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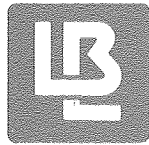
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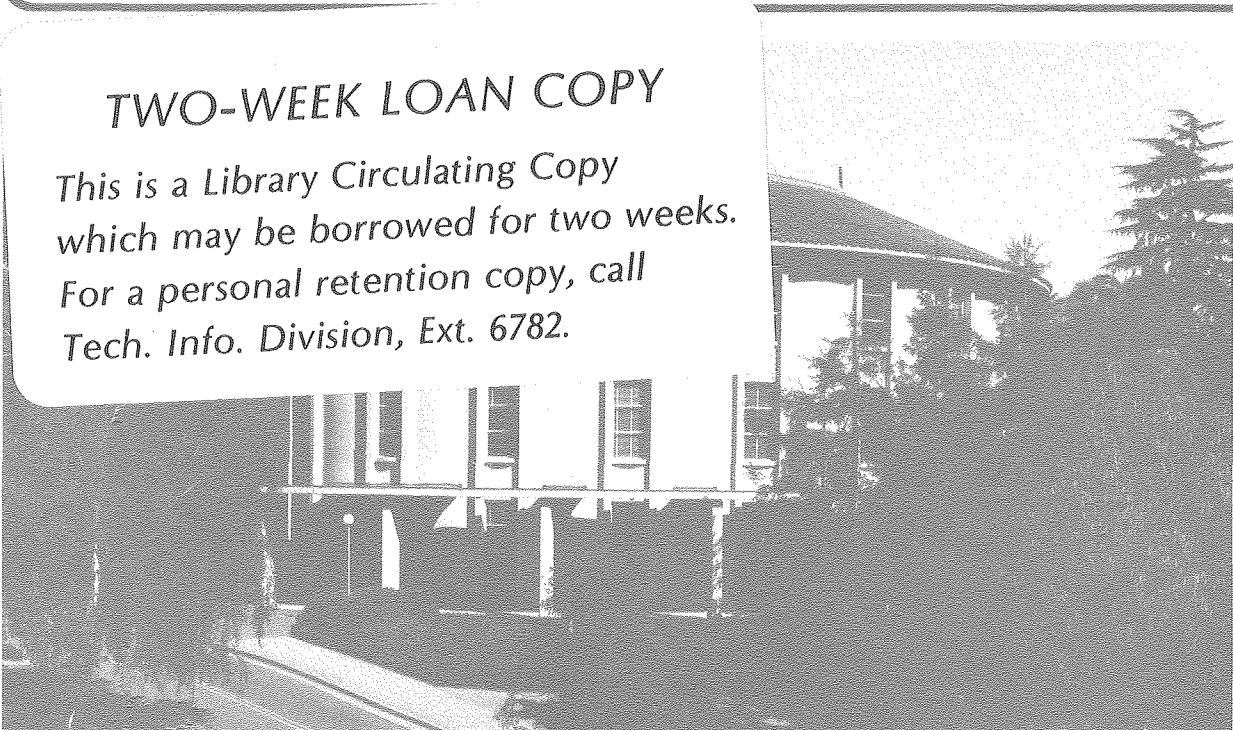J.P. Smith, A.C. Thompson, and M.P. Klein

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DISCLAIMER

THE STATE OF MANGANESE IN THE PHOTOSYNTHETIC APPARATUS:

FIRST VIEW OF THE MANGANESE SITES BY X-RAY ABSORPTION SPECTROSCOPY

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ABSTRACT

Manganese atoms have long been implicated as essential ingredients in photosynthetic oxygen evolution. Heretofore they have eluded direct observation. We report the first direct observation, by X-ray Absorption Spectroscopy, of the Mn sites in chloroplasts isolated from _Spinacia oleracea_. The manganese in chlorplasts is commonly thought to exist in two pools. The major pool, corresponding to two-thirds of the manganese, can be reversibly released with concomitant loss of oxygen evolving capacity, and has thus come to be assigned as the active pool. The role of the remanant one-third, or tightly bound pool is moot. Our analysis of the Extended X-ray Absorption Fine Structure of the active pool is consistent with a bridged dimeric structure involving two manganese atoms separated by about 2.7 Å. The distance between manganese and bridging ligands is about 1.8 Å. Analysis of the edge region suggests that the manganese in the active pool exists in oxidation states somewhat higher than Mn(II).

The photosynthetic evolution of oxygen by green plants and blue-green algae has been the subject of extensive study during the past halfcentury. One of the results of these studies has been the demonstration that manganese is essential for oxygen evolution to occur. Plants deprived of manganese in their growth media evolve oxygen at markedly reduced rates or not at all.[1]

Experiments by Joliot,[2] and subsequently by others, revealed that the pattern of oxygen evolution induced by a series of brief, saturating flashes of light was periodic, modulo four, with little or no oxygen appearing after the first, fifth, etc. flash, and maximal yield on the third, seventh, etc. flashes. Kok et al.[3] provided a kinetic model to account for this periodic pattern of oxygen evolution. In that model, some intermediate, called S, cycles from the S_0 to the S_4 state, advancing one step for each photon absorbed. Upon reaching the terminal step, water is split and oxygen released.

