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INVESTIGATIONS OF BONDING, STRUCTURE, AND
QUANTITATIVE ANALYSIS IN BIOLOGICAL SYSTEMS
BY MEANS OF X-RAY PHOTOELECTRON SPECTROSCOPY

Leo Nichols Kramer
(Ph. D. Thesis)

July 1971

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Investigations of Bonding, Structure, and Quantitative Analysis
in Biological Systems by Means of X-ray Photoelectron Spectroscopy

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ABSTRACT

X-ray photoelectron spectroscopy (XPS) has been used to obtain information concerning the structure and bonding in several biological and biologically related systems; it has also been shown to be useful for quantitative analysis of grain proteins. Correlations between charge and Fe3P and S2P electron binding energies (B.E.s) have been established for a series of iron and sulfur compounds. These correlations, along with the XPS spectra of model iron-sulfur complexes have been used to help interpret the XPS spectra of the non-heme iron proteins. The resulting evidence supports postulated structures. In a similar manner the bonding and nature of the Prussian blue complexes were investigated. It was concluded that the Prussian blues are covalently bonded "super-complexes" rather than ionic compounds. The nature of magnesium in chlorophyll found in photosynthetic membranes is also investigated by BPS. No significant difference can be determined

between the XPS spectra of magnesium contained in illuminated and non-illuminated photosynthetic membranes. Finally, the quantitative and qualitative analysis of nitrogen and sulfur in cereal grains have been carried out in order to demonstrate XPS as a method for rapid screening of grain proteins.

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There are many people to whom I owe thanks for their help in completing this work. In particular, Dr. Mel Klein and Professor Melvin Calvin provided many useful suggestions and advice concerning the work presented in this thesis. Their guidance greatly helped develop creative initiative. To Dr. Chuck Fadley, Dr. Jack Hollander, and Dr. Tica Novakov I am grateful for their aid with many of the experimental details of this work and for helpful discussions concerning the unknowns of photoelectron spectroscopy. This work could never have been completed without Mr. Charles Butler maintaining and constructing essential spectrometer equipment. To my coworkers, I am indebted for many stimulating discussions, especially to Dr. David Hendrickson and Mr. Alex Sun. Many laboratory personnel helped in constructing and maintaining the equipment used for this work and of special importance in this were Joe Katz and Gene Minor.

For providing me with greater insight, sensitivity and more valuable knowledge than any graduate education can provide I owe my greatest thanks to the Little Prince^{*} and the person who introduced me to him. Without this experience, I may never have made my most important discovery.

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* By Antoine de Saint Exupéry

I. INTRODUCTION

There are a considerable number of spectroscopic techniques available to obtain information related to the electronic bonding and structure characteristic of chemical systems. However, X-ray photoelectron spectroscopy (XPS) has the unique versatility of yielding direct electron energy measurements from virtually any atomic species. This technique was first developed by Siegbahn and coworkers, and has been used to study solids, gases, and frozen liquids.^{1,2} A variation of this spectroscopy, molecular photoelectron spectroscopy,³ uses vacuum ultraviolet radiation instead of X-rays and is applicable primarily to the valence shell electronic structure of molecules.

The basic photoelectron experiment involves irradiation of a sample with photons of energy $h\nu$ and analysis of the resulting photoelectron energy distribution emitted from the sample. Knowledge of the photon energy and determination of the photoelectron kinetic energy ($E_{K.E.}$) yields the electronic binding energy ($E_{B.E.}$) according to the following basic relationship:

$$E_{B.E.} = h\nu - E_{K.E.} \quad (1)$$

This process is illustrated for X-ray photoelectron spectroscopy in Figure 1. In this figure an electron from the atomic 2s energy level is shown being photoejected into the energy continuum of the spectrometer vacuum chamber. Measurement of

the energy distribution of electrons being photoemitted from the sample surface is carried out in a double-focussing magnetic spectrometer as represented in Figure 1. This system permits measurement of small changes in electron binding energies with a reproducibility of about 0.2 eV in many cases.

Changes in the electronic environment of an atom, resulting from a change in ligands or formal oxidation state, has been shown to correspondingly change the core electron binding energies. These "chemical shifts" can be interpreted with theoretical physical concepts. Theoretical interpretations of chemical shifts in sulfur, nitrogen, and carbon compounds have been carried out by Siegbahn, et al.^{1,2} using both empirical charge correlations and molecular orbital calculations. Fadley, et al.,⁴ using atomic orbital calculations with corrections for lattice effects presented an explanation for chemical shifts in ionic compounds. Semi-quantitative molecular orbital calculations on nitrogen and phosphorous compounds were carried out by Hendrickson, et al.^{5,6} Jolly and Hendrickson⁷ have related thermodynamic data to chemical shifts in electron binding energies. More recent approaches to the interpretation of X-ray photoelectron spectra have been presented in several other papers.⁸⁻¹¹

Besides chemical shifts of core energy levels, XPS has been used to investigate molecular orbitals of gases,^{2,12} transition metal d bands,^{1,13} and splittings in core electron binding energies.^{13,14,15,16} The splittings originate from electron correlation effects of unfilled valence orbitals

upon core levels^{13,15,16} and probable ligand field effects on core $P_{3/2}$ levels.¹⁴

In order to establish a comparative perspective of this XPS technique with other scientific probes and the information obtainable from them, a generalized "crystal-field" Hamiltonian of a single ion is presented in equation (1).¹⁷ The physical nature of the Hamiltonian's perturbational terms and their related spectroscopic probes are briefly discussed.

$$H = H_o + H_v + H_{ls} + H_{ss} + H_n + H_q + H_{sm} + H_{lm} \quad (1)$$

The first term of equation (1) can be represented as

$$H_o = \sum_{a=1}^n \frac{\bar{p}_a^2}{2m} - \frac{Ze^2}{r_a} + \sum_{a>b=1}^n \frac{e^2}{r_{ab}} \quad (2)$$

where these terms describe the kinetic energy, the electron-core potential, and the electron-electron repulsion, respectively. In this expression $\bar{p}_a$ is the linear momentum of the ath electron, r_a its distance from the nucleus and r_{ab} the distance between the ath and bth electrons. This term includes all the interactions within the free ion.

Electronic behavior is affected by the strong electric fields set up by the surrounding ligands. This is expressed by the second term of equation (1)

$$H_v = \sum_a -eV(x_a, y_a, z_a) \quad (3)$$

where V is the potential of the electric field set up by the ligands and X_a, Y_a, Z_a are the coordinates of the a th electron of the partly filled shell.

The magnetic interaction between the electron spins, s_k , and the orbital moments, l_k , give rise to the third term in equation (1)

$$H_{ls} = \sum_i \rho(r_i) l_i \cdot s_i \quad (4)$$

H_V and H_{ls} may be of comparable magnitude depending on the strength of the ligand field.

These first three terms of H , equations (2), (3) and (4) determine the electron energies (on the order of 1 to 1000 eV) measured through the XPS technique. Of course atomic absorption, X-ray absorption and emission, electronic absorption and fluorescence, and electron spectroscopies such as electron impact give information concerning electron energies described by these terms.^{17,33} However, except for some electron spectroscopies, these techniques involve energy transitions between bound states, and the information obtainable is correspondingly restricted. None of the above mentioned spectroscopies is as versatile and direct in measurement of electron energy levels as photoelectron spectroscopy.

The remaining terms in the generalized Hamiltonian are magnetic perturbations which make a negligible contribution to the total electron energy. These are, in order of appear-

ance in equation (1), spin-spin, nuclear-spin, quadrupole and external magnetic interactions. The greatest energies involved in these terms are the result of spin-spin interactions with transitions on the order of 10^{-4} eV. Methods of investigation include EPR, NMR, and Mossbauer spectroscopies.¹⁷ However, these techniques require some special characteristics of the system, such as unpaired electron spin, in order for them to be applicable. The XPS technique is not so restricted. Application of various techniques mentioned above and a discussion of their spectral interpretation is presented in the section dealing with the non-heme iron proteins.

Because of the versatility of XPS we have been able to apply this technique to a wide range of biologically related problems. Information has been obtained about the nature of the iron-sulfur moiety in the non-heme iron proteins as well as that of magnesium in chloroplast systems. Through correlations between calculated charge and electronic binding energy, information can be obtained as to the nature of the bonding in these systems. Rapid quantitative analysis of nitrogen and sulfur in grain proteins has also been possible by suitable application of this XPS technique.

II. EXPERIMENTAL EQUIPMENT AND METHODS

The X-ray photoelectron spectrometer used in these experiments is illustrated schematically in Figure 2 and is described in detail elsewhere.^{18,19} This spectrometer is

constructed from two air-cored iron-free solenoids, which give a field form varying approximately as $1/\sqrt{r}$ permitting "double-focussing" of electrons (i.e. both radially and axially). Its energy resolution is $\frac{\Delta E}{E} = 0.06\%$ full width at half maximum intensity. The solid angle of source subtended by the resolution baffle is approximately 0.12% of 4π , and the source and detector slits are approximately 0.04 cm wide and 1 cm high.

The primary components of the spectrometer system are described below. X-rays are produced from a specially constructed X-ray tube with interchangeable anodes (usually constructed from magnesium or aluminum) giving characteristic K α X-rays (with energies on the order of 1200 to 1400 eV). Several source holder arrangements are available. A solid sample holder¹⁹ and a low temperature transfer chamber were used in our experiments. This accessory transfer chamber is shown in Figure 3 and the use of this chamber is described below. After dispersion in the spectrometer, the electrons are counted by an open-window Bendix channeltron electron multiplier. The solenoid current and data acquisition and recording system are controlled by a PDP-8 computer with associated electronics.

The accessory transfer chamber mentioned above was used for all work with protein solutions. In using this chamber, the solution is placed in a shallow-cupped, gold-plated sample holder and is frozen at approximately -50°C while under an atmosphere of pure dry nitrogen in a dry box. This

clean atmosphere prevents condensation of extraneous vapors on the surface of the solid solution. Such condensates could drastically decrease the solute spectral intensity since only those electrons originating within a few hundred angstroms of the surface escape elastically and enter the spectrometer. The frozen sample, still under the inert atmosphere, is transferred to the spectrometer by means of the portable chamber fitted with a suitably valved airlock. The sample temperature is then reduced to the desired value by radiative heat transfer to a surrounding surface maintained at 77°K, which acts as a trap for extraneous gases in the spectrometer. The transfer container is then evacuated and the sample moved into position in the spectrometer. A more complete description of the use of this technique is presented elsewhere.²⁰

Experimental data were curve-fitted by a least-squares computer program which fitted either Gaussian or Lorentzian functions to the XPS spectra.²¹

III. BINDING ENERGY DETERMINATIONS USING AN EXTERNAL CARBON REFERENCE

All electronic binding energies ($E_{B.E.}$ s) reported in this manuscript have been referenced to the Cls B.E. of the carbon film deposited on the sample surface. Without referencing of this type what one actually determines from the XPS experiments is

$$E_{B.E.} = E_{X-ray} - E_{K.E.} + \psi_{wf}$$

where the term, ψ_{wf} , is the work function contribution to the energy, which will either accelerate or retard the photoelectrons entering the spectrometer.* Further, when comparing the binding energies between two samples one determines

$$\Delta E_{B.E} = \Delta(E_{X\text{-ray}} - E_{K.E.}) - \Delta\psi_c$$

where $\Delta\psi_c$ is the contact potential between the two samples and represents the difference between the two work functions of the samples.** In using an external reference film it is assumed $\Delta\psi_c$ is negligible between the sample and the surface

*The "true work function" at the surface of an electrical conductor is defined²² as the difference between the electrochemical potential, $\bar{u}$, of the electrons just inside the surface and the electrostatic potential, $-e\phi$, of an electron in the vacuum just outside it

$$\psi_{wf} = -e\phi - \bar{u}$$

**When two electrical conductors are connected electrically, electrons will flow from one to the other until equilibrium is attained. There then occurs a difference of potential between a point above the surface of one of the conductors with respect to a point above the surface of the other. This "contact potential" is the difference between the two work functions of the samples:²²

$$\Delta\psi_c = \psi_f' - \psi_f''$$

film, only if this assumption is true, is accurately known relative B.E.s obtainable.

Ideally a reference material for measuring electron B.E.s should reflect all external effects on the B.E. of the sample being investigated. The only reference meeting this requirement is an atom of constant chemical environment within the molecules investigated. In practical terms, an external reference must usually be used, and the best one can expect from such a reference is a measure of gross external phenomenon acting upon the sample's electron energies—such as charging effects and variations in the static magnetic field.

All references should be able to reflect the effects of variations in magnetic fields but adequate determination for charging phenomenon requires good physical and electrical contact between reference and sample. This can be accomplished by deposition of a thin film of reference material on the sample surface. Furthermore, electrons in high dielectric materials will feel only a partial effect of surface charging. This attenuation is due to internal polarization screening, and the net charge inside a dielectric is²³

$$Q_{\text{net}} = \frac{Q}{K}$$

where Q is the charge on the surface, and K is the dielectric coefficient of the sample. The use of metal films (with a $K = 1$) may overcompensate for charging effects since metallic

$$H_{ab} = \int \psi_a^* H \psi_b d\tau$$

and E_1 is the orbital energy. In the modified method used in this manuscript, diagonal matrix elements are approximated by valence orbital ionization potentials (VOIP's). For the ionization $C_x^q \rightarrow C_y^{q+1}$ the VOIP is computed from the formula²⁶

$$\text{VOIP}(q) = I(q) + E(C_y) - E(C_x)$$

where q is the charge on the atom, $I(q)$ is the ground state ionization potential of C_x^q and the E 's correspond to the weighted mean of energies of all multiplet terms arising from a given configuration relative to the ground state of the atom or ion in question. These diagonal elements were adjusted for partial charges by²⁷

$$H'_{aa} = H_{aa} - 2q_i$$

where q_i is the charge on atom "i". This adjustment is made in an attempt to compensate for the effect of the potential of the surrounding ligand atoms upon the central atom. Off-diagonal matrix elements are approximated by Cusach's formula²⁸

$$H_{ab} = \frac{\int_{ab}}{2} (H_{aa} + H_{bb}) (2 - |\int_{ab}|)$$

Slater-type orbitals were used as a minimum basis set for these calculations. These orbitals are wave functions describing the motion of a single electron in a central field in which

the potential energy is given by²⁹

$$V(r) = -\frac{Z_{nl}}{r} + \frac{n^*(n^*-1)}{2r^2}$$

The quantity n^* is an effective principal quantum number and Z_{nl} is an effective nuclear charge. These Slater orbitals were also adjusted according to²⁷

$$u_i = u_i^o + \frac{0.35 q_i}{n^*}$$

where u_i is the adjusted orbital exponent of the exponential Slater function. The Mulliken population analysis³⁰ can be illustrated by a simple example of a two atom M.O.

