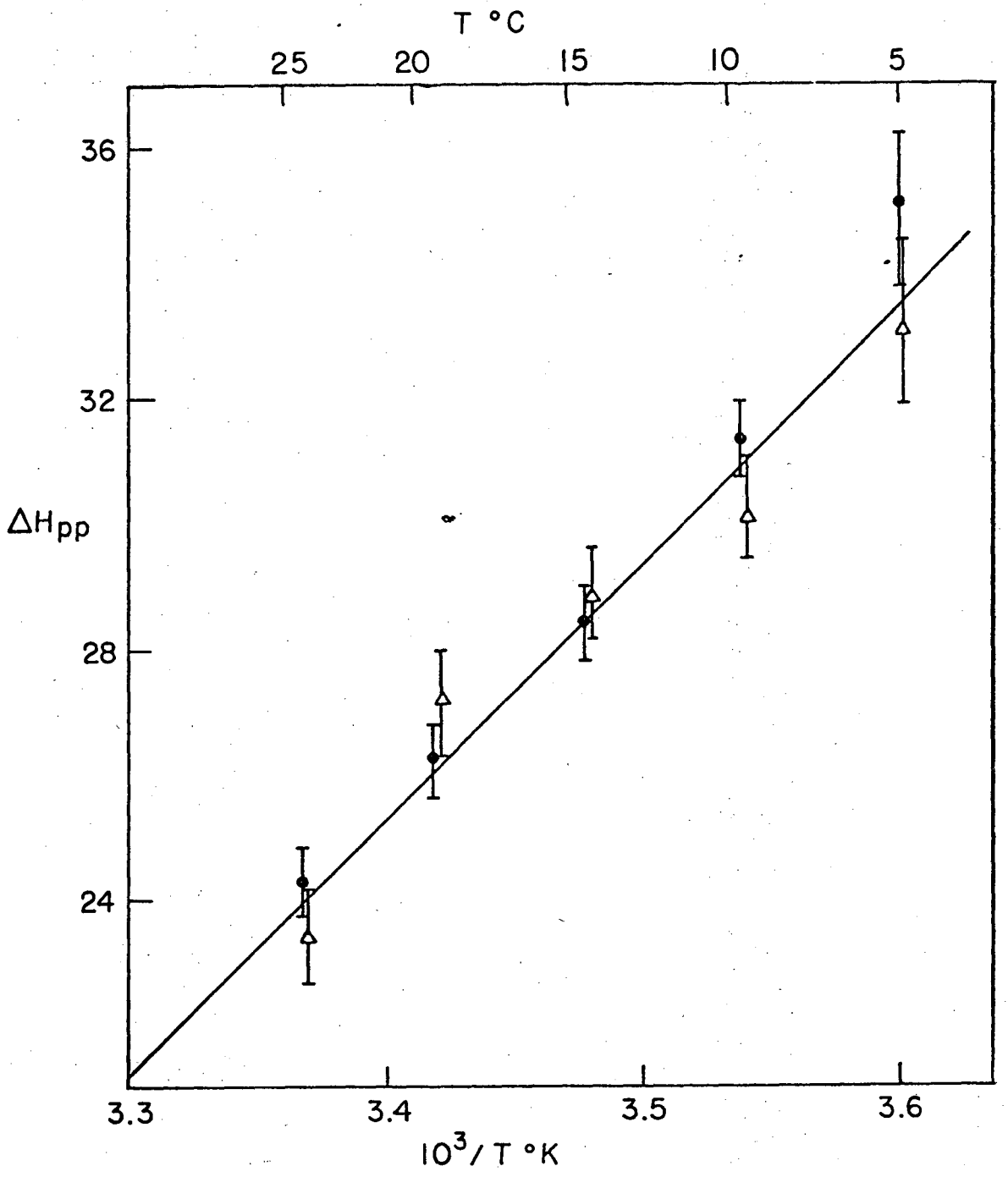

Fig. 4.