During the development of understanding of the various aspects and pathways in photosynthesis it has come to be recognized that four photons acting on photosystem II are necessary to produce the four oxidizing equivalents required to oxidize two molecules of water to produce one oxygen molecule. Blankenship and Sauer[4] proposed a schematic model in which a pair of manganese atoms, somehow juxtaposed, could cycle from a Mn(II)-Mn(II) state to a Mn(IV)-Mn(IV) state, at which epoch a concerted four electron-four proton oxidation of two water molecules occurs.

Such was the general state of knowledge of the water splitting site when the research reported here was inaugurated. In the photosynthetic apparatus, where the chloroplasts contain several types of chlorophyll, hemoproteins, caretenoids, and other pigments, the very weak optical

transitions which might be attributed to manganese are totally masked. Electron paramagnetic resonance (EPR) is frequently employed to study Mn but attempts to observe the Mn sites in chloroplasts have been uniformly unsuccessful except when the Mn is released from its native environment.

Treatment of the usual preparations of chloroplasts by alkaline tris (hydroxymethyl)aminomethane (Tris) releases about two-thirds of the bound Mn into an inner membrane phase where the Mn becomes EPR visible as Mn^{2+} and indistinguishable from the hexaquated ion.[5a] Upon such treatment the capacity for oxygen evolution is abolished or markedly attenuated. Removal of the Tris by dialysis followed by light, restores oxygen evolving capacity concomitant with the loss of the Mn^{2+} EPR signal.[5b]

With this background it is apparent that some spectroscopic method which would permit the direct observation of the Mn sites, in situ or in vivo, could be of great significance in the investigation of this important, yet thus far invisible, site. Such a method is to be found in X-ray spectroscopy using synchrotron radiation. Several recent reports[6] have been presented which discuss the types of information which can be acquired using X-ray spectroscopy and hence we give only a brief recapitulation of the salient features as they apply to the problem under discussion.

Each element has characteristic energies at which X-ray absorption occurs for each of its respective inner shells. We shall be concerned here with the K-edge absorption of Mn near 6.5 KeV. The precise energy and the shape of the absorption edge can frequently be related to the oxidation state and site symmetry about the metal under study. Modulations in the absorption coefficient at higher energies, the now well known Extended X-ray Absorption Fine Structure, EXAFS, reflect the microstruct-

ural environment of the ligands cordinated to the element under study. Thus, X-ray absorption spectroscopy provides, in principle, a means to determine the oxidation states and ligation of Mn and changes which might ensue upon light excitation.

METHODS AND MATERIALS

The common inorganic and organic compounds of manganese were obtained from standard commercial sources. Others were synthesized in our laboratory by published procedures or were gifts from colleagues. Chloroplasts from spinach were prepared by published procedures (Blankenship and Sauer[4,5]). All chloroplast samples were centrifuged, and as much water was removed as possible in order to prepare the samples as a moist paste. Tris-treated samples were prepared according to the method of Blankenship and Sauer[4,5]. This treatment releases about two-thirds of the manganese into an aqueous phase within the thylakoid membranes. Such preparations are hereafter designated "Tris-treated chloroplasts". Tris-treated chloroplasts were also subjected to osmotic rupture by suspension in distilled water. They were then centrifuged and washed with sucrose buffer (0.4M sucrose, 0.05M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.6, 0.01 M NaCl) to remove the loosely bound manganese and provide specimens which contained only the tightly bound manganese. All of the chloroplast samples were painted, as thick pastes, onto Millipore* filters. Some of the samples, observed in the solution state, were contained in plastic cuvettes with thin windows of Kapton**, a polycarbonate. Powders of manganese standards were mixed with cellulose powder and pressed into a suitable form by a press as used for preparation of solid samples for IR spectroscopy.

Data Acquisition. Data were acquired at several of the beam lines at the Stanford Synchrotron Radiation Laboratory. The best energy resolution was obtained at the EXAFS station on Beam Line I utilizing its Si (220) crystals. For some other runs, the higher fluxes from the focussed beam line were employed. For the reference compounds, where the signal-tonoise ratios were adequate, spectra were obtained in the absorption mode.[7] All of the spectra from the chloroplasts were obtained in fluorescence mode. In this mode, the excitation function for X-ray fluorescence character- istic of the element under study, in this case Mn, is measured.[8] The small concentration of Mn in the chloroplast samples, ca 100 M, required the use of the more sensitive fluorescence method. In fact, the method was developed with the experiments on the photosynthetic apparatus in mind. Many hours of signal averaging were required to obtain EXAFS data of even modest quality. A three element array of Si detectors was employed to increase the counting rates.

Data Analysis. Two classes of data are being sought in these experiments. The first concerns the precise energy at which the onset of K-edge absorption occurs. This energy will be related to the oxidation state or states of the Mn. The second class of data includes the EXAFS oscillations, whose Fourier transforms yield apparent radial distances between the Mn atoms and their immediate neighbors and finally, simulations of the EXAFS oscillations which yield accurate distance information and some indication of the elemental identity and number of ligands.

The details of the data analysis methods are contained in references 9 and 10 and in a paper currently in preparation [11]. For determination of edge position, the edge region was fitted to an analytic function and

analytic first and second derivatives were determined. Fourier transformation of EXAFS, over a range of $4\text{-}11\mathring{A}^{-1}$, yields a radial distribution function with one or more peaks. Their positions in R-space are the apparent distances in $\mathring{A}$, between absorber and neighbor atoms. Isolation by a suitable window function of individual R-space peaks corresponding to single distances or unresolved multiple distances were inverse Fourier transformed for use in the programs which were used to fit the oscillations to those predicted by the theoretical back-scattering amplitude and phase shift functions of Teo and Lee [12]. The identity of the scatters within each R-space shell were guessed and the resulting simulations iteratively compared with the data until the errors were minimized. After achieving satisfactory results with each isolated shell, the entire interferograms were simulated using the composite of back scatterers and their respective parameters determined from the isolated peaks as the starting values.