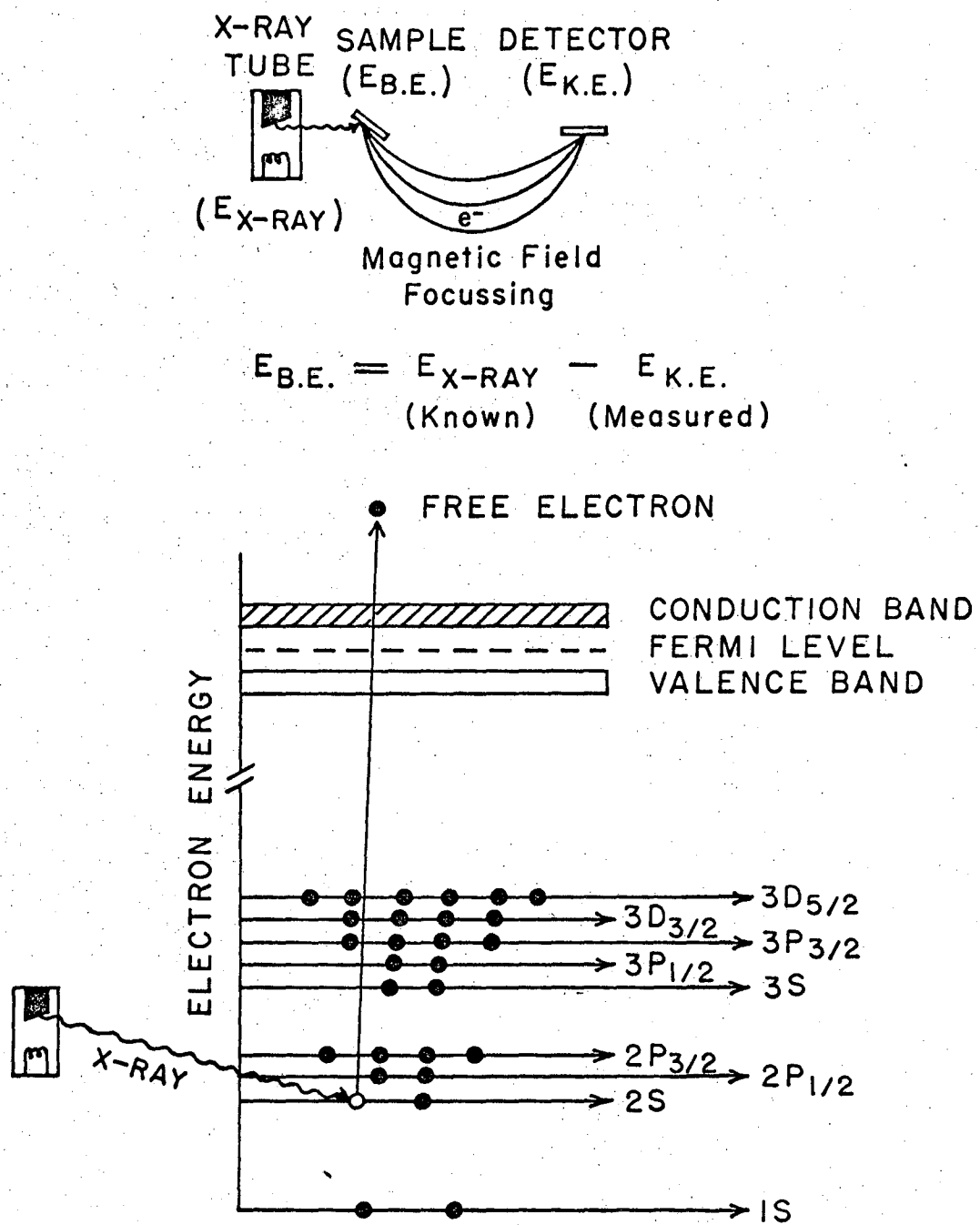
$$\psi = C_i \phi_i + C_j \phi_j$$

Squaring this wave function and integrating gives

$$N = NC_i^2 + 2NC_i C_j S_{ij} + NC_j^2$$

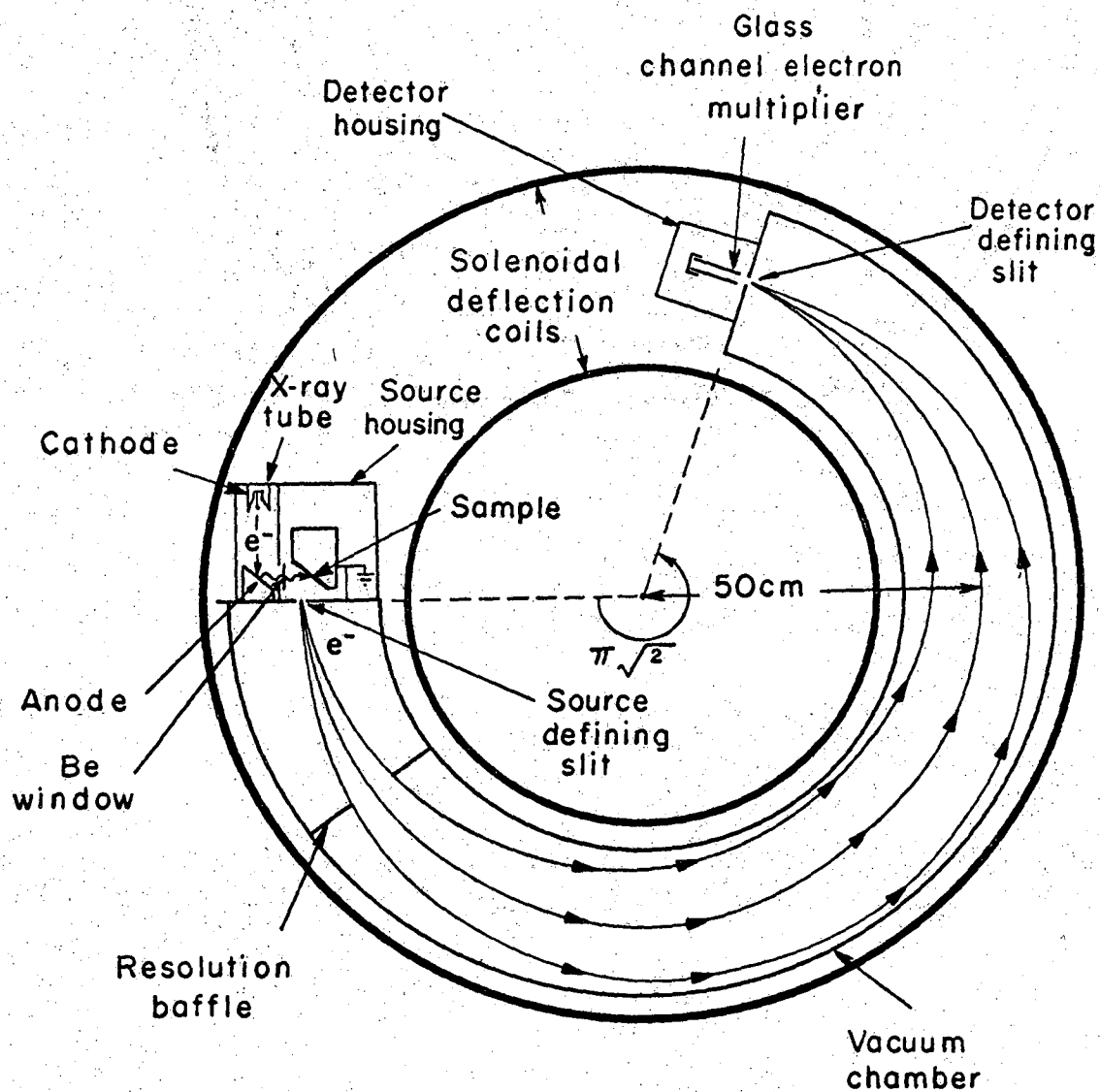
where N is the number of electrons in the molecular orbital, NC_i^2 and NC_j^2 are the net atomic populations and $2NC_i C_j S_{ij}$ is the overlap population. The net atomic population plus 1/2 of the overlap population is defined as the gross atomic population. The net charge on an atom is calculated by summing over all occupied molecular orbitals. The entire procedure was reiterated until charge self-consistency was attained.

There are many limitations to this semiempirical M.O. method. It neglects important contributions to electron energy which include electron-correlation, relaxation and relativistic effects. An analysis of the approximations of the extended Huckel formalism and its use in calculating parameters of transition metal complexes has been described by Blyholder and Coulson³¹ and Basch et al.³² However lacking a more comprehensive method for calculating electron energies of large molecular systems (considering the practical limitations of computer time and expense), we use this method to help interpret the XPS spectra of transition metal complexes.



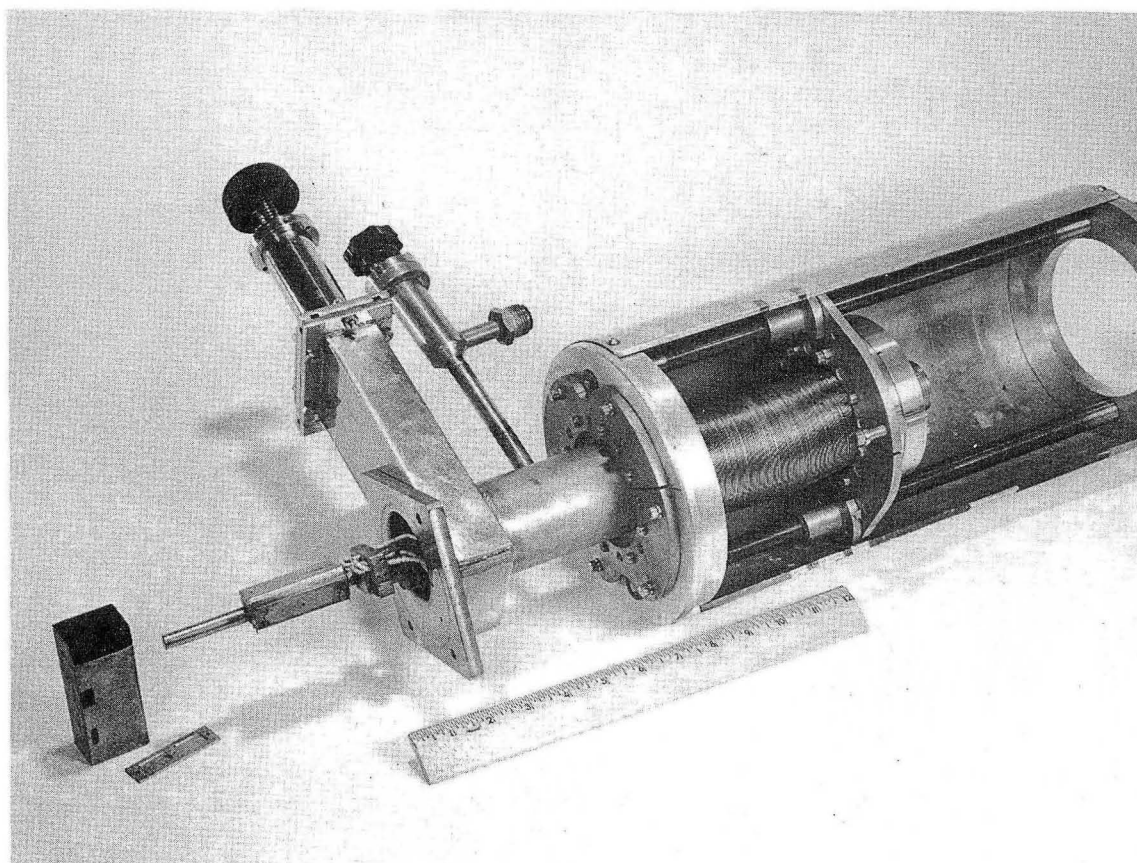
XBL 705-5218

Figure 1. X-ray photon shown photoejecting an electron into energy continuum, illustrating the X-ray photoelectron experiment.



XBL694-2402

Figure 2. Schematic illustration of X-ray spectrometer used in this work.



XBB 715-2008

Figure 3. Low temperature transfer chamber, showing sample block in extended position. The gold plated sample holder and a copper box which surrounds the sample block and acts as a trap for extraneous vapors are shown next to the chamber.

Table I

Compound	Fe3P B.E. Uncorrected	Fe3P B.E. Corrected	ClS B.E.	Au4f B.E.s
tape	--	--	288.7 eV	87.3 83.7
$K_3Fe(CN)_6$	59.6	54.9	288.7 eV	87.2 83.6
$Fe_2O_3(1)$	62.1	54.9	291.2 eV	87.3 83.7
$Fe_2O_3(2)$	61.4	54.7	290.7 eV	87.3 83.7
Ferric phosphate broad		--	290.6 eV	87.4 83.7

REFERENCES

1. K. Siegbahn, et al., "ESCA - atomic, molecular, and solid state structure studied by means of electron spectroscopy" (Almqvist and Wiksells, Uppsala, 1967).
2. K. Siegbahn, et al., "ESCA - applied to free molecules" (North-Holland Publishing Co., Inc., Amsterdam, 1969).
3. D. W. Turner, et al., "Molecular Photoelectron Spectroscopy" (Wiley, New York, 1970).
4. C. S. Fadley, S. B. M. Hagstrom, M. P. Klein, and D. A. Shirley, J. Chem. Phys., 48, 3779 (1968).
5. D. N. Hendrickson, J. M. Hollander and W. L. Jolly, Inorg. Chem., 8, 2642 (1969).
6. M. Pelavin, D. N. Hendrickson, J. M. Hollander, and W. L. Jolly, J. Phys. Chem., 74, 1116 (1970).
7. W. L. Jolly and D. N. Hendrickson, J. Am. Chem. Soc., Vol. 22, No. 7 (1970).
8. H. Basch and L. C. Snyder, Chem. Phys. Letters, 3, 333 (1969).
9. U. Gelius, B. Roos and P. Siegbahn, Chem. Phys. Letters, 4, 471 (1970).
10. M. E. Schwartz, Chem. Phys. Letters, 6, 631 (1970); 7, 78 (1970).
11. L. N. Kramer and M. P. Klein, Chem. Phys. Letters, 8, 183 (1971).
12. T. D. Thomas, Lawrence Radiation Laboratory Report UCRL-18961.

13. C. S. Fadley and D. A. Shirley, Phys. Rev. Letters, 21, 980 (1968); C. S. Fadley and D. A. Shirley, J. Appl. Phys., 40, 1395 (1969).
14. T. Novakov and J. M. Hollander, Phys. Rev. Letters, 21, 1133 (1969); Bull. Am. Phys. Soc., 14, 524 (1969).
15. J. Hedman, et al., Phys. Letters, 29A, 178 (1969).
16. C. S. Fadley, et al., Phys. Rev. Letters, 23, 1397 (1969).
17. H. A. O. Hill and P. Day (ed.), "Physical Methods in Advanced Inorganic Chemistry" (Interscience Publishers, London) 1968.
18. K. Siegbahn, C. Nordling and J. M. Hollander, Lawrence Radiation Laboratory Report UCRL-10023 (1962).
19. C. S. Fadley, Ph.D. Thesis, Lawrence Radiation Laboratory Report UCRL-19535 (1970).
20. L. N. Kramer and M. P. Klein, J. Chem. Phys., 8, 3620 (1969).
21. This program was arranged by C. E. Rugge Lederer, of the Lawrence Radiation Laboratory.
22. C. Herring and M. H. Nichols, "Reviews of Modern Physics," Vol. 21, #2 (1949).
23. D. Corson and P. Lorrain, "Introduction to Electromagnetic Fields and Waves," (W. H. Freeman and Co., San Francisco) 1962.
24. R. Hoffmann and W. N. Lipscomb, J. Chem. Phys., 36, 2179, 3489 (1962).
25. R. Hoffmann, J. Chem. Phys., 39, 1397 (1963).

26. H. Basch, A. Viste and H. B. Gray, Theoret. Chim. Acta, 3, 458 (1965).
27. P. C. van der Voorn and R. S. Drago, J. Am. Chem. Soc., 88, 3255 (1966).
28. H. Cusachs, J. Chem. Phys., 44, 835 (1966).
29. F. L. Pilar, "Elementary Quantum Chemistry," (McGraw-Hill Book Co., New York) 1968.
30. R. S. Mulliken, J. Chem. Phys., 23, 1833, 1841, 2338, 2343 (1955).
31. G. Blyholder and C. A. Coulson, Theoret. Chim. Acta, 10, 316 (1968).
32. H. Basch, et al., J. Chem. Phys., 44, 10 (1966).
33. R. S. Berry, "Electronic Spectroscopy by Electron Spectroscopy," Annual Reviews of Physical Chemistry, H. Eyring, Ed., (Annual Reviews, Inc., Palo Alto, California) 1969, Vol. 20.
34. L. N. Kramer and M. P. Klein, J. Chem. Phys., 51, 3618 (1969).

Section V. XPS of Non-heme Iron Proteins: Investigation
of the Iron-sulfur Moiety in Non-heme Iron
Proteins by Means of X-ray Photoelectron
Spectroscopy.

The non-heme iron proteins, collectively referred to as the ferredoxins, have received considerable attention in recent years because of their unusual properties. The biochemical interest derives from their extreme values of redox potential which nature has exploited by their incorporation into several electron transport chains. The biochemical investigations have been summarized in recent reviews (1,2). Several types of physical measurements have been performed, the objectives of which have been to elucidate those features of the molecular, atomic and electronic structure responsible for the biochemical properties. Table I lists some pertinent physical properties of these proteins. The chemical and physical investigations on these proteins have been reviewed by Tsibris and Woody (3). The work reported here, employing a relatively new class of physical measurement, was engendered in the same spirit of probing further into the structure-function relationship of these intriguing molecules.

When this work was initiated it was known that the proteins contained non-heme iron, acid labile sulfur and cysteine sulfur. The absorption spectra of the intact proteins exhibited features which changed in a characteristic and reversible manner upon reduction (or oxidation). Since these features were totally absent in the apo-proteins, it was concluded that the iron and probably certain of the sulfurs were at the active site. Subsequently, the elegant EPR experiments deriving from Beinert and collaborators (4,5) which employed isotopes of iron and sulfur, and replacement of the labile sulfur with selenium, showed conclusively that these elements were indeed at the EPR site and presumably at the active site. There has thus evolved a picture that the active site(s) contain an iron-sulfur complex or clusters.