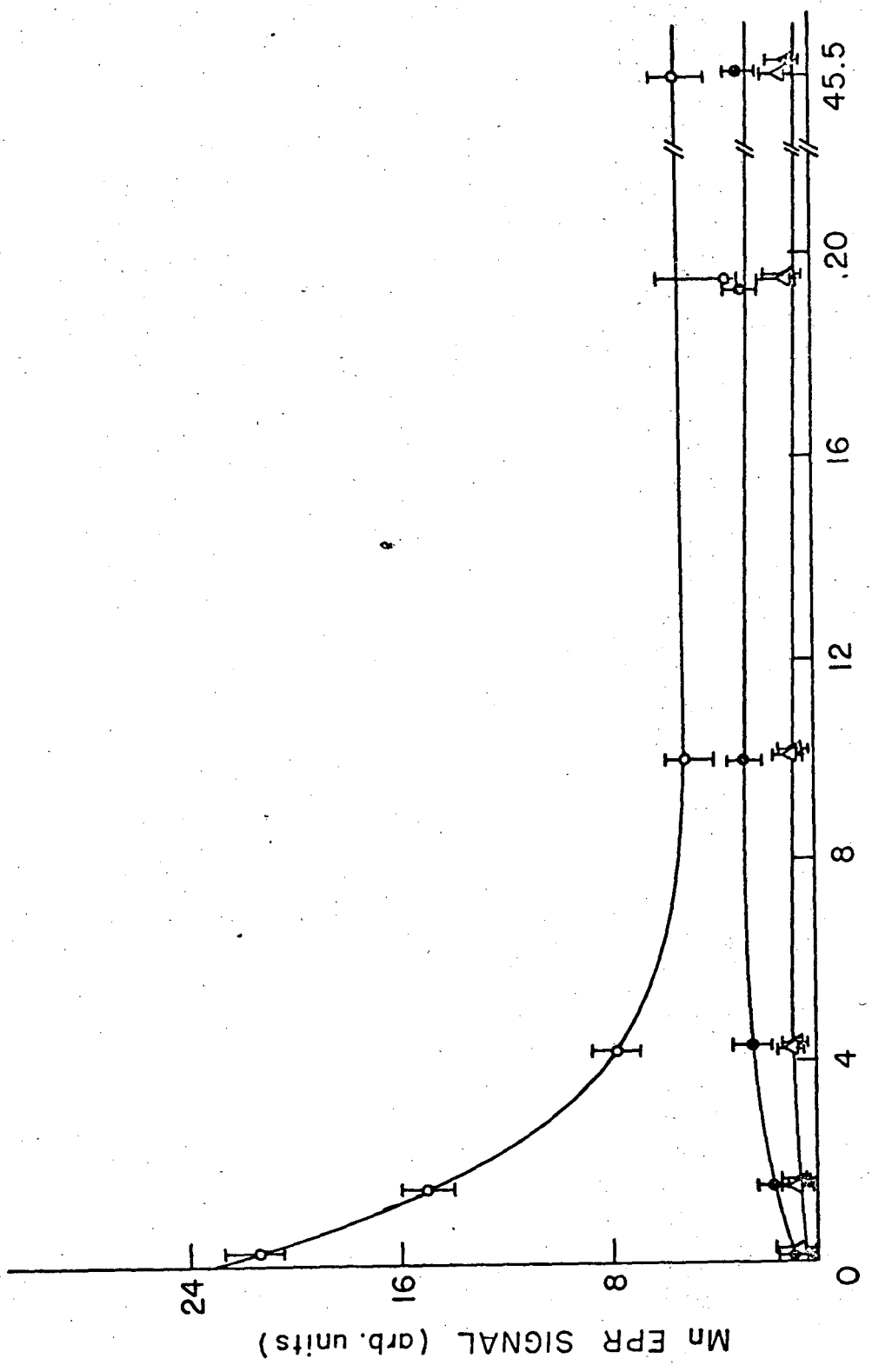

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Fig. 5.