<div align="center">RESULTS</div>

Figure 1 shows the edge region for five samples, <u>viz</u>, active chloroplasts (labeled X), <u>Tris</u>-treated chloroplasts(labeled Y), <u>Tris</u>--treated, osmotically shocked chloroplasts (labeled Z), Mn(III)acetylacetonate (labeled 11), and aqueous $MnCl_2$ (labeled 9). Figure 2 is a plot of the inflection point energy <u>vs</u> the "coordination charge" calculated for each of a series of compounds of known structure and oxidation state, as well as the experimentally measured energy points for the three types of chloroplast samples.

Figures 3a and 3b show Fourier transform R-space data for the active chloroplast samples obtained from the EXAFS data weighted by k^0 and k^3, respectively. R-space is shifted from the true distance by approximately

-0.45 A. Figures 3c and 3d show similar data obtained from the inactive or Tris-treated-osmotically shocked chloroplast samples. Figures 3e and 3f are R-space data for a weighted difference between the active and inactive chloroplast preparations.

DISCUSSION

As there have been no previous direct observations of the manganese sites in the photosynthetic apparatus, there is as yet no proof that they are involved directly in the water splitting reactions. If we assume that they are so involved, and there is ample indirect evidence to support such a conjecture, two questions are immediate: 1) what is the oxidation state or states of the "active" pool of manganese? and 2) what are the ligands of manganese and how are they juxtaposed?

As the interpretation of the edge region is, in part, dependent upon the EXAFS results, we will discuss the EXAFS first. Figures 3a and 3b show the R-space Fourier transforms of the EXAFS of samples of active chloroplasts weighed by k^0 and k^3, respectively. It is observed that there are three R-space peaks, R>1Å, in this sample. The large increase in the size of the peak at R_{eff} = 2.3Å upon the k^3 weighting suggests that this peak represents backscattering by a neighbor of large atomic number. The k^3 weighting also has the effect of slightly increasing the resolution of the peaks between 1 and 2A.

The R-space data for the shocked or inactive chloroplasts are shown in Figures 3c and 3d. It is apparent that significant changes in the ligation of Mn are reflected, especially in the peaks between 2 and 3Å.

Figures 3e and 3f show the R-space data for the difference between the spectra of the active and one-third of the Tris-shocked chloroplast

spectra. There are two major peaks and one of lesser amplitude in the k^3 transform.

The analysis of these data proceeded along the following lines. We assumed that the Mn is composed of two pools and the EXAFS of the intact chloroplasts is their composite. The difference between the active and the Tris-shocked samples is taken to represent the photosynthetically active pool. The k^3 transform, shown in Figure 3f was isolated into three separate R-space peaks by suitable window functions and each inverse transformed to k-space. The difference between the k^0 and k^3 transforms indicates that the peaks at lower R_{eff} arise from low-Z elements such as C, N, or O, while the third peak at R_{eff} = 2.3Å arises from a heavier element such as one from the first row of the transition metals. The Fourier-isolated and inverse transformed peaks were then iteratively fitted against sinusoids whose amplitude and phase functions were obtained from the theoretical treatment of Teo and Lee.[12] The parameters determined from the minimization of these single peak fits were then used as the starting values for fitting the entire spectrum. In this case, the features between 1Å < R_{eff} < 2.6Å were isolated and inverse transformed and then fitted. The fitting results are given in Figure 4 and in Table I.

It is thus evident that a valid fit for the active pool of Mn results from a coordination environment in which there are approximately two low Z ligands at 1.81Å ; several additional low Z ligands at 2.14Å and a higher Z ligand at 2.72Å. These are true interatomic distances. The backscattering amplitudes and phase functions do not permit a distinction between Mn and Fe but they clearly exclude S and P. As there are no reports of the Tris-treatment releasing Fe, we thus assume that the

partner transition element is Mn. Such ligation is completely consistent with a di-μ-oxo bridged dimer complex.

We have obtained and analyzed the EXAFS for three di-u-oxo bridged Mn dimers. The crystal structure of one of them has been determined[13] and used to guide our analysis of the EXAFS of the other two. The analyses are in good accord with that given here for the structure of the "loosely bound", active pool of Mn in the chloroplasts as presented above.

We have also examined the EXAFS features for the remnant, tightly bound Mn observed in the Tris-shocked chloroplast samples. Due to technical difficulties, only a rudimentary analysis was performed. The results will not be presented here.

Results for the Edge Region. In 1967 Batsanov, et al.[14] showed that a linear correlation between the X-ray edge energy and a quantity called the "coordination charge" could be obtained for simple compounds of Mn.

This relationship has been used in X-ray photoelectron spectroscopy[15] and more recently in X-ray absorption spectroscopy by Cramer, et al.[16], where such a correlation was found to obtain for simple complexes of Mo.