The problem which we addressed was to examine the iron and sulfur moieties by X-ray photoelectron spectroscopy (XPS). This method permits a study of each element specifically and individually, and is capable of providing some structural and bonding information rather more directly than is accessible from any other contemporary form of spectroscopy. We have applied this method to four non-heme iron proteins: rubredoxin, "high potential iron protein" (HIP), clostridial ferredoxin, and spinach ferredoxin. These four were chosen as representative of all the currently known iron-sulfur proteins. XPS data from model complexes which correlate well with theory are used to aid in the interpretation of the protein spectra.

The first section of the paper describes the physical method. The second discusses the experimental procedures. We then present the experimental results, followed by a discussion and interpretation, and conclude with a summary of the findings.

METHODS

When a sample is irradiated with light of sufficient energy, electrons may be ejected and their kinetic energy is given by the well known Einstein relation,

$$K.E. = h\nu - B.E.,$$

where K.E. is the kinetic energy of the electrons, h is Planck's constant, ν is the frequency of the light quantum, and B.E. is the binding energy of the electrons in the sample. If the photon energy is increased sufficiently, by using X-rays, electrons may be ejected from the inner or core levels of the constituent atoms of the sample. Since photon or X-ray

energies are known to high accuracy, the binding energies may be determined with precision by measurement of the kinetic energies. The measurements may be performed with a variety of devices, but most commonly magnetic and electrostatic deflection analyzers are employed. The overwhelming majority of the energy levels of the elements across the periodic table have been determined in this manner.

The method is outlined schematically in Figure 1. In this figure are sketched the discrete energy levels of a particular atom in a compound as well as those levels which are a collective property of the compound as a whole and make up its valence band or molecular orbitals. An X-ray photon is shown lifting an electron from the 2S level of this atom into the continuum of energies, or, equivalently, removing it to infinity. At this point the electron enters the spectrometer, wherein it is brought to a focus and impinges on a detector when the electron has the correct energy (more rigorously the correct momentum). The energy range is scanned by varying the strength of the magnetic field and thus a spectrum is traced out by recording the number of electrons reaching the detector at each value of the magnetic field in complete analogy with mass spectrometry. This method was developed and brought to its present state of refinement by the group at the Institute of Physics at the University of Uppsala, Sweden (6).

Since all atoms are constructed in the same manner, it is possible to photoeject their electrons, and by a suitable choice of exciting X-ray energy and particular atomic level one can distinguish among the different constituent atoms of the sample. It is apparent, then, that calibration against a sample of known elementary composition will permit

a qualitative and quantitative analysis of the unknown sample. The absolute sensitivity of the method is very high, although it is not especially suitable for detecting small amounts of one element in the presence of a large excess of other elements.

More recently it has been shown that a given element in different chemical configurations exhibits chemical shifts of its binding energies (7). These chemical shifts thus extend significantly the utility of the method so that not only the total quantity of a given element may be determined, but also the type or types of chemical bonding situation in which the atom is located. The origins and theoretical foundations for these chemical shifts are rather well understood (7,8). An intuitive understanding of the chemical shifts may be derived from the following simple argument. In a neutral atom the electron binding energies are determined by the attractive potential between the electrons and the positive nucleus and the repulsive interactions among the electrons. In a normal complex the valence electrons are partially donated to or accepted from the ligating atoms. The net effect in a particular complex is a decrease or increase of the net charge on the atom under study relative to the charge on this atom in other complexes. This change of net charge is accompanied by an increase or decrease of the binding energies of the core electrons. More formal and detailed arguments will be found in the literature references and will be presented in the discussion section.

EXPERIMENTAL PROCEDURE

Spinach ferredoxin was prepared by the method of Tagawa and

Arnon (9). Clostridial ferredoxin and clostridial apoferreredoxin were obtained from Prof. J. C. Rabinowitz, HIPIP from Dr. R. G. Bartsch, and rubredoxin from Dr. W. Lovenberg.

All spectra were produced and analyzed in the Berkeley iron-free photoelectron spectrometer (10). In order to obtain spectra from proteins in a state approximating the solution and to obviate the interfering effects of $\text{SO}_4^{=}$ accompanying $(\text{NH}_4)_2\text{SO}_4$ precipitation, the samples were run in frozen buffered aqueous solution. An accessory transfer chamber, which allowed controlled cooling of the samples, was used in the spectrometer source housing (11). The solutions were frozen to about -90°C before submitting them to the spectrometer vacuum of about 10^{-5} torr. Each ferredoxin spectrum represents the best data obtained from experiments with from three to five different samples of the same protein. Spectra composed of several peaks were resolved by curve-fitting with lorentzian lines of restricted half-widths by means of a computer program described elsewhere (12).

X-ray doses of 10^5 r. are known to decompose cystine and cysteine in aqueous solution at room temperature (13). Radiation decomposition of amino acids in the dry, solid state in air is also known to occur (13). Experiments were carried out to determine the radiation dose to which the samples in our spectrometer were subjected. Exposure of a radiation safety film badge to the conditions of a typical experiment indicated a maximum dose rate of approximately 10^3 rads/hr. To determine the effect of this quantity of radiation on the proteins, samples of spinach ferredoxin, frozen to dry ice temperatures, were subjected to a dose of 3×10^4 rads of Co^{60} γ -rays. These experiments showed that the biological activity of the irradiated sample, as determined by the rate of NADP

reduction (9), decreased approximately 10 to 15% from an unirradiated control sample. As all of the spectra presented in this paper were obtained within 12 hours, bulk decomposition does not appear to be a serious factor.

The purity of the ferredoxin samples, from which XPS spectra were obtained, was checked optically. All samples had purity ratios within 10% of the optimum literature values. The optical purity ratios of the samples, before and after XPS spectra were obtained, appeared to remain constant to within 10%. However, XPS spectra result from the top few hundred angstroms of the sample surface (7), and degradation in only these layers would be difficult to detect by an optical method (since only a fraction of the sample would be affected). These top layers are submitted not only to an X-ray flux of about 1000 rads/hr, but are also susceptible to freeze-drying effects and possibly surface reactions (although measures were taken to avoid the freeze-drying phenomenon) (11). The spectra presented here were consistently reproducible using about three different samples for each protein, and the corresponding spectra did not deteriorate significantly with time. Thus, the proteins investigated by this XPS method did not explicitly show the effects of decomposition.

EXPERIMENTAL RESULTS

Figure 2 shows the S2P photoelectron spectra of the four non-heme iron proteins under investigation and also that of clostridial apoferreredoxin. This apoferreredoxin was prepared in the laboratory of Dr. J. C. Rabinowitz by removing the iron and labile sulfur from clostridial ferredoxin, leaving only cystine groups in the protein (14).

The corresponding Fe3P photoelectron spectra are shown in Figure 3. These spectra have been adjusted by subtracting out a constant background from the raw data. It should be noted that the typical Fe3P spectrum displays a half-width of about 2.8 eV. However, the apparent broadening in the iron spectra of these proteins is not necessarily due to the existence of chemically or magnetically different iron species, but may result from the very poor statistical resolution of the Fe3P photoelectrons from these proteins.

Table II summarizes the quantitative results of these iron and sulfur spectra. The estimated error to the measured B.E.s is the scatter of values obtained from several experiments with each protein. The area ratios have also been estimated by considering all experimental spectra but, nevertheless, are only a rough estimate and are not to be taken as precisely accurate.

These non-heme iron proteins were reduced using $\text{Na}_2\text{S}_2\text{O}_3$ (or, in the case of HIPIP, oxidized with $\text{K}_3\text{Fe}(\text{CN})_6$) and investigated as frozen solutions. However, no significant change in the photoelectron spectra could be detected upon reducing or oxidizing the proteins. However, in many cases the reduced proteins showed significant decomposition after exposure to the experimental conditions and thus unequivocal interpretation of the effects of reduction upon the XPS spectra was not possible.

Possible model ligating systems of the iron-sulfur complex in the non-heme iron proteins are presented in Tables III and IV. Table III contains the measured Fe3P and S2P B.E.s and calculated charges of synthesized model iron-sulfur complexes. Table IV contains the calculated iron and sulfur charges of hypothetical model iron-sulfur complexes. The data of Tables II, III, and IV are summarized by Figure 4, in which the B.E.s of

the non-heme iron proteins are represented as short lines within the semicircles. The uncertainty in these B.E.s is represented by the length of these lines. Model complexes from Table IV are abbreviated by the notation M.C. in Figure 4.

To provide background information for use in the interpretation of the protein spectra, the iron and sulfur photoelectron spectra of a diverse series of compounds of known stoichiometry and structure were collected. In a previous publication we have presented these results together with an interpretation based upon the extended Hückel theory (15). These data are presented in Table V and Figure 5. We will consider these model data in the Discussion section.

DISCUSSION

The photoelectron spectrum of a chemical system should give insight into the electronic structure of that system, since changes in chemical environment of an atom will correspondingly change the B.E.s of its core electrons (7). When interpreting our XPS data we use the assumption that similar atomic binding energies represent similar chemical environments. Although this assumption is necessary, it is not sufficient alone for interpreting XPS spectra. Molecular orbital calculations of atomic binding energies or parameters related to B.E.s in synthesized and hypothetical model complexes can also be helpful in substantiating the interpretation of the XPS spectra. We have established, in a separate publication (15), direct correlations between electron B.E. and atomic charge, calculated by means of extended Hückel theory, for a diverse series of iron and sulfur compounds.

Atomic charges and binding energies of the model complexes were calculated using the extended Hückel method formulated by Hoffmann (16,17), with modifications to the Coulomb integrals and Slater exponents such that iteration to charge self-consistency could be obtained. This method does not include electron repulsion terms explicitly, and uses empirical parameters for evaluating the elements of the secular determinant. Table V shows a direct correlation between calculated B.E. and calculated charge. From this observation it seems reasonable to expect similar results for attempts to correlate measured electron B.E.s with either calculated charge or calculated electron B.E.s. However, the correlations are admittedly somewhat artificial since the simple extended Hückel calculations are insensitive to some important contributions to electron energy which include electron correlation, relativistic and relaxation effects. Because of its success in predicting relative B.E.s in organic complexes (15), the extended Hückel formalism offers the best method available to calculate charge and binding energy for such large molecular systems as we deal with here, despite its limitations mentioned above.

Using these correlations and the XPS data from a number of inorganic iron-sulfur complexes we have interpreted the XPS spectra from the non-heme iron proteins. Where applicable, we also compare the results of other spectroscopic techniques with those of XPS.

The sulfur data are especially interesting. Rubredoxin, with no labile sulfur atoms and 4 cysteine sulfurs, shows one S2P photoelectron peak at 162.7 eV, which is relatively close to the value for S2P photoelectrons from clostridial apoferrredoxin. Correlations between B.E. and calculated charge (as shown in Figure 5) indicate that a B.E. of 163 eV corresponds

to a sulfur charge of about zero. This is consistent with the apoferreredoxin, which contains cysteine sulfurs. However, X-ray diffraction results (19) indicate the four sulfurs of oxidized rubredoxin are bound to iron in a tetrahedral arrangement, and the XPS of inorganic complexes show such sulfurs to exhibit typically an S2P photoelectron peak at about 161.5 eV (see Table III and Figure 4). Consequently, the rubredoxin S2P B.E. of 162.7 eV must be interpreted as an unusual bonding situation where the cysteine sulfur is weakly bonded to neutral iron such that little or no charge is effectively transferred between the two atoms, or, considering item 1c of Table IV, as cysteine sulfur bonded to iron in a positively charged complex wherein any negative charge on the sulfur would be neutralized. The peak at about 168 eV in the rubredoxin spectrum corresponds to the S2P electrons from the $\text{SO}_4^{=}$ ion, which is apparently present with this protein.

HIPIP, with four labile sulfur atoms and four cysteine sulfurs, yields two sulfur photoelectron peaks of approximately equal intensity at B.E.s of 162.9 eV and 161.5 eV. The value at 162.9 eV corresponds closely to that of the S2P photoelectrons from rubredoxin and is given a similar interpretation. As illustrated in Figure 4, the value of 161.5 eV corresponds more nearly to the typical value of an iron-bonded sulfur found in the iron-sulfur complexes of Table III. The sulfur associated with this B.E. is assumed to be the "labile" sulfur and to be bonded only to the iron and not to the protein. The third S2P peak at highest B.E. is assumed to originate from an oxidized sulfur species not associated with the active site.

Spinach ferredoxin, with two labile sulfur atoms and five cysteine sulfurs, exhibits a spectrum which can be decomposed into at least two

S2P photoelectron peaks in the approximate ratio of 5 to 2 (as determined by a computer analysis). Through a lorentzian curve fit, these peaks are determined to occur at 163.1 eV and 161.1 eV, respectively. The value at 163.1 eV is taken to correspond to that of rubredoxin and is interpreted similarly, while the value at 161.1 eV again corresponds more nearly to the sulfur of inorganic iron-sulfur complexes and is assigned to the "labile" sulfur.

Clostridial ferredoxin, with six to eight labile sulfurs and six to eight cysteine sulfurs, also can be decomposed into at least two photoelectron peaks in the approximate ratio of 4 to 3. It should be noted, however, that in at least one experiment with this protein, an intensity ratio of 1 to 1 was found. It is not certain which ratio is the more accurate. The B.E. value of 163.2 eV is interpreted in a manner similar to that of rubredoxin, while the value at 161.5 eV corresponds again to an iron-bonded sulfur found in the iron-sulfur complexes.

Curiously, the XPS spectrum of ammonium sulfate precipitated clostridial ferredoxin displayed only one S2P peak at 161.3 eV aside from a sulfate S2P peak at 168 eV. This could possibly be due to a difference in bonding between solution and crystallized ferredoxin. There is also the possibility that the spectral intensity at approximately 163 eV is due at least in part to cysteine groups in denatured protein since no samples attained 100% purity and surface decomposition would not be detected directly. Finally, it should again be noted that the intensity ratios given for the above protein spectra are only rough estimates based on the assumption that only two peaks are present; thus quantitative interpretation of sulfur content cannot be made with certainty.

Due to the ambiguities of peaks position and the unspecified origin of spectral broadening, interpretation of the iron data is somewhat less certain than is the sulfur data. Rubredoxin, containing only one iron atom, yields a single Fe3P peak of normal half-width. The Fe3P B.E. of 54.6 eV is unusually high compared to those of the iron-sulfur complexes listed in Table III and Figure 4. EPR investigations of oxidized rubredoxin indicate that the iron is in the ferric state, and since X-ray diffraction data give a structure which would not be expected to delocalize charge to a great degree, the high Fe3P B.E. is compatible with the EPR interpretation. A complex such as shown in 1c of Table IV could give an XPS spectrum corresponding to that of rubredoxin, since the high positive charge on the iron should result in a relatively high Fe3P B.E., while the zero charge on the sulfur would correspond to an S2P B.E. of approximately 163 eV. HIP, clostridial ferredoxin and spinach ferredoxin have somewhat similar Fe3P B.E.s ranging from 53.0 to 53.6 eV. As seen in Figure 4, these values are much more consistent with those of the iron-sulfur complexes presented in Tables III and IV. X-ray diffraction data (21,22) indicate that these latter three proteins contain iron associated with S in Fe-S clusters. This type of chemical environment would most likely result in electron delocalization, and consequently it would be difficult to assign a specific oxidation state to the iron of these proteins. This electron delocalization would be consistent with the low Fe3P B.E.s exhibited by the proteins. Such close association of irons is also suggested by EPR and magnetic susceptibility studies (23-25). Interpreting EPR data from spinach ferredoxin (clostridial ferredoxin has a similar EPR spectra), Brintzinger et al. (26) devised a model of an

iron-sulfur center as two iron-centered tetrahedra joined at an edge. Gibsen et al. (27), interpreting the same data, assumed that the active moiety contains, in the oxidized form, two high-spin Fe^{3+} irons which are anti-ferromagnetically coupled to form a singlet state. It has also been reported that oxidized clostridial ferredoxin gives a magnetic susceptibility per iron which shows a linear increase with temperature over the range 6 to 65°C, indicating strong anti-ferromagnetic coupling between irons (24). If the S'' charge on model complex #2 from Table IV were more positive (and thus have a higher $S2P$ B.E.) through perhaps a charged complex, then this type of model would be compatible with the XPS spectra of spinach and clostridial ferredoxin and HIPIP. The $\text{Fe}3P$ photoelectron peak broadening can possibly be due to slightly different chemical environments of the iron atoms or to core polarization effects (12). Core polarization phenomena are due to electron exchange interactions between unpaired valence electrons and the electrons of the inner atomic shells. The net result is a splitting of core electron binding energies of several electron volts. Information concerning spin state and ligand arrangement is derived from the intensities and separation of the split photoelectron lines. However, the $\text{Fe}3P$ peak broadening may simply result from the poor statistical resolution; thus, no definite interpretation can presently be attributed to this broadening.