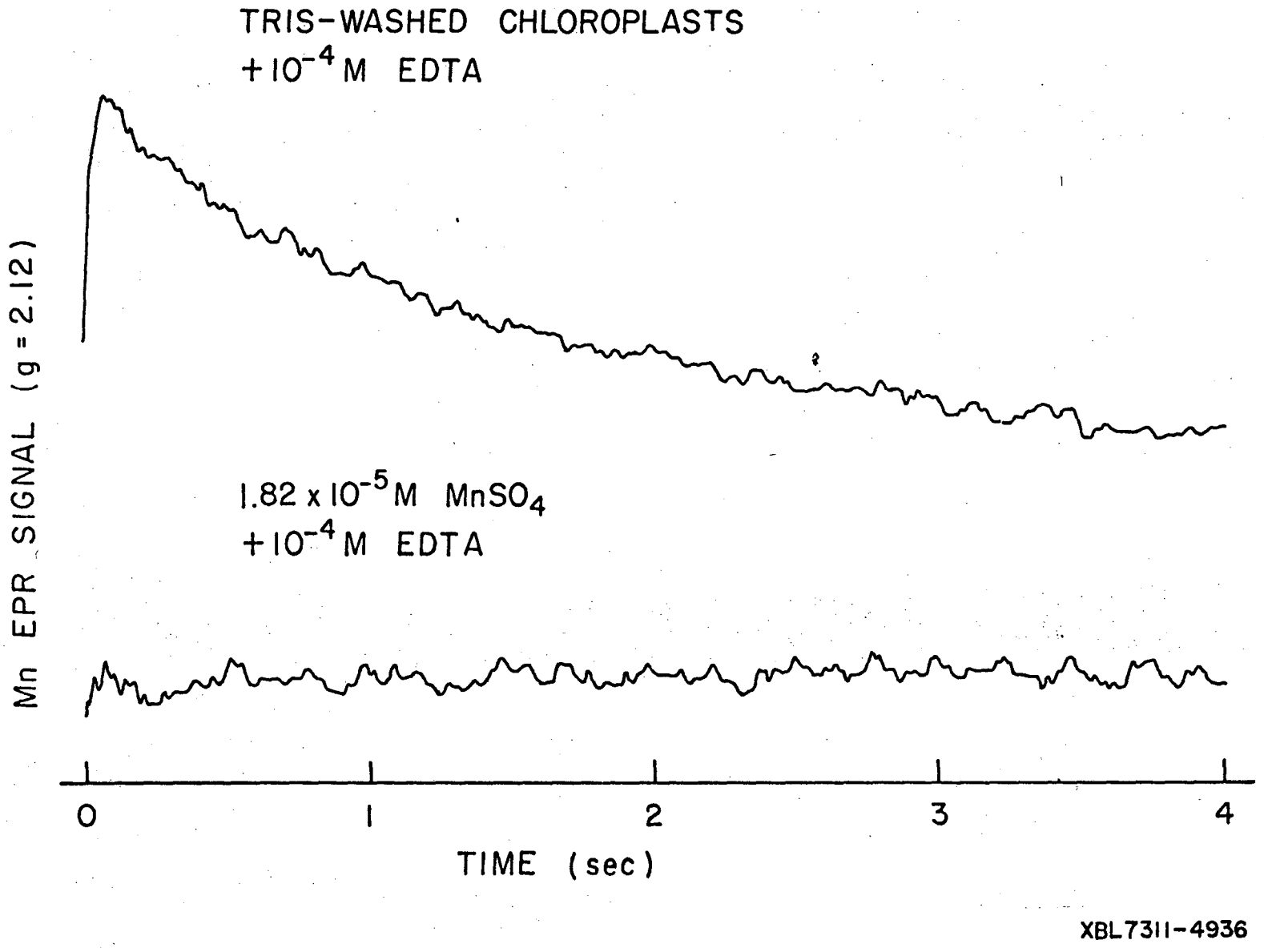

TRIS-WASHED CHLOROPLASTS
+10⁻⁴ M EDTA
1.82 x 10⁻⁵ M MnSO₄
+10⁻⁴ M EDTA
Mn EPR SIGNAL (g = 2.12)
0
1
2
3
4
TIME (sec)
XBL7311-4936

Fig. 6.