The edge energies together with their corresponding "coordination charges" for a variety of manganese compounds are presented in Figure 2 and Table II. The details of the calculations will be presented elsewhere.[19] Figure 2 demonstrates that there indeed exists a linear relationship between the K-edge energy and coordination charges for the Mn compounds included.

Also included in Table II and shown in Figure 3 are the values of the edge energies of the three chloroplast samples.

Examination of Figure 2 shows a general grouping of the Mn(II) compounds and then a gap before encountering the Mn(III) compounds.

Closer examination shows five compounds with the same calculated coordination charge but exhibiting different edge energies (entries 6-10). This apparent inconsistency arises from the relative crudeness of the coordination charge calculations which does not distinguish between carbonyl, hydroxyl oxygen, or water, for example. Their bonding properties are, however, distinctly different. See Table II.

We have found that a reasonable prediction for the coordination charge, and hence the oxidation state or states of the Mn contained in an unknown sample can be obtained from the data in Figure 2 if the unknown does not contain Mn in multiple sites with different oxidation states and that significant pi-binding is absent.

The Chloroplast Edges. In Figure 1 it can be seen that qualitative changes in the Mn edge region of the chloroplast samples occur upon treatment with Tris. The active chloroplast sample displays an edge spectrum which is qualitatively similar to that displayed by Mn(III) acetylacetonate. Upon treatment with Tris, the shape of the edge region changes; a new feature appears at lower energy and this feature is that displayed by Mn^{2+}. Such an observation is not unanticipated. The work of Blankenship and Sauer showed that upon Tris treatment an aqueous Mn^{2+} EPR signal appears.[5]

In Figure 1 is also displayed the edge region of the "shocked" chloroplasts. This sample is meant to reflect the pool of tightly bound manganese which remains after Tris-treatment. We are obliged to assume that neither the ligation nor oxidation state of this pool is changed upon these treatments. The edge features and energy are quite typical of Mn(II) complexes and are well matched by the edge spectrum of Mn(II)acetylacetonate (not shown).

If we subtract from the spectrum of the active chloroplasts a spectrum of the shocked chloroplast weighted by the fraction 0.33, we should obtain the spectrum of the "loosely bound" pool of Mn (not shown). That is, the pool which may be reversably released and reabsorbed by the Tris-treatment.

If we first consider the Tris-washed sample, it is apparent that the edge energy corresponds to a Mn(II) species. The shape of the edge region also corresponds to such a species which exhibits the characteristic high, broad peak exemplified by the aqueous Mn^{2+} spectrum.

The spectrum of the Tris-washed, osmotically shocked samples exhibits an edge energy and the post-edge peak characteristic of Mn(II). In this instance the post-edge peak is wider than that observed for aqueous Mn^{2+}. Such an increased width may be attributed to heterogeneity in oxidation state, in ligation, or both.

We turn now to the edge spectra of the active chloroplast and of the synthesized "loosely bound" pool of Mn. Both edges exhibit broad post-edge features. Such width is often displayed by complexes with significant distortion of the first coordination sphere either from heterogeneity of ligand types or ligand distances, or heterogeneity of sites or both. The edge energies appear to argue against an oxidation state of +2 because our calculations show that a Mn(II,II) complex with the observed coordination charge would require the presence of four-oxygen ligands or three oxygen and one nitrogen ligand, each in the first coordination sphere. Referring to the results of the EXAFS studies presented above in which it is shown that the dominant structure about Mn is that of a di-u-oxo bridged Mn dimer, the probability of either suggestion is remote.

Accordingly, the data are consistent with a system in which at least some of the Mn exists in a state of oxidation higher than Mn(II). Some reference to the known chemistry of di-u-oxo bridged dimers is helpful in this regard. Extensive electrochemical studies have been performed[18] on the three di-u-oxo Mn dimers for which we have edge and EXAFS data. These include Mn(III)Mn(IV)bipyridine$_4$, Mn(III)Mn(IV)phenanthroline$_4$, and MN(IV)Mn(IV)phenanthroline$_4$. The electrochemical studies indicate stable oxidation state differences of one but not two electrons. Thus the most likely possibilities for oxidation states are (II,III), (III,III), (III,IV), etc. Comparisons of the spectrum of the synthesized "loosely bound pool" with those of model compounds reveal that the former has the same K-edge energy as have Mn(III,IV)bipyridine$_4$ and Mn(III,IV)phenan-throline$_4$. The edge shapes are rather different which perhaps results from significant disorder in ligand distances for the chloroplast data. We interpret these findings to suggest an upper limit on the oxidation state of the chloroplast Mn as (III,IV).

A Mn(II,III) complex with average ligation by four oxygen atoms and one nitrogen atom yields a coordiation charge of 0.28. A Mn(III,III) complex with a mixture of five oxygen atoms and one nitrogen atom as ligands yields a coordination charge of 0.37, suprisingly close to the value of 0.36 deduced for the loosely bound pool of manganese. Substitution of one or more water molecules for oxygen atoms can lead to higher edge energies and hence an over estimation of deduced coordination charge and oxidation state.

The inadequacies of the calculational method together with the quality of the chloroplast data do not permit a distinction between the

Mn(II,III) and Mn(III,III) states at this time. Thus we consider them both to be reasonable candidates for the majority species.