The observation that both the iron and "inorganic" sulfur peaks are absent in the XPS spectra of apoferredoxin is consistent with the optical data, i.e., these constituents are associated with the chromophoric group in the native protein. Unlike optical spectroscopy experimental difficulties mentioned above have thus far precluded assessment of the effects of reducing agents on the XPS spectra of the non-heme iron proteins.

CONCLUSION

The XPS data of the non-heme iron proteins have been interpreted with respect to hypothetical and synthesized iron-sulfur complexes. Where applicable these interpretations have been shown to be compatible with results from other spectroscopic techniques. Essentially we have found that the XPS data from rubredoxin, exhibiting relatively high B.E.s for the iron and sulfur atoms and only one sulfur peak, are compatible with a structure similar to item 1c of Table IV. On the other hand, the XPS data from the other three proteins investigated, exhibiting relatively low B.E.s for the iron atoms and two sulfur peaks, are more compatible with a structure similar to item 2 of Table IV. These interpretations are admittedly somewhat generalized, but can be used as a foundation for more conclusive work in the future.

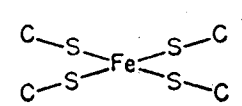
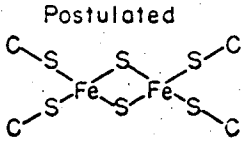
This report has demonstrated the feasibility of using XPS for studying biological systems. Experimental limitations of spectral resolution and intensity should be overcome in order to yield much more information than presently is possible. For example, valuable information concerning ligand arrangements about an atom, electron distribution and spin state can be obtained by studying core polarization phenomenon of suitable complexes (12). One must always be concerned about the detrimental effects of radiation upon biological systems, and better methods should be developed to monitor the purity of a sample's uppermost layers. With improvements in equipment and technique, this spectral method should prove a valuable tool in probing biological systems.

REFERENCES

1. MALKIN, R., AND RABINOWITZ, J. C., Ann. Rev. Biochem., 36, 113 (1967).
2. KIMURA, T., Struct. Bonding (Berlin), 5 1 (1968).
3. TSIBRIS, J.C.M., AND WOODY, R. W., Coordination Chemistry Reviews, Elsevier Publishing Co., Amsterdam, 1970.
4. DER VARTANIAN, D. V., ORME-JOHNSON, W. H., HANSEN, R. E., AND BEINERT, H., Biochem. Biophys. Res. Commun., 26, 569 (1967).
5. SHETHNA, Y. I., WILSON, P. W., HANSEN, R., AND BEINERT, H., Proc. Nat. Acad. Sci. U.S.A., 52, 1263 (1964).
6. HAGSTROM, et al., in K. SIEGBAHN (Editor), Alpha- Beta- and Gamma-Ray Spectroscopy, Vol. 1, North-Holland, Amsterdam, 1968, p. 845.
7. SIEGBAHN, K., et al., ESCA--atomic, molecular, and solid state structure studied by means of electron spectroscopy, Almquist and Wiksella, Uppsala, 1967.
8. FADLEY, C. S., HAGSTROM, S.B.M., KLEIN, M. P., AND SHIRLEY, D. A., J. Chem. Phys., 48, 3779 (1968).
9. TAGAWA, K., AND ARNON, D., Nature, 195, 537 (1962). We are indebted to Mr. Alexander Sun for his patient instruction and aid in preparing spinach ferredoxin.
10. SIEGBAHN, K., NORDLING, C., AND HOLLANDER, J. M., Lawrence Radiation Laboratory Report, UCRL-10023.
11. KRAMER, L. N., AND KELIN, M. P., J. Chem. Phys., 51 #8, 3618 (1969).
12. FADLEY, C. S., Ph.D. dissertation, University of California, Berkeley, 1970, Lawrence Radiation Laboratory Report, UCRL-19535. (This program was developed by Claudette Ruge of the Lawrence Radiation Laboratory.)
13. GREENSTEIN, J. P., AND WINITZ, M., Chemistry of the Amino Acids, Vol. 1, John Wiley and Sons, Inc., New York, 1961, p. 683.

14. MALKIN, R., AND RABINOWITZ, J. C., Biochem. Biophys. Res. Commun., 23, 822 (1966).
15. KRAMER, L. N., AND KLEIN, M. P., Chem. Phys. Letters, 8 #2, 183 (1971).
16. HOFFMANN, R., AND LIPSCOMB, W. N., J. Chem. Phys., 36, 2179, 3489 (1962).
17. HOFFMANN, R., J. Chem. Phys., 39, 1397 (1963).
18. VAN DER VOORN, P. C., AND DRAGO, R. S., J. Amer. Chem. Soc., 88, 3255 (1966).
19. HERRIOTT, J. R., STEKER, L. C., JENSEN, L. H., AND LOVENBERG, W., J. Mol. Biol., 50, 391 (1970).
20. CASTNER, T., et al., J. Chem. Phys., 32, 668 (1960).
21. STRAHS, G., AND KRAUT, J., J. Mol. Biol., 35, 503 (1968).
22. SIEKER, L. C., AND JENSEN, L. H., Biochem. Biophys. Res. Commun., 20, 33 (1965).
23. BEINERT, H., AND SANDS, R. H., Biochem. Biophys. Res. Commun., 3, 41 (1960).
24. ORME-JOHNSON, W. H., AND BEINERT, H., Biochem. Biophys. Res. Commun., 36, 337 (1969).
25. POE, M., PHILLIPS, W. D., McDONALD, C. C., AND LOVENBERG, W., Proc. Nat. Acad. Sci. U.S.A., 65, 797 (1970).
26. BRINTZINGER, H., PALMER, G., AND SANDS, R. H., Proc. Nat. Acad. Sci. U.S.A., 55, 397 (1966).
27. GIBSON, J. F., et al., Proc. Nat. Acad. Sci. U.S.A., 56, 987 (1966).

TABLE I

PROTEIN	M.W.	(1) No. of Fe (2) No. of S _i (inorg.) (3) No. of S _o (cysteine)	REDOX POTENTIAL	EPR	X-RAY DIFFRACTION (Fe-S moiety)
RUBREDOXIN	6,000	(1) 1 Fe (2) 0 S _i (3) 4 S _o	-57 mV	Oxidized: 4.3, 9 Reduced: (none)	
SPINACH FERREDOXIN	12,000	(1) 2 Fe (2) 2 S _i (3) 5 S _o	-430 mV	Oxidized: (none) Reduced: 1.89, 1.94, 2.01	Postulated 
HIPIP	10,000	(1) 4 Fe (2) 4 S _i (3) 4 S _o	+330 mV	$g_{II} = 2.12, g_I = 2.04$ Oxidized: (none) Reduced: (none)	Unknown
CLOSTRIDIAL FERREDOXIN	6,000	(1) 6-8 Fe (2) 6-8 S _i (3) 8 S _o	-410 mV	Oxidized: (none) Reduced: 1.89, 1.96, 2.00	Unknown

XBL714-5123

Summary of important physical characteristics of the four non-heme iron proteins under investigation.

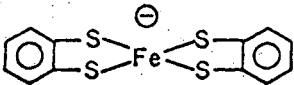
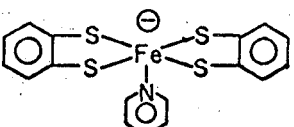
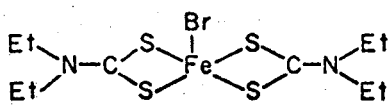
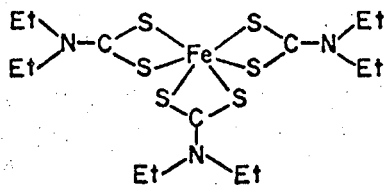
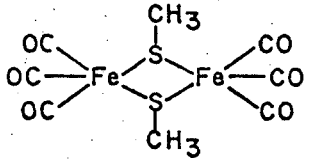
TABLE II

PROTEIN	STOICHIOMETRY OF Fe-S _i MOIETY	Fe3P B.E.	AREA RATIO	S2P B.E.	AREA RATIO
OXIDIZED RUBREDOXIN	1 Fe-O S _i 4 Cysteines	54.6 ± .4 eV	1	162.7 ± .3 eV	1
REDUCED HIPIP	4 Fe-4 S _i 4 Cysteines	53.0 ± .4 eV	1	162.9 ± .3 eV 161.5 ± .3 eV	1 1
OXIDIZED CLOSTRIDIAL FERREDOXIN	X Fe-X S _i 8 Cysteines (x = 6 to 8 atoms)	53.4 ± .4 eV	1	163.2 ± .3 eV 161.5 ± .4 eV	(approx) 4 3
OXIDIZED SPINACH FERREDOXIN	2 Fe-2 S _i 5 Cysteines	53.6 ± .5 eV	1	163.1 ± .4 eV 161.1 ± .4 eV	(approx) 5 2
APO CLOSTRIDIAL FERREDOXIN	0 Fe-0 S _i 4 Cystines	— — — —	—	163.2 ± .2 eV	1

XBL 714-5124

Summary of the quantitative results from the XPS spectra of the non-heme iron proteins. The area ratios are only approx. values derived from lorentzian functions fitted to the XPS data.

TABLE III

MODEL COMPOUNDS	Fe3P B.E.(eV)	CALC. CHARGE	S2P B.E.(eV)	CALC. CHARGE
FeS_2	53.0	0.45	161.5	-0.22
FeS	—	0.43	160.7	-0.43
	53.2	0.29	161.4	-0.43
	52.9	—	161.4	—
KFeS_2	—	0.35	161.1	-0.46
	54.0	0.82	161.4	-0.3
	53.5	0.95	161.5	-0.3
	54.9	—	162.3	—
$(\text{C}_5\text{H}_5\text{FeS})_4$	54.0	—	162.1	—

XBL 714-5125

Measured Fe3P and S2P B.E.s and calculated charges of synthesized model iron-sulfur complexes. The B.E.s are reproducible to within .3 eV and the charges are self-consistent to within .05 charge units.

TABLE IV

	MODEL COMPLEX	CALC. Fe CHARGE	CALC. S' CHARGE	CALC. S" CHARGE	BOND DIST. #1 (Å)	BOND DIST. #2 (Å)
(1)	H₃C-S-①S'②CH₃ H₃C-S-Fe-S-CH₃ (a) Tetrahedral, neutral (b) Tetrahedral, +2 complex (c) Tetrahedral, +3 complex	0.37	-0.22	—	2.3	1.82
		0.63	-0.06	—	2.3	1.82
		0.71	0	—	2.3	1.82
(2)	H₃C-S-①S'②S''-CH₃ H₃C-S-Fe-S-Fe-S-CH₃ Tetrahedral, neutral	0.66	-0.31	-0.32	2.24	2.44
(3)	CH₃ H₃C-S-①S'②S''-CH₃ H₃C-S-Fe-S-Fe-S-CH₃ Tetrahedral, neutral	0.64	+0.15	-0.23	2.24	2.44
(4)	H₃C-N-H H-N-Fe-S'①②S''-CH₃ H₃C-S-Fe-S-Fe-S-CH₃ H₃C-N-H Pseudotetrahedral, neutral	0.3	-0.6	+0.1	2.3	2.48

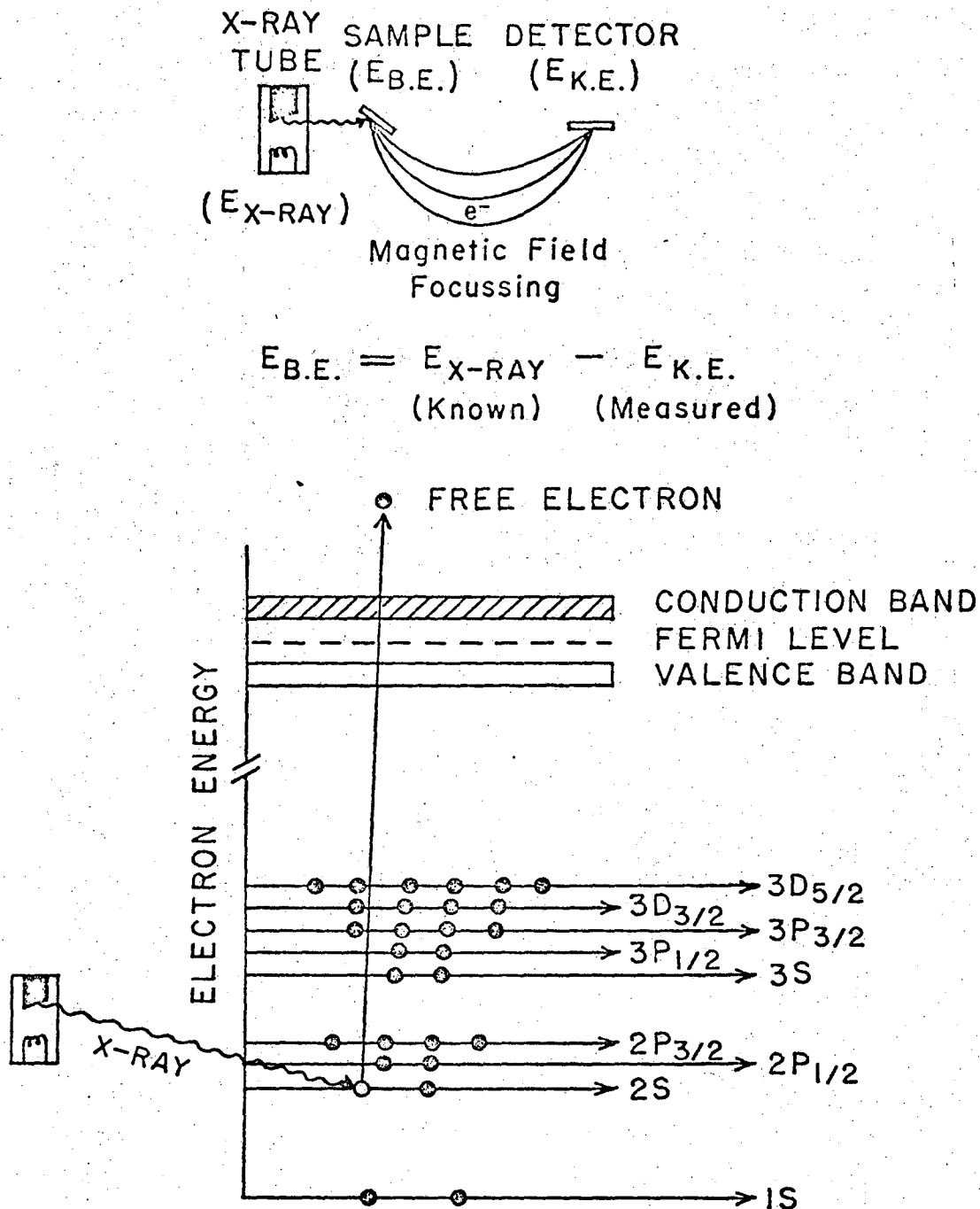
XBL 714-5126

Calculated iron and sulfur charges of hypothetical model iron-sulfur complexes. The charges are self-consistent to within .05 charge units.