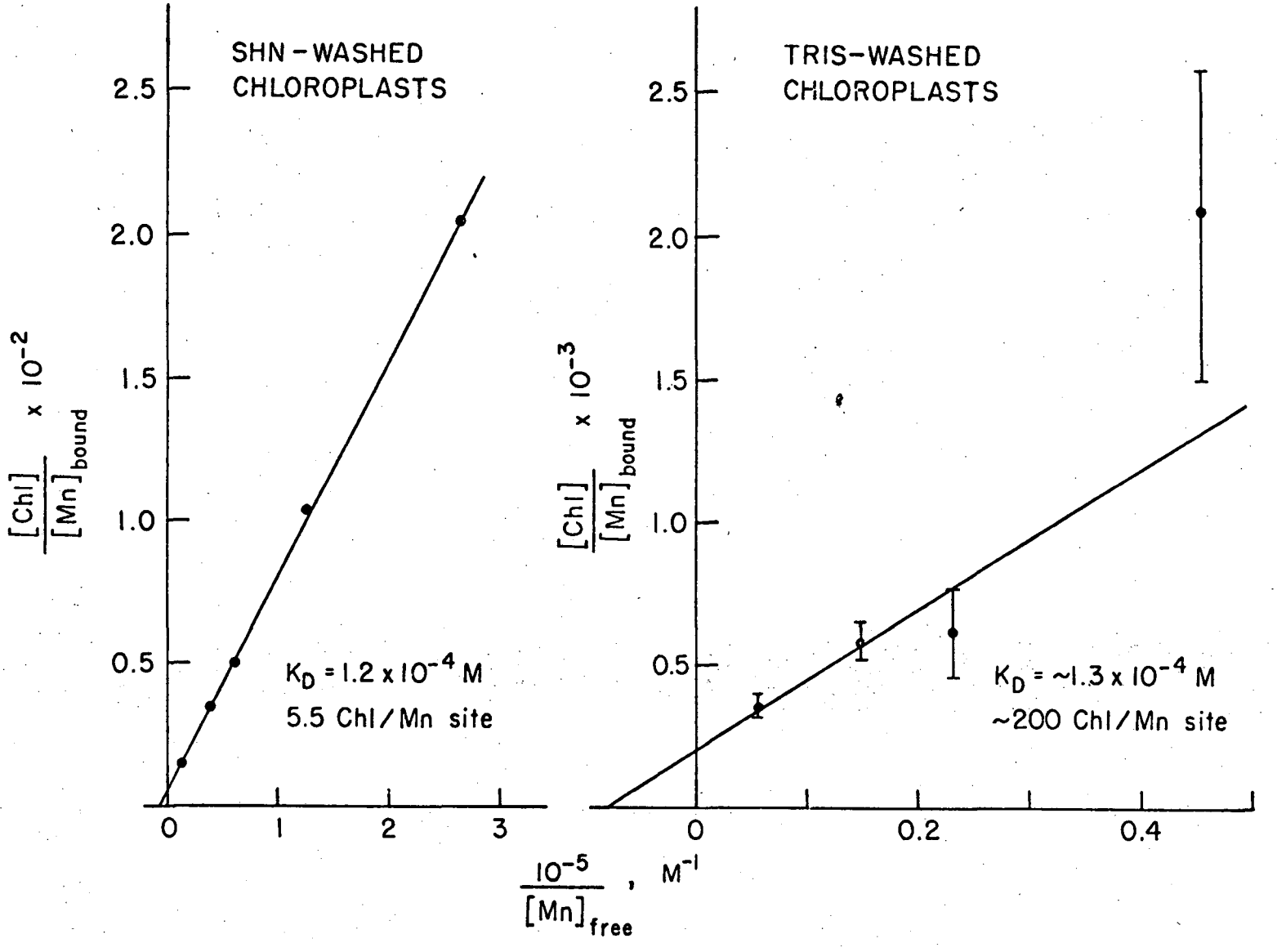
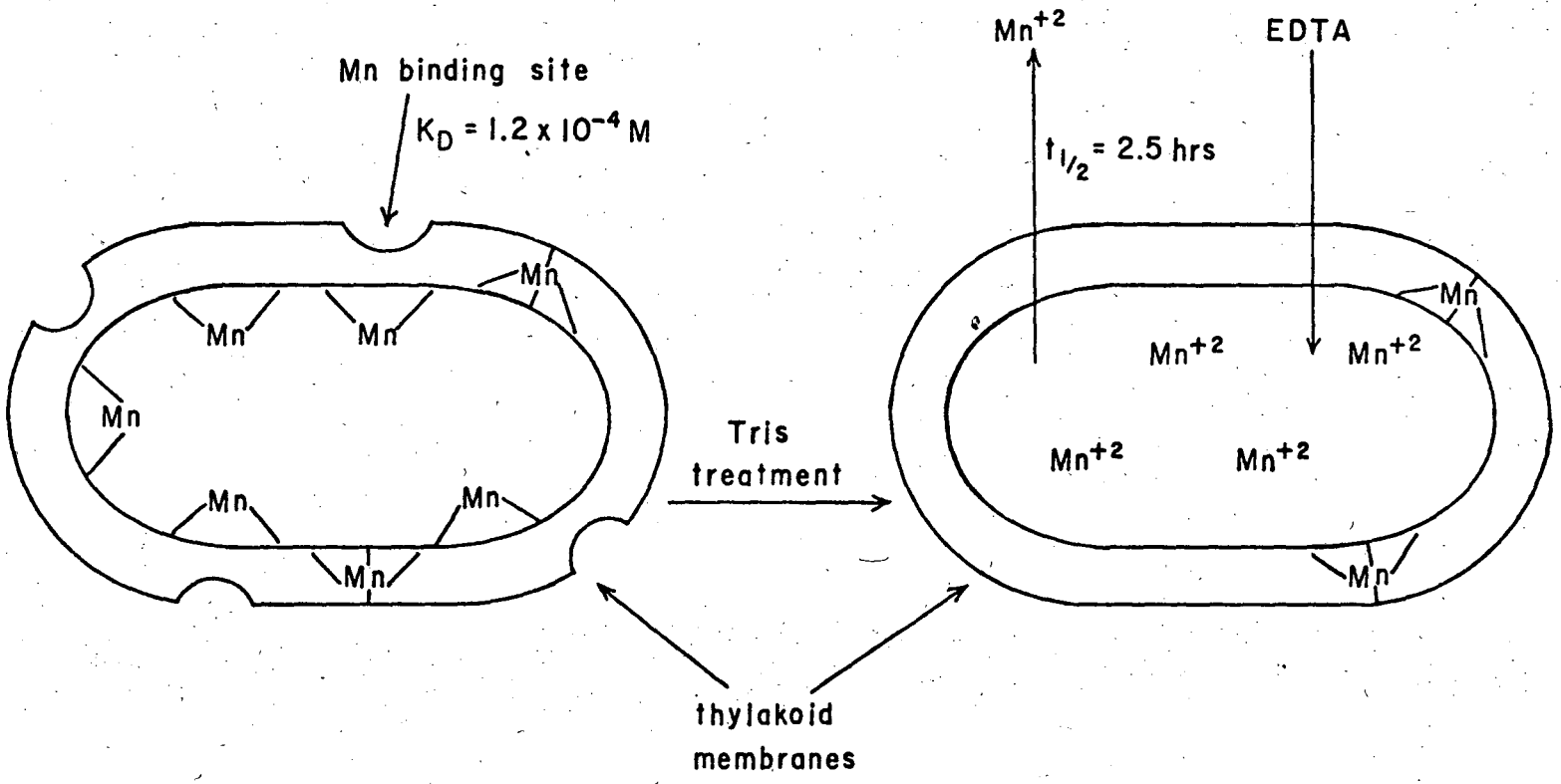

SHN - WASHED
CHLOROPLASTS
TRIS-WASHED
CHLOROPLASTS
$\frac{[Chl]}{[Mn]_{bound}} \times 10^{-2}$
$\frac{[Chl]}{[Mn]_{bound}} \times 10^{-3}$
$K_D = 1.2 \times 10^{-4}$ M
5.5 Chl/Mn site
$K_D = \sim 1.3 \times 10^{-4}$ M
~200 Chl/Mn site
$\frac{10^{-5}}{[Mn]_{free}}$, M^{-1}
XBL7311-4938

Fig. 7.



XBL7311-4937

Fig. 8.

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