CONCLUSION

On the basis of manganese release experiments following zero through four flashes of light, and casting their findings into Kok's model, Wydryzynski and Sauer[19,4b] have been able to obtain ratios of the S-states and a consistent description for the corresponding oxidation states of manganese. Their results are compatible only with a model in which the S-states of Kok[3] contain two manganese atoms in a cooperative unit. The S-states correspond, then to S_0 = Mn(II,II), S_1 = Mn(II,III), S_2 = Mn(III,III), S_2^* = Mn(III,IV), S_3 = Mn(II,III)O_2H_2, and S_4 = Mn(III,III)O_2H_2. The ratio of S_1/S_0 = 3 in the dark.

The EXAFS simulations of the data for the active pool of Mn are in accord with a bridged Mn dimeric species while the edge data are consistent with this complex existing in the (III,III) oxidation state. At this juncture we cannot exclude contributions of (II,III) or (III,IV) states, nor is it necessary to do so.

Our interpretations of our data are thus not only compatable with those of Wydrzynski and Sauer but provide the first direct evidence that the manganese atoms occur as paired species.

What has been demonstrated here is that at long last there is a means for directly probing the Mn sites in the photosynthetic apparatus and establishing their oxidation states and coordination environments. The data and results presented here were obtained under conditions of relatively low photon fluxes, at SSRL. With the advent of some operation of the SPEAR storage ring dedicated to synchrotron radiation research, together with advanced instrumentation to permit us increased sensitivity,

we are engaged in repeating some of the work reported here and to seeking the putative changes in oxidation state and manganese coordination following one through four flashes of light. Observation of such changes would then firmly establish the connection between the Mn sites and the water splitting reactions in photosynthesis. Detailed results of this work will appear elsewhere.[20,21]

ACKNOWLEDGEMENTS

Table 1. EXAFS Curve Fitting Results

Sample

Active Chloroplasts		Fitting Atom	Fitting Error	Distance $\overset{\circ}{A}$	Number of Atoms
EXAFS range 4-11$\overset{\circ}{A}^{-1}$					
	1)	O	0.0013	1.851	1.4
		N		2.046	3.3
		Mn		2.703	0.44
	2)	N	0.0036	1.789	1.0
		O		2.207	0.6
		Mn		2.688	0.45
Chloroplast Difference EXAFS range 4-11$\overset{\circ}{A}^{-1}$					
	1)	O	0.0063	1.812	0.92
		N		2.139	0.32
		Mn		2.694	0.23
	2)	N	0.0067	1.809	0.94
		O		2.077	0.43
		Mn		2.706	0.29
Mn(3,4)BiPy (fits to single isolated Peaks)		O	0.193	1.812[a]	1.84
		Mn	0.016	2.677[a]	0.90

a Crystallographic distances: Mn-O = 1.814$\overset{\circ}{A}$, Mn-Mn = 2.716$\overset{\circ}{A}$

Table 2. Manganese Coordination Charges and X-ray K-Edge

Energies for Model Complexes and Chloroplast Samples

Label	Compound	Coordination Charge (e's)[d]	K-Edge (eV)[e]
1	α-MnS	-3.04	6542.15
2	Mn(II) (S-phenyl)$_4$ P(phenyl)$_4$	-1.36	6545.11
3	MnCl$_2 \cdot 4H_2O$	-1.02	6546.97
4	Mn$_2$LCl$_2 \cdot 2H_2O$[a]	-0.67	6546.81
5	Mn$_2$LBr$_2 \cdot 2CH_3OH$[a]	-0.70	6546.63
6	Mn(II)C$_2$O$_4 \cdot 2H_2O$	-0.46	6546.82
7	Mn(II) (CH$_3$CO$_2$)$_2 \cdot 4H_2O$	-0.46	6547.91
8	Mn(II) (AcAc)$_2 \cdot 2H_2O$[b]	-0.46	6547.41
9	Mn^{+2} (aqueous)	-0.46	6548.54
10	Mn(II)$_2$ P$_2$O$_7$	-0.46	6547.18
11	Mn(III) (AcAc)$_3$[b]	0.54	6550.70
12	Mn$_2$O$_3$	0.54	6549.55
13	Mn(IV,IV) Phen[c]	0.86	6551.04
14	α-MnO$_2$	1.54	6552.06
x	Active Chloroplasts	0.18 (18)	6549.1
y	"Tris treated"	-0.35 (18)	6547.9
z	"Tris-treated, osmotically shocked"	-0.04 (18)	6548.6

a. Where L is the dianion of 11,23-dimethyl-3,7,15,19-
tetraazatricyclo-(19.3.1.1^{9,13})hexacosa-1(25),2,7,9,11,13(26),
14,19,21,23-decaene-25,26-diol.

b. AcAc = acetyl acetonate

c. Di-μ-oxo-tetrakis (1,10 phenanthroline) dimanganese (IV,IV) perchlorate

Table 2 (cont'd)

d. Calculated by themethod given in the text for refernce compounds and
    inferred for the chloroplast sampes by their positions on Figure 2.

e. Error estimated to be $\pm$ 0.2eV for reference compounds and $\pm$ 0.3 eV for
    chloroplast samples.

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FIGURE CAPTIONS

Figure 1. Mn K absorption edges. X) intact chloroplasts; Y)

Tris-treated chloroplasts; Z) Tris-treated osmotically shocked

chloroplasts; 9) Aqueous $MnCl_2$; 11) Mn(III) acetylacetonate.