TABLE V

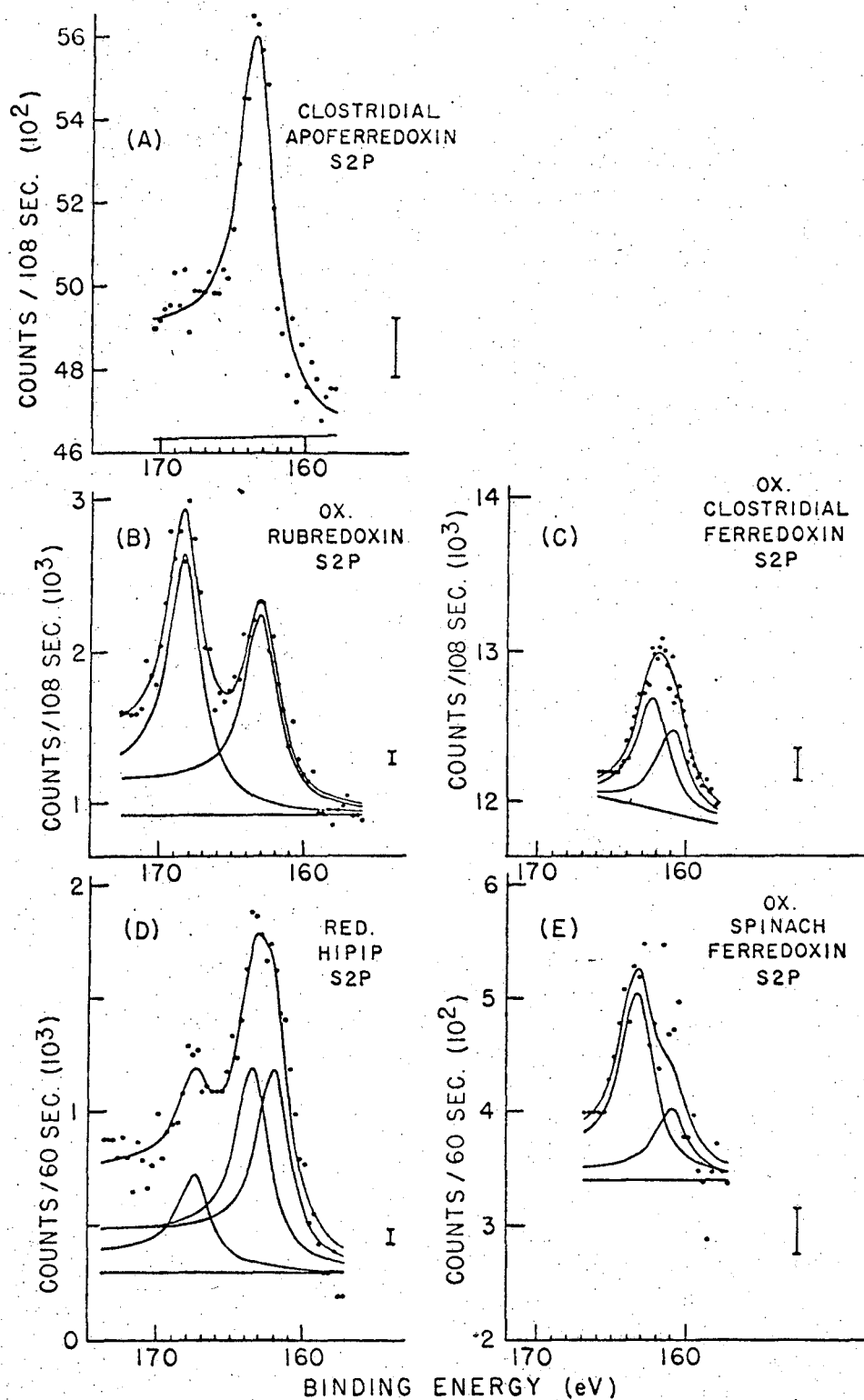
Measured and calculated electron binding energies and calculated charges for a diverse series of iron and sulfur compounds. The B.E.s are reproducible to within .3 eV and the charges are self-consistent to within .05 charge units.

No.	Molecule	Measured Fe3P B.E. (eV)	Calc. Fe3P B.E. (eV)	Calc. iron charge	Measured S2P B.E. (eV)	Calc. sulfur charge
1	FeF_6 (K_3FeF_6)	57.7	56.0	+1.81		
2	FeO_4^- (K_2FeO_4)	57.7	56.1	+1.79		
3	$\text{Fe}(\text{H}_2\text{O})_6^{+3}$ ($\text{Fe}_2(\text{SO}_4)_3 \cdot$ $(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$)	56.6		+1.51		
4	$\text{Fe}(\text{H}_2\text{O})_6^{+2}$ ($\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$ $\cdot 6\text{H}_2\text{O}$)	54.2		+0.86		
5	$\text{Fe}(\text{CN})_6^{-3}$ ($\text{K}_3\text{Fe}(\text{CN})_6$)	55.0		+1.24		
6	$\text{Fe}(\text{CN})_6^{-4}$ ($\text{K}_4\text{Fe}(\text{CN})_6$)	54.0		+1.03		
7	Fe(metal)	52.4	52.0(def.)	0(def.)		
8	$\text{Fe}(\text{C}_5\text{H}_5)_2$	53.7	54.5	+1.00		
9	$\text{Fe}(\text{C}_5\text{H}_5)_2^+ \cdot (\text{NO}_2)_3\text{C}_6\text{H}_2\text{O}^-$	54.9		+1.36		
10	$\text{Fe}(\text{CO})_5$	54.0	54.7	+1.02		
11	$\text{Fe}_2(\text{CO})_9$	54.6		+1.30		
12	FeS_2	53.0	53.3	+0.45	161.5	-0.22
13	$\text{Fe}(\text{S}_2\text{CNETet})_3$	53.5		+0.95	161.5	-0.3
14	$\text{FeBr}(\text{S}_2\text{CNETet})_2$	54.0	54.1	+0.82	161.4	-0.3
15	$\text{Fe}(\text{S}_2\text{C}_6\text{H}_3\text{CH}_3)_2^- \cdot \text{N}(\text{n-C}_4\text{H}_9)_4^+$	53.2		+0.29	161.4	-0.43
16	Ferrichrome A	54.9	55.5	+1.53		
17	Hemin Cl	54.2		+1.04		
18	Fe^{+3}Cl Phthalocyanine	54.4		+1.46		
19	SO_4^-				167.8	+1.86
20	SO_3^-				166.4	+1.40
21	CH_3SOCH_3				165.5	+0.70
22	CH_3SSCH_3				162.7	-0.13
23	CH_3SH				162.7	+0.09
24	FeS			+0.43	160.7	-0.43
25	KFeS_2			+0.35	161.1	-0.46



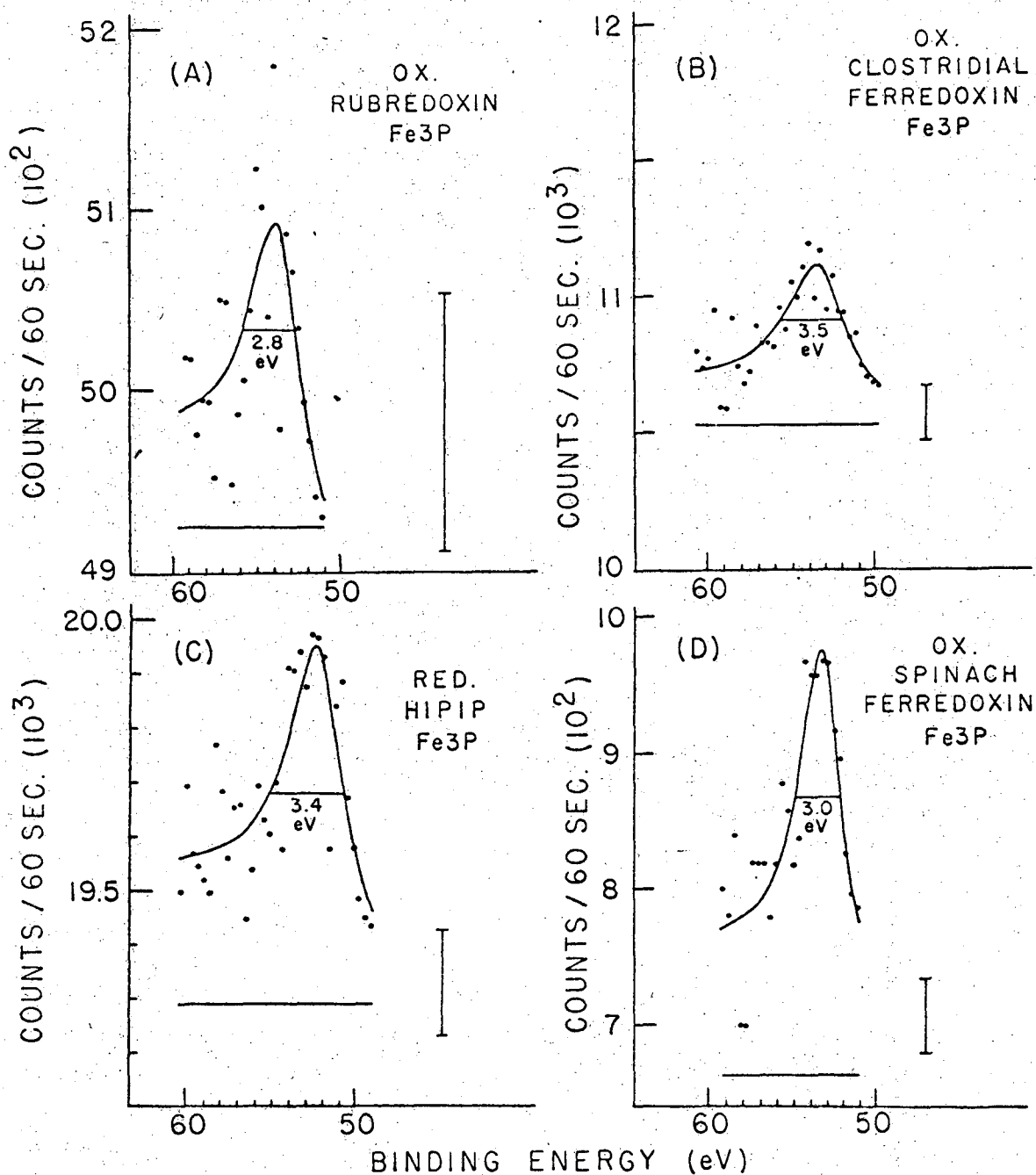
XBL 705-5218

Figure 1. Schematic outline of X-ray photoelectron method. A 2S electron is shown being ejected into the energy continuum by an X-ray photon.



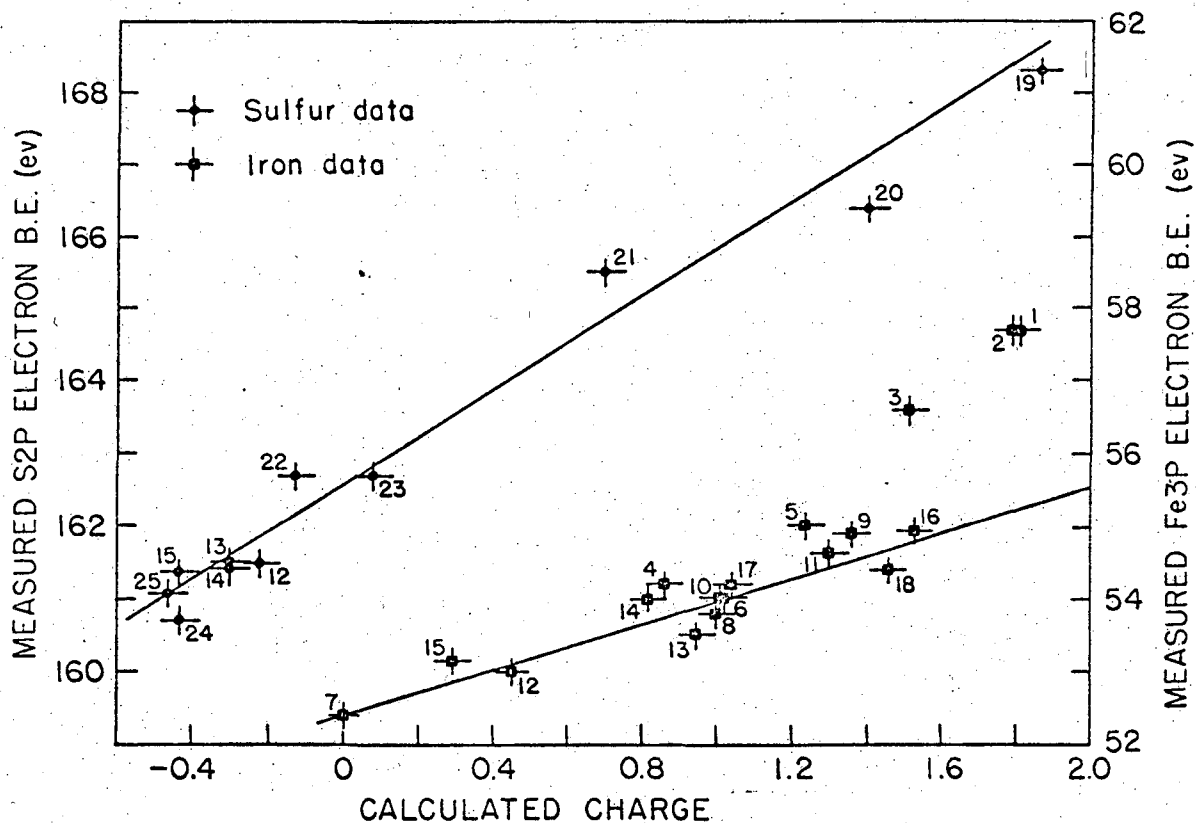
XBL714-5127

Figure 2. S2P photoelectron spectra of the four non-heme iron proteins under investigation and also that of clostridial apoferredoxin. The spectra have been fitted with lorentzian curves by means of a least squares computer analysis.



XBL714-5128

Figure 3. Fe3P photoelectron spectra of the four non-heme iron proteins under investigation. The spectra have been fitted with lorentzian curves by means of a least squares computer analysis.



XBL 708-5303

Figure 5. Plot of measured Fe3P B.E. and S2P B.E. versus calculated charge in iron and sulfur compounds chosen to represent a diversity of electronic environments. The line drawn through the iron data points is a least squares fit to the data from neutral molecules. The numbers associated with the data points correspond to the compounds listed in Table V.

APPENDIX

In order to obtain an overall view of the type of information obtainable from XPS as compared to other spectroscopies, presented below is a brief description of the information resulting from the main spectroscopic probes used to investigate the ferredoxins.

As reported in the main text, the EPR spectra of the non-heme iron proteins exhibit characteristic g values. These spectra can be interpreted with respect to the following hamiltonian:

$$(1) H = g\beta\hbar\vec{S} \cdot \vec{H}_D - H_D$$

$$\text{where } H_D = D[S_z^2 - 1/3 S(S+1)] + E(S_x^2 - S_y^2).$$

H_D is the zero field splitting arising from spin-orbit coupling and is dependent on the symmetry of the ligand field. This coupling modified the normal free electron g values to give g values characteristic of the system.

Measurement of magnetic susceptibility, X_M , of the non-heme iron proteins can be related to the spin state of the active moiety. A typical relationship between magnetic susceptibility, X_M , and spin magnetic moment, U_S , is as follows:

$$(2) X_M^{\text{corr}} = \frac{NU_S^2/3k}{T}$$

$$(3) U_S = g \sqrt{S(S+1)}$$

where the constants have their usual significance.

Mössbauer spectroscopy has been applied to several non-heme iron proteins. One can obtain information about the chemical environment and oxidation state of an iron species by determining the nuclear isomer shift which is due to the electrostatic interaction between the charge

distribution of the nucleus and that of electrons which have a finite probability at the nucleus (e.g., "S" electrons). Another parameter is the nuclear quadrupole splitting which is the result of the interaction of the nuclear quadrupole moment with the gradient of the electric field at the nucleus. The field gradient is caused by ligands and empty valence shells. Finally one can measure the magnetic hyperfine interaction,

$$(4) H_M = -g_N B_N \vec{I} \cdot \vec{H}$$

which is the interaction of the nuclear magnetic moment, I , with a magnetic field, H . The effective magnetic field acting on the nucleus arises from the electrons of the atom itself and is usually called the internal field. The main contribution to this field is given by the Fermi contact interaction, i.e., direct coupling between the nucleus and the unpaired S-electron density. One can also use an external magnetic field.

VI. INVESTIGATION OF THE BONDING AND NATURE OF THE PRUSSIAN
BLUE COMPLEXES USING X-RAY PHOTOELECTRON SPECTROSCOPY
AND E.H. M.O. CALCULATIONS

There has been much controversy in the past years over the nature of the iron atoms in the Prussian blue complexes. This problem has been reviewed recently in a paper by Wilde, et al.¹ The nominal chemical formulas of soluble and insoluble Prussian blue are $\text{KFe}(\text{Fe}(\text{CN})_6)$ and $\text{Fe}_4(\text{Fe}(\text{CN})_6)_3$, respectively.² However, these formulas are difficult to confirm because of the presence of adsorbed impurity ions^{1,15,16}

Although many investigations have attempted to characterize these complexes,¹⁻¹⁴ the actual constituents and the nature of bonding involved are still not unequivocally established. There are several possible structures which are compatible with X-ray and magnetic susceptibility data.¹ However, the generally accepted structure of the Prussian blues is a face-centered-cubic structure with either potassium ions or ferric ions occupying the interstices in the lattice. There is also uncertainty about the type of bonding between the iron and cyanide groups. Some investigators consider the Prussian blues to be supercomplexes,^{1,17} while others^{4,18} consider the Fe-N bond to be mainly ionic.