Figure 2. Plot of the "cordination charge" calculated by the method

described in the text, against the measured energy of the Mn

K-edge for 14 compounds and complexes (labelled 1-14). The

edge energies for the three chloroplast samples X, Y, and Z,

are entered on the line least-square fitted to points 1 - 14.

See Table 2 for a description of the compounds.

Figure 3. Fourier transforms of the EXAFS for three chloroplast data

sets. Transforms of k^0 (k) and of k^3 (k) are shown for each

set. a and b for intact chloroplasts, c and d for

Tris-treated osmotically-shocked chloroplasts, e and f for the

difference between the data for intact chloroplasts and

one-third that of the Tris-treated osmotically shocked

chloroplasts. The latter simulation is intended to represent

the loosely bound active pool of manganese.

Figure 4. Fourier filtered EXAFS of the difference spectrum (...) and a

computed simulation (———). The parameter for the simulation

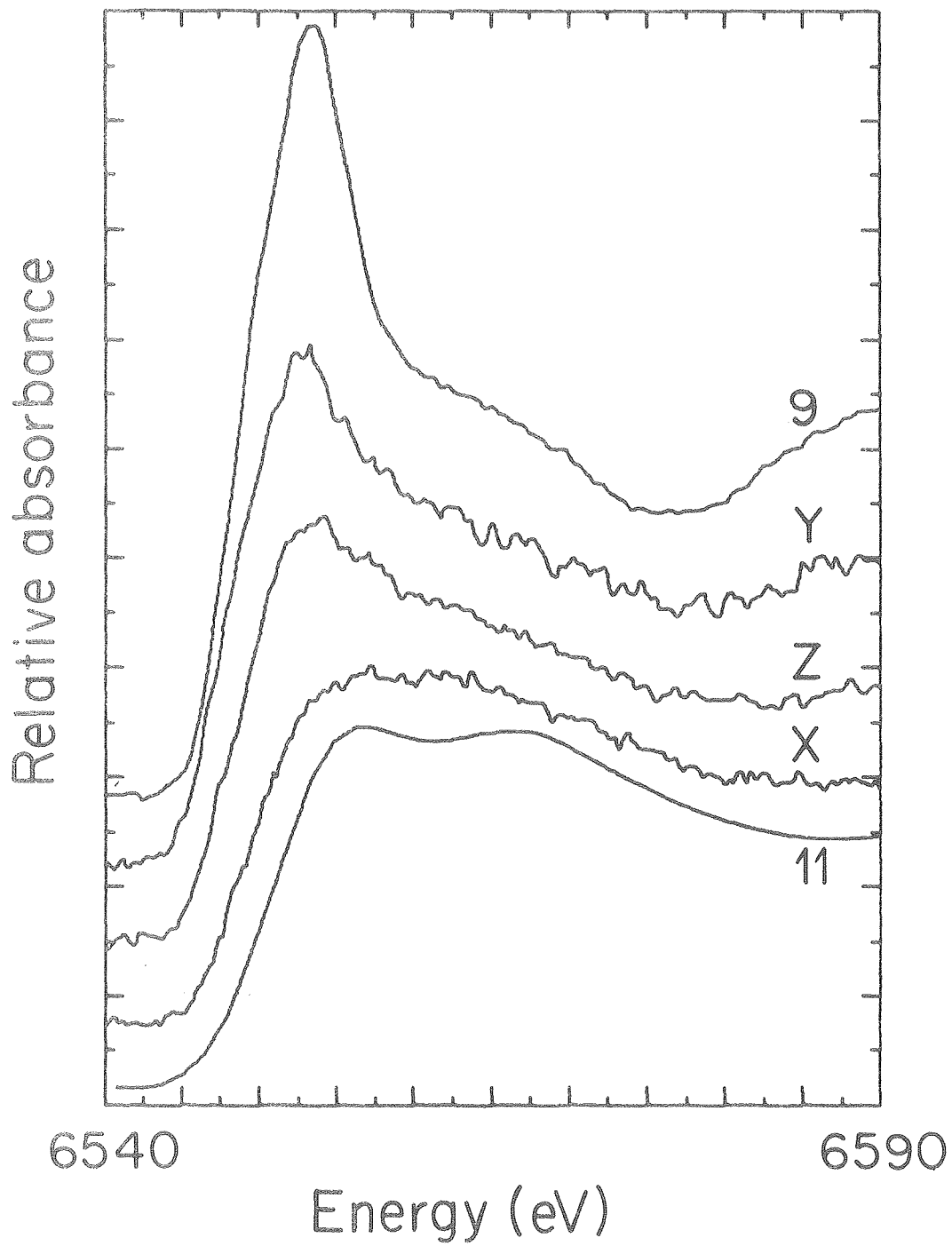are given in Table I.