In order to help elucidate these uncertainties of the Prussian blue complexes, we have used a method of investigation which can obtain direct information about the electronic environment of an atom; i.e., photoelectron spectroscopy.

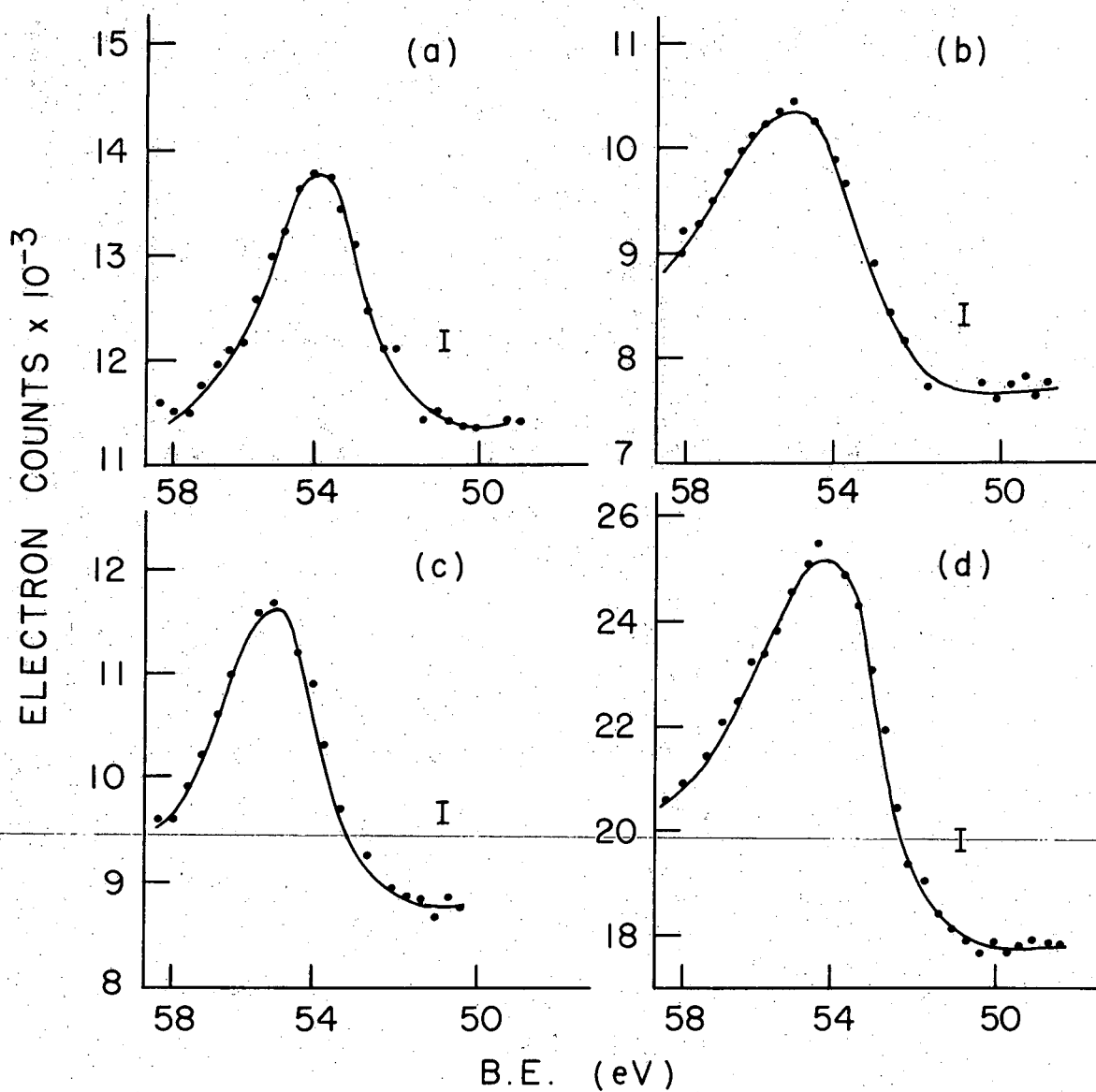
Recently, chemical shifts of inner-core electron binding energies have been observed in a variety of iron complexes,⁹ and have been related to the atomic charge by using self-consistent charge (S.C.C.) extended Huckel molecular orbital (M.O.) calculations.²⁰ Specifically, we have compared the photoelectron spectra of the Prussian blues and the calculated charges of a hypothetical approximation to these complexes with those of the iron cyanides.

Both insoluble and soluble Prussian blue were prepared by the method outlined in reference 4. The spectra were produced and analyzed in the Berkeley iron-free electron spectrometer.²¹ Figure 1 shows the photoelectron spectra of the iron cyanides and Prussian blues. Figures 2 and 3 show least squares fits to the Prussian blue data by Lorentzian curves restricted only in width, intensity, and number. For both Prussian blues only two peaks were assumed to contribute to the experimental data, with ratios of 1:1 for the soluble Prussian blue and 3:4 for the insoluble Prussian blue. The widths at half-maximum were restricted to that of the spectra of the iron cyanides (i.e., 2.8 eV). Table I lists the relative electron binding energies of the nitrogen 1S and iron 3P electrons in these compounds. Also listed in Table I are the calculated charges on the iron and nitrogen atoms. The calculated atomic orbital overlaps between the iron and carbon and the iron and nitrogen in the Prussian blue model are listed in Table II. Figure 4 shows the model used for the Prussian blue calculation; the values in Tables

I and II were determined for those atoms marked with the corresponding primes in Figure 4. Bond distances were obtained from reference 10, (Fe-C and Fe-N distances being 1.85 and 2.07 Å, respectively).

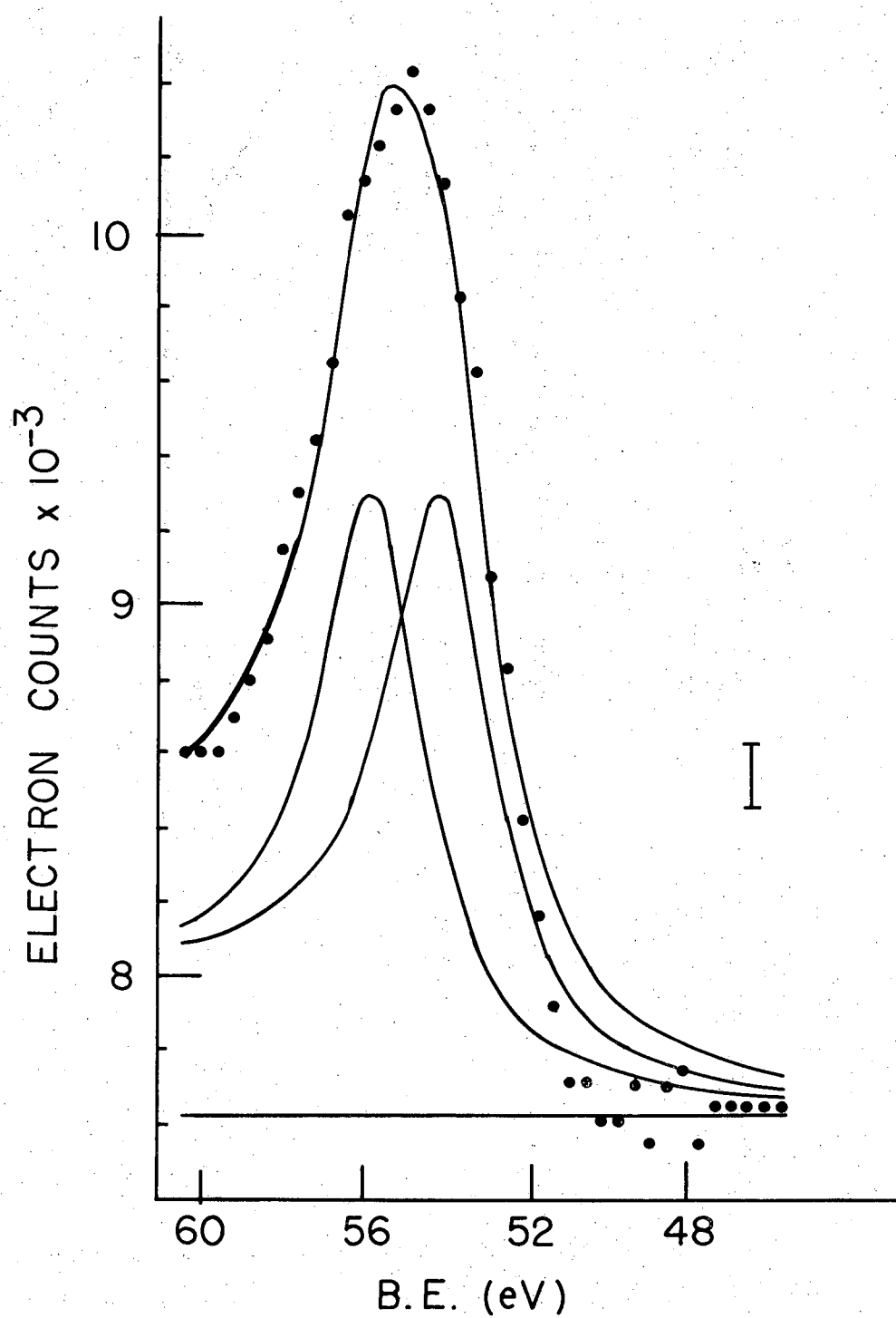
Although the Prussian blue spectra have been fitted with only two peaks, there is no conclusive evidence that this represents the true origin of the observed spectra. In fact, Wilde, et al.¹ have pointed out that iron impurities on the order of 10% can be expected in even the most carefully prepared samples of Prussian blues. Furthermore, the commonly accepted structure of insoluble Prussian blue includes interstitial Fe(III) ions surrounded by water molecules. If this is in fact true, the photoelectron spectra of these Fe(III) ions could fall anywhere within the Prussian blue spectra and not necessarily in the same position of the nitrogen-surrounded Fe(III) ions. Consequently, the postulated decompositions of the Prussian blue spectra shown in Figures 2 and 3 represent only a possible interpretation, which is probably more significant for the soluble Prussian blue complex than for the insoluble complex.

The method of charge calculation using extended Hückel theory has been described elsewhere.²⁰ Owing to the large number of electrons involved, we limited our Prussian blue representation to the hypothetical structure shown in Figure 4. Although this structure may not give the best quantitative charge values, the values should have at least qualitative significance with respect to other calculated charges using



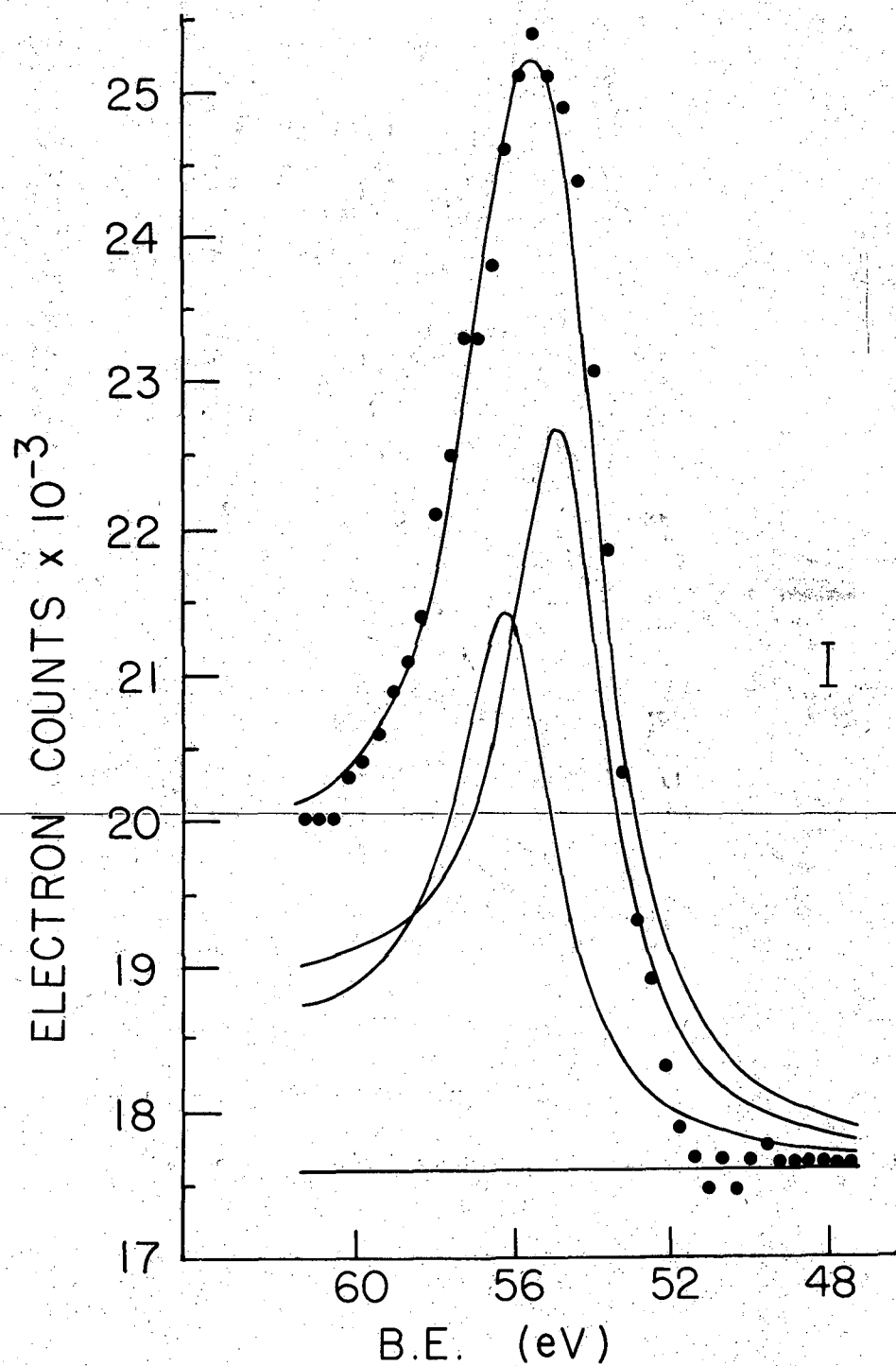
XBL717-5257

Figure 1. Fe3P photoelectron spectra of the Prussian Blue and iron cyanide complexes using Al K α irradiation.
 (a) $K_4Fe(CN)_6$. (b) Soluble Prussian Blue.
 (c) $K_3Fe(CN)_6$. (d) Insoluble Prussian Blue.



XBL 717-5258

Figure 2. Computer fit to soluble Prussian blue data by two equivalent Lorentzian curves.



XBL717-5261

Figure 3. Computer fit to insoluble Prussian blue data by two Lorentzian curves in the ratio of 3 to 4.