Figure 1   XBL796-4868
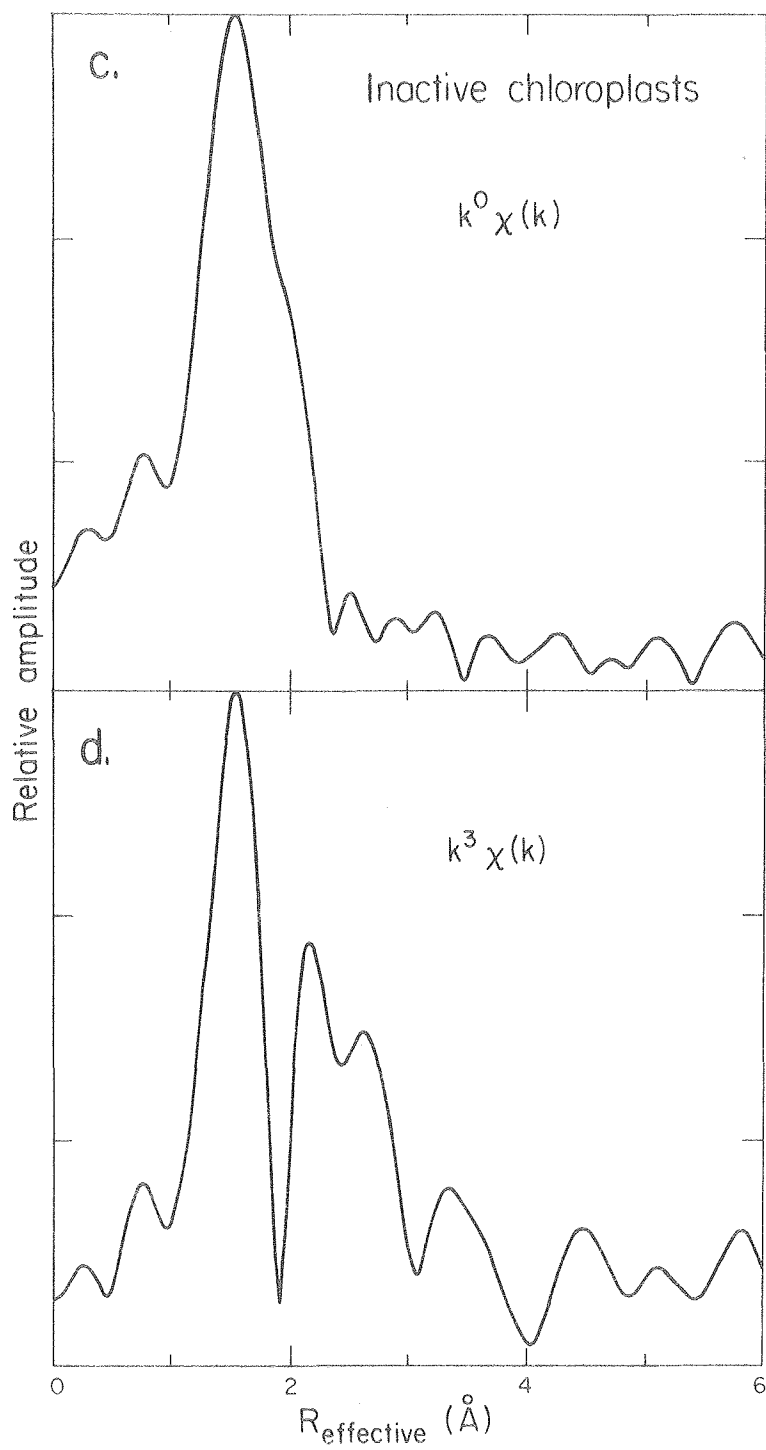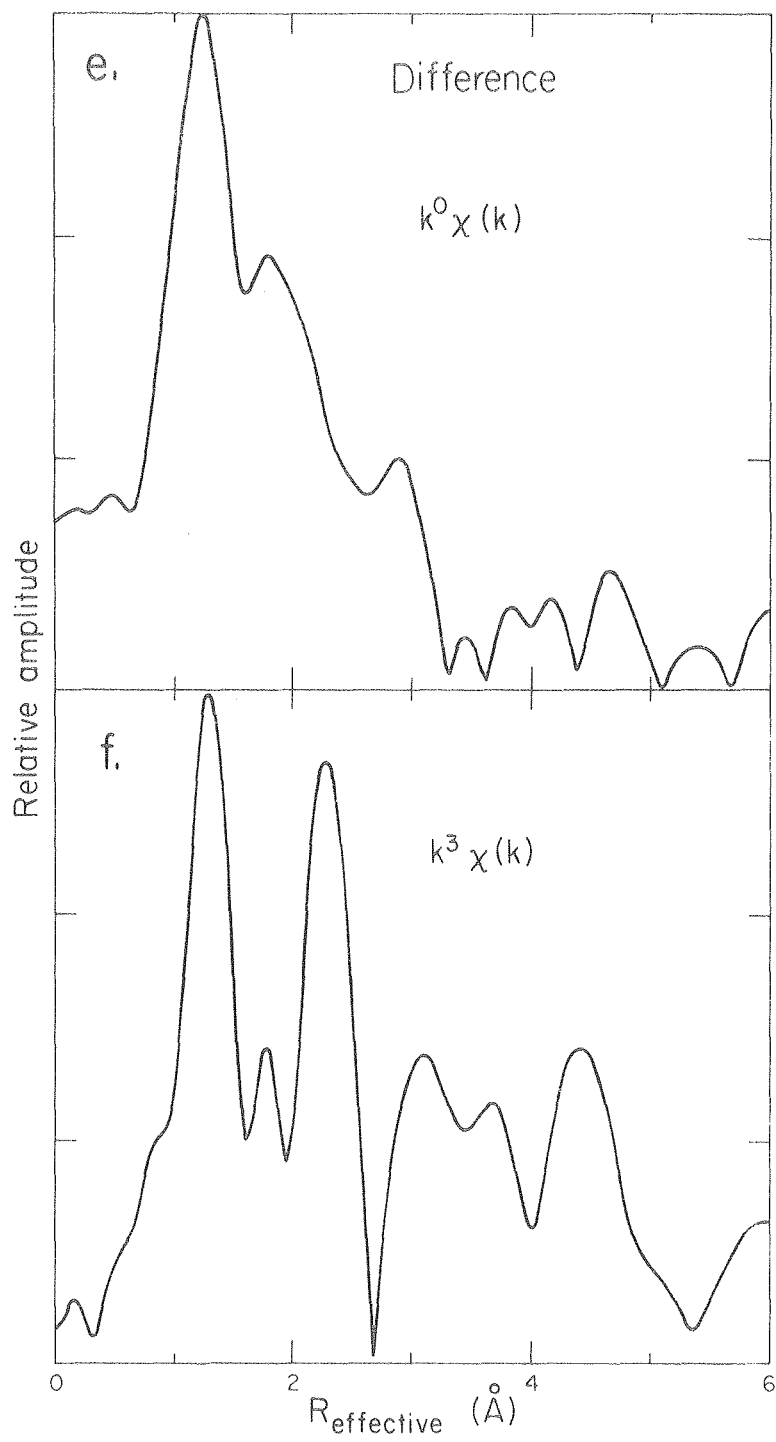
Fig. 3 c & d