Table I

Measured Relative Binding Energies of N(1S) and Fe(3P)
Electrons and the Corresponding Calculated
Charges on the Atoms under Investigation

Compound	Fe(3P) B.E.(eV)	Fe(3P) Calc. charge	N(1S) B.E.(eV)	N(1S) Calc. charge
$K_4Fe(CN)_6$	54.0	$1.02 \pm .05$	397.1	-0.84 ± 0.05
$K_3Fe(CN)_6$	55.0	$1.22 \pm .05$	397.0	-0.80 ± 0.05
$KFeFe(CN)_6$	56.0	(Fe') $1.65 \pm .07$	397.1	-0.79 ± 0.07
	54.6	(Fe'') $1.23 \pm .07$		
$Fe_4[Fe(CN)_6]_3$	56.4	(Fe') $1.65 \pm .07$	397.2	-0.79 ± 0.07
	55.0	(Fe'') $1.23 \pm .07$		

Table II
Calculated Atomic Overlaps between the Corresponding
Fe-C and Fe-N Atoms Shown in Figure 4

	Fe' - C' Overlap				Fe'' - N' Overlap			
	C2s	C2Pz	C2Px	C2Py	N2x	N2Pz	N2Px	N2Py
Fe 3d _z ²	-.0834	0	.0939	0	-.0640	0	-.0767	0
Fe 3d _{xz}	0	.1246	0	0	0	-.0764	0	0
Fe 3d _{x²-y²}	.1444	0	-.1627	0	.1108	0	.1328	0
Fe 3d _{yz}	0	0	0	0	0	0	0	0
Fe 3d _{xy}	0	0	0	.1246	0	0	0	-.0764
Fe 4s	.3917	0	-.1281	0	.3216	0	.1135	0
Fe 4p _z	0	.2297	0	0	0	.1634	0	0
Fe 4p _x	.5479	0	-.1160	0	.4799	0	-.1367	0
Fe 4p _y	0	0	0	.2297	0	0	0	.1634

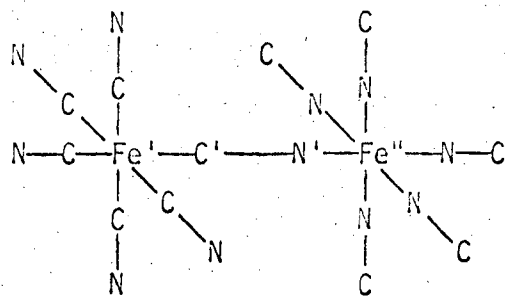


Figure 4. Hypothetical approximation to the Prussian blue structure (used for molecular orbital calculations).

this extended Huckel method. The calculations were carried out with the only restrictions being that the total charge of the two iron atoms should be +5, and each CN group should carry one extra electron, giving the total complex a -6 net charge. The most striking result of this calculation is that the greater charge is found on the carbon-surrounded iron atom, which has been considered to have only a +2 oxidation state as opposed to a +3 oxidation state for the other iron atom. In fact, there is a significant difference between the calculated iron charges in the hypothetical complex amounting to about 0.4 charge units (the difference between the ferro- and ferricyanides is 0.22 charge units). Compared with the 1.4 eV peak separation in the computed Prussian blue spectra, this computed charge difference is reasonable. Furthermore, the computed charge on the nitrogen atoms in both the Prussian blues and iron cyanides are very nearly the same within the accuracy of the computations. Consequently, one expects, and actually does obtain similar chemical shifts for the nitrogen electrons. The overlap matrices between the Fe-C and Fe-N atoms are shown in Table II. The qualitative significance of this data is that the strength of interaction between the Fe-N atoms is comparable to that between the Fe-C atoms. Hence, in assuming a Prussian blue structure based on Figure 4, one would not expect ionic bonding to play a significant role, except perhaps for interstitial counterions.

Table III presents the analytical results for the Prussian blues. The first column in this table contains the

results from net chemical analysis of the Prussian blues. Normalization of these results indicate that there is about 8% excess iron in the insoluble Prussian blue and almost 30% excess iron in the soluble Prussian blue. This excess may be attributed to iron impurities. Impurities can cause erroneous interpretation of XPS spectra. Consequently, the evidence presented by this method cannot give an unequivocal answer as to the nature of the iron species in the Prussian blues. However, on the assumption that the major portion of the XPS spectra results from the Prussian blue samples, the most certain observation one can make from the Prussian blue spectra is that there is more than one distinguishable iron species in both complexes. Because of the lack of peak resolution, the photoelectron spectra of the Prussian blue complexes cannot unequivocally support any one structure. They can only suggest reasonable possibilities. Furthermore, the molecular orbital calculations are necessarily crude and their value can only be judged from the consistency of their predictions, (e.g., as presented in a previous work).²⁰ Nevertheless, there is significant potential for this type of investigation into the electronic and structural environments of many chemical complexes.

Table III

A. Soluble Prussian Blue

Element	Theoretical wt. %	Experimental wt. %
C	15	12
N	18	13
Fe	23.7	29.5

B. Insoluble Prussian Blue

Element	Theoretical wt. %	Experimental wt. %
C	16	17.8
N	19	21.8
Fe	29.5	37

REFERENCES

1. R. E. Wilde, S. N. Ghosh, and B. J. Marshall, Paper presented at the 159th National Meeting of A.C.S., Houston, Texas, February 27, 1970.
2. B. M. Chadwick and A. G. Sharpe, Adv. Inorganic Chem. Radiochem., 8, 83 (1970).
3. L. M. Epstein, J. Chem. Phys., 36, 2731 (1962).
4. E. Fluck, W. Kerler and W. Newirth, Angew. Chem. Intern. Ed. Engl., 2, 277 (1963).
5. J. F. Duncan and P. W. R. Wigley, J. Chem. Soc., 1120 (1963).
6. K. Maer, Jr., M. L. Beasley, R. L. Collines, and W. O. Milligan, J. Am. Chem. Soc., 90, 3201 (1968).
7. A. Ito, M. Suenaga, and K. Ono, J. Chem. Phys., 48, 3597 (1968).
8. J. F. Keggin and F. D. Miles, Nature, 137, 577 (1936).
9. A. K. Van Bever, Rec. Trav. Chim., 57, 1259 (1938).
10. H. B. Weiser, W. O. Milligan and J. B. Bates, J. Phys. Chem., 46, 99 (1942).
11. M. B. Robin, Inorg. Chem., 1, 337 (1962).
12. P. S. Braterman, J. Chem. Soc. A, 1471 (1966).
13. D. Davidson and L. A. Welo, J. Phys. Chem., 32, 1191 (1928).
14. R. M. Bozorth, H. J. Williams and D. I. Walsh, Phys. Rev., 103, 572 (1956).
15. H. Holtzman, Ind. Eng. Chem., 37, 855 (1945).
16. H. B. Weiser, W. O. Milligan and J. B. Bates, J. Phys. Chem., 42, 945 (1938); 45, 701 (1941).

17. J. C. Bailer, ed., "The Chemistry of the Coordination Compounds," Reinhold Publ. Co., New York, N.Y., 1956, p. 275.
18. D. E. Knapp, Ph.D. Dissertation, Kent State University, Ohio (1967).
19. L. N. Kramer and M. P. Klein, J. Chem. Phys., 51, 3618 (1969).
20. L. N. Kramer and M. P. Klein, to be published.
21. K. Siegbahn, C. Nordling and J. M. Hollander, UCRL-10023 (1962).

VII. X-RAY PHOTOELECTRON INVESTIGATION OF MAGNESIUM AND ITS
NATURE IN CHLOROPHYLL SITUATED IN PHOTOSYNTHETIC
MEMBRANES

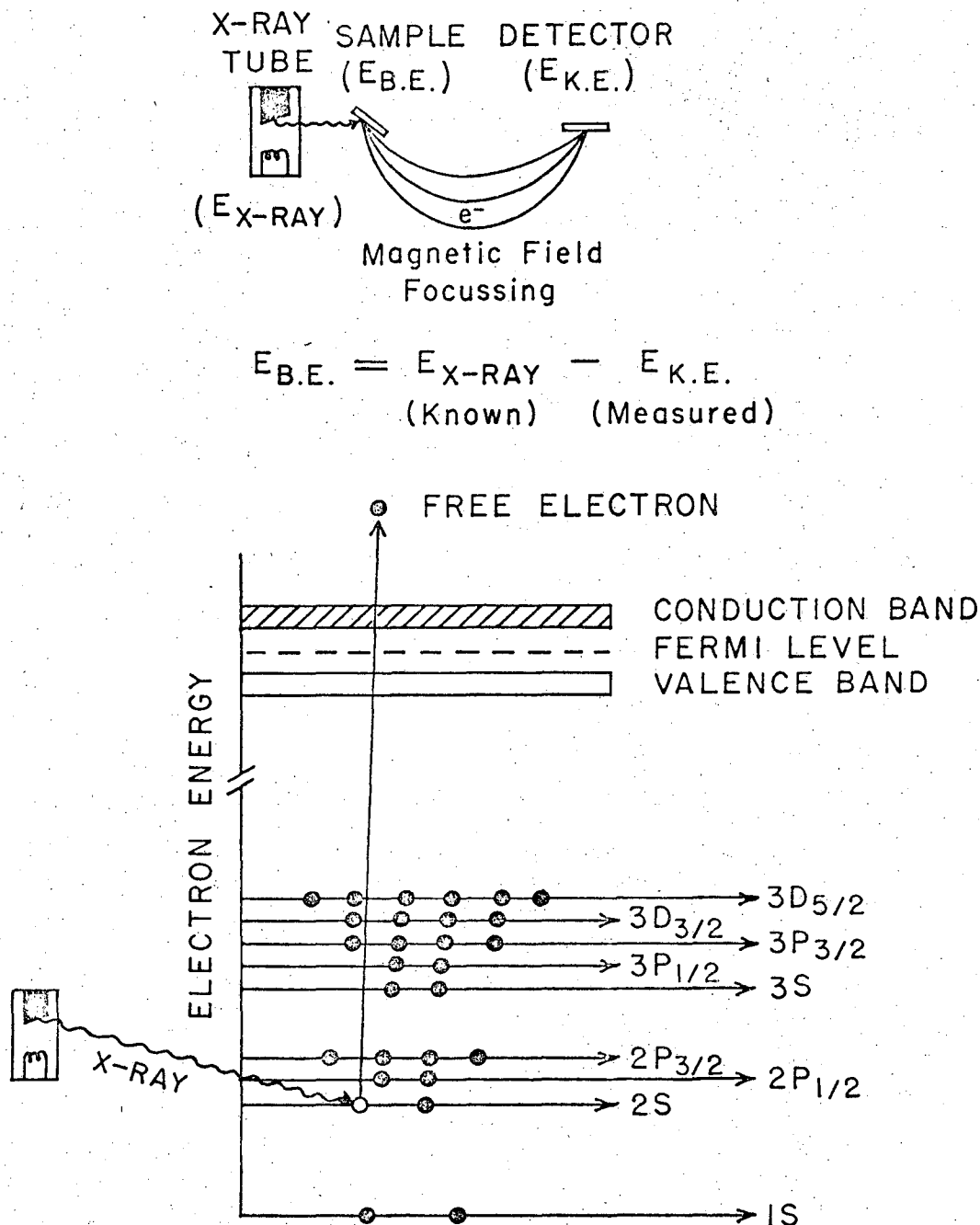
The role of chlorophyll molecules in the photosynthetic process has long been a subject of intensive investigation.^{1,2} However, the nature and role of magnesium in biologically active chlorophyll has not yet been unambiguously determined.

In order to help elucidate this problem, we have used a spectroscopy, X-ray photoelectron spectroscopy (XPS), which can give direct information concerning the electronic environment of magnesium (or of any element, for that matter). This technique has previously been described elsewhere,³ and only a brief sketch will be given here. Essentially, photoelectron spectroscopy involves bombarding a sample of the material to be investigated with low energy X-rays (e.g., Al(K α) X-rays with an energy of about 1400 eV) and analyzing the kinetic energy of the resulting photoelectrons. From this information, one can obtain the binding energy (B.E.) of the core electrons. These binding energies have been shown to exhibit chemical shifts³ due to a change in chemical (i.e., electronic) environment of the atom under investigation. This technique is illustrated in Figure 1.

In order to help interpret the photoelectron spectra of the chlorophyll magnesium found in biological systems, we have established a chemical shift series from a representative sampling of common magnesium complexes. Figure 2 illustrates

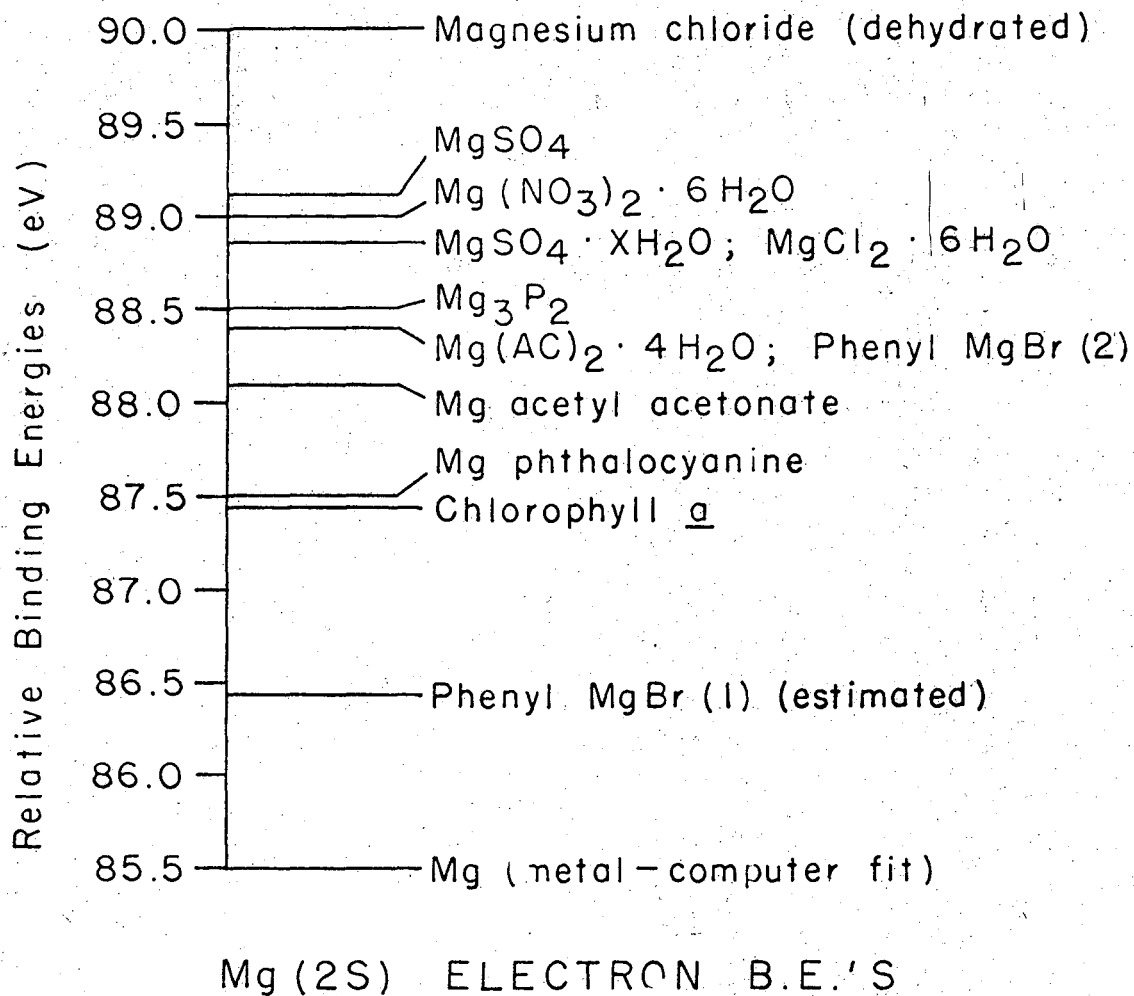
this series. In analyzing this data one should keep in mind that the values given are at best only relative, and solid state effects such as surface charging and work function variations can cause significant changes in the measured binding energy values. However, the values reported in Figure 2 have thus far been reproduced to approximately 0.2 eV.