XBL 7910-5032 A

Fig. 3 e & f

XBL 7910-5028A

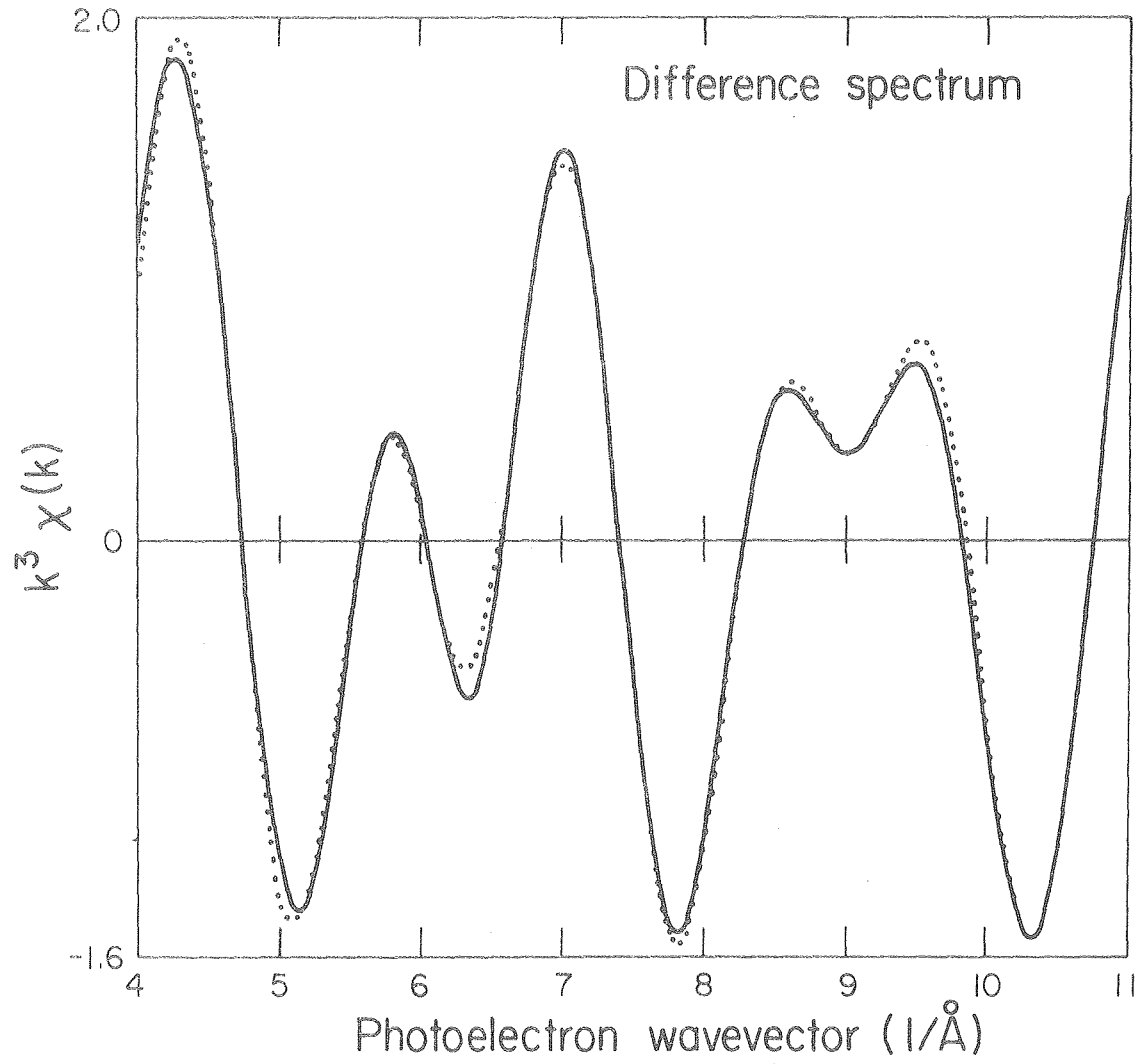Figure 4   XBL 7910-5027