A few comments are necessary to clarify the values shown in Figure 2. The Mg (2S) B.E. of magnesium metal was obtained by a computer fit of Lorentzian functions to the photoelectron spectra of magnesium foil; this is shown in Figure 3. The foil spectra showed a broadened peak, apparently corresponding to overlap of MgO and Mg metal spectra. A computer least squares fit by two Lorentzian curves of equal width resolved the foil spectra into components at 87.5 eV (corresponding to MgO) and at 85.5 eV (presumably from Mg metal). The photoelectron spectrum of Phenyl MgBr Grignard in frozen ether solution is shown in Figure 4; a broadened peak is observed. Due to the equilibrium of chemical species in Grignard reagents, $(R_2Mg + MgX_2 \rightleftharpoons 2 R MgX \rightleftharpoons (R MgX)_2)$, multiple peaks are indeed expected. Figure 5 shows the photoelectron spectra of magnesium chloride (dehydrated) and of $MgCl_2 \cdot 6H_2O$ frozen at $-50^\circ C$ to prevent dehydration in the vacuum system of the spectrometer. The dehydrated magnesium chloride spectrum was obtained after allowing the frozen magnesium chloride sample to dehydrate at room temperature while in the spectrometer sample chamber. The difference in



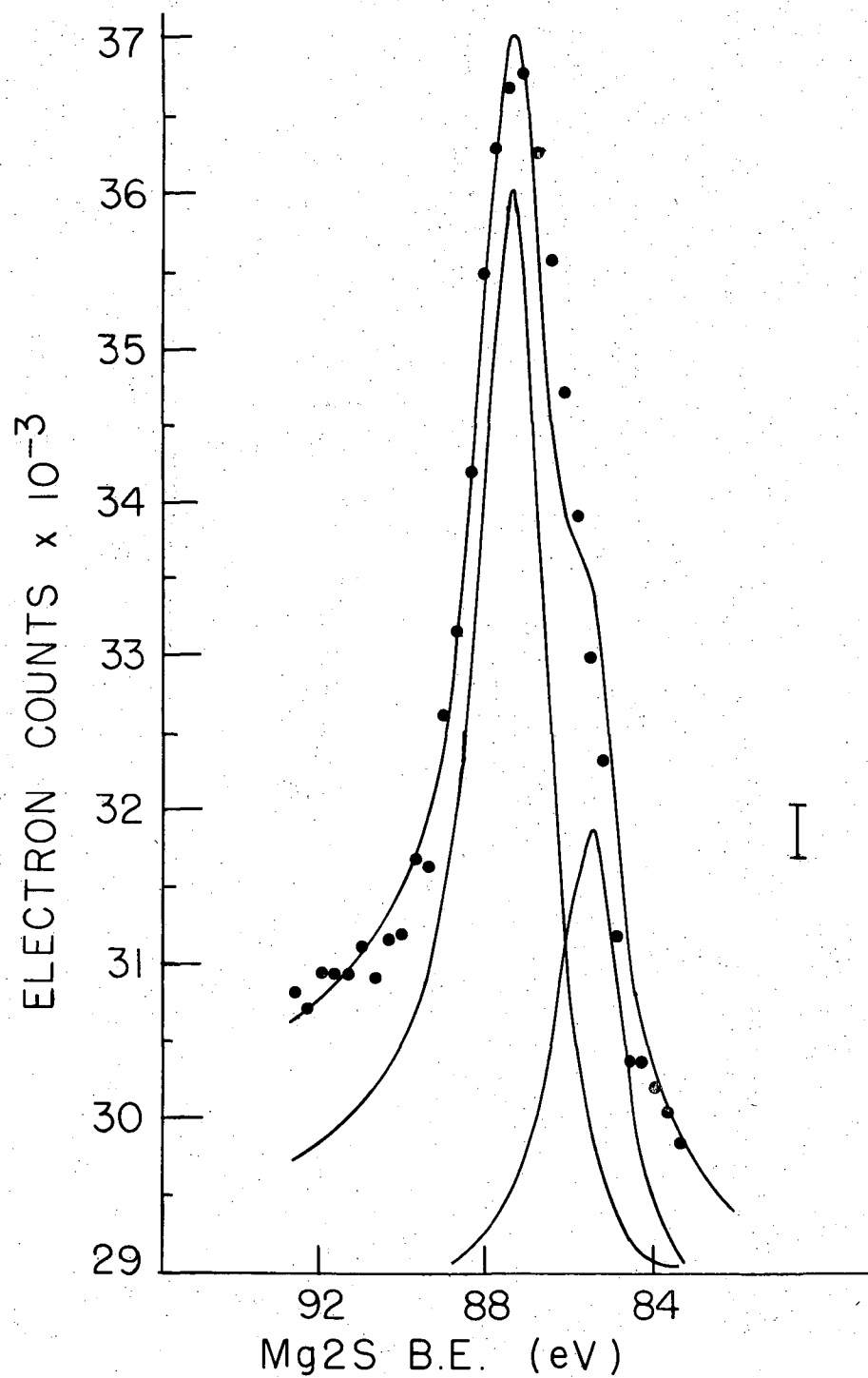
XBL 705-5218

Figure 1. Essential features of the X-ray photoelectron technique used for this investigation. X-rays from an aluminum anode cause emission of photoelectrons from the atomic core of an irradiated sample. The kinetic energy of these electrons are then analyzed by a double-focussing magnetic field in the Berkeley iron free spectrometer.¹¹ Unless otherwise specified, AlK α X-rays were used for all spectra presented in this report.



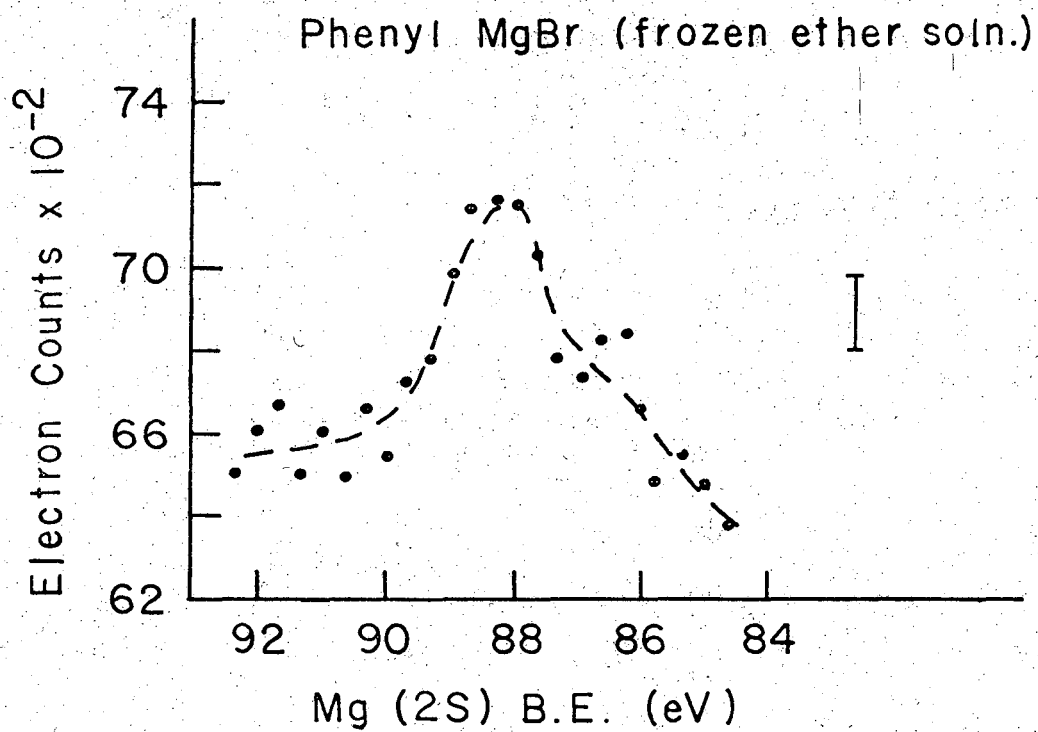
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Figure 2. Chemical shift diagram of a representative sampling of magnesium compounds. Peak width at half-maximum is about 2.1 eV; energy measurements are reproducible to about 0.2 eV.



XBL717-5260

Figure 3. Least squares fit of two Lorentzian functions to the X-ray photoelectron spectrum of magnesium foil. The only restriction was that the two peaks have equal half-widths of 2.1 eV.



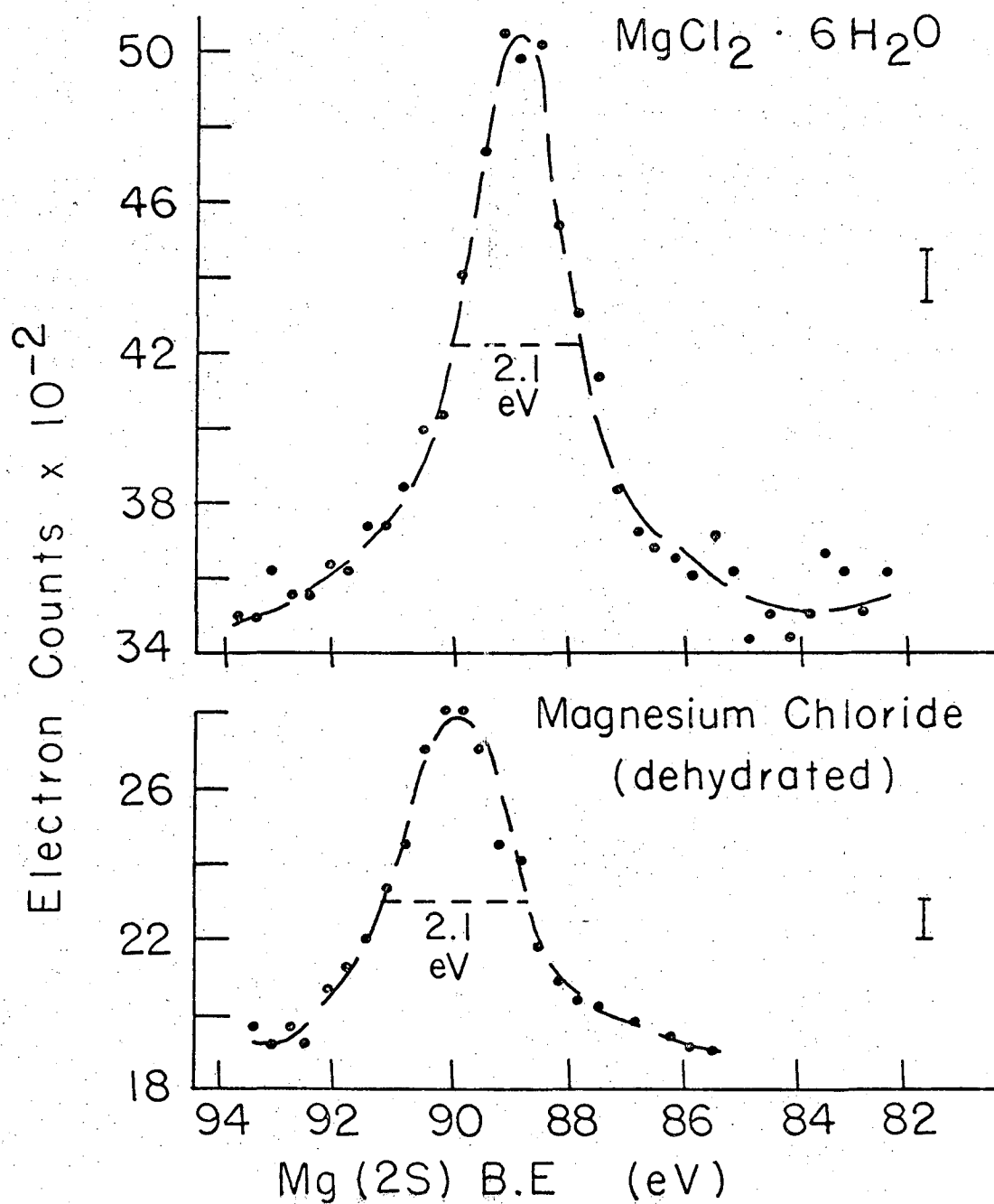
XBL 7010-5466

Figure 4. X-ray photoelectron spectra of PhenylMgBr in ether solution. MgK α X-rays were used.

B.E. between the two compounds of about one eV is attributed then to the loss of water of hydration. This shift is qualitatively consistent with that observed in iron compounds with varying numbers of water molecules in the coordination sphere.⁵ The shift towards higher B.E. with loss of water molecules indicates an increase in lattice potential and/or the loss of electron density contributed by the water molecules to the magnesium complex. It should also be noted that the position of chlorophyll a on the chemical shift series indicates at least partial covalent character associated with the magnesium atom, as is illustrated below. This suggests that the electronic environment of magnesium is coupled with that of the surrounding porphyrin molecule and, consequently, would be affected by molecular excitations during the photosynthetic process. The Mg (2S) spectrum of chlorophyll a (shown in Figure 6) also exhibited a secondary peak at about 91 eV, which is attributed to the background spectrum.

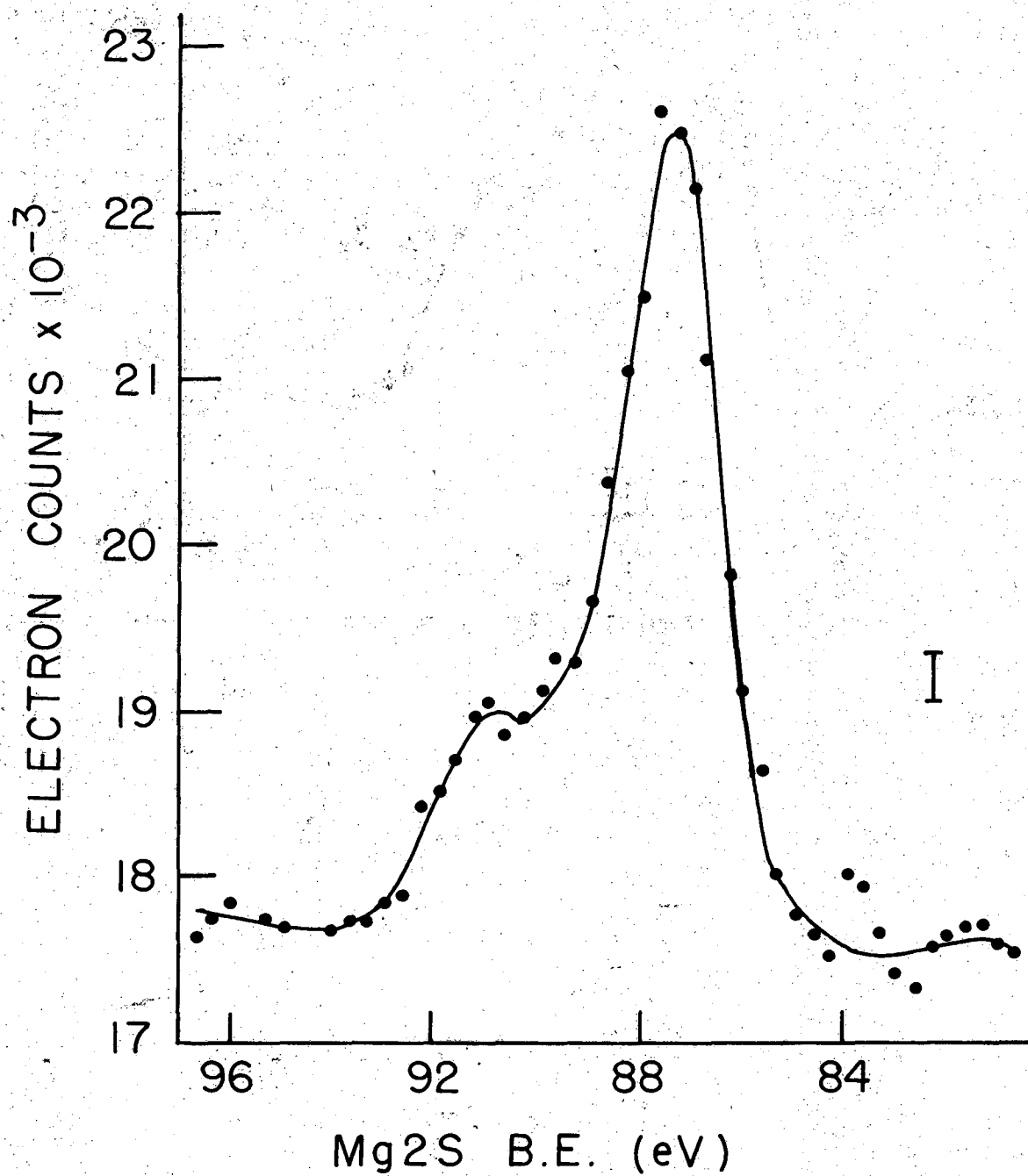
The general validity of these chemical shifts have been checked through independent extended Huckel calculations. This method of calculation and its limitations have been discussed elsewhere.⁶ Table I compares the calculated charges of magnesium metal, Mg phthalocyanine, and $\text{Mg}(\text{H}_2\text{O})_6^{++}$. The observed trend is in agreement with the chemical shifts of Figure 2. Whether one assumes a configuration of sp or s^2 for the magnesium valence electrons makes a difference in the quantitative, but not qualitative, relation between charge values calculated by this method.

In order to obtain photoelectron spectra of chlorophyll magnesium in a biological environment, we first attempted to investigate spinach chloroplasts. Spinach chloroplasts were prepared by the method of Jensen and Bassham.⁷ One would expect difficulty in obtaining a magnesium photoelectron spectrum from whole chloroplasts for several reasons: (1) the relatively low concentration of magnesium atoms would make detection difficult; (2) the variety of magnesium in different chemical environments would tend to wash out any specific magnesium spectrum; (3) the chloroplast membrane would inhibit electrons from reaching the sample surface; and (4) low temperature work, as is necessary for investigation of intact chloroplast suspensions,⁸ results in a loss of spectral intensity after a few hours in the spectrometer (because of experimental limitations in maintaining a clean sample surface at low temperature). Nevertheless, several attempts were made to obtain a magnesium spectrum from intact, frozen chloroplasts, frozen chloroplasts broken through sonication, and also freeze-dried chloroplasts. Preliminary experiments indicated that the chloroplast preparations exhibited a magnesium photoelectron spectrum somewhat similar to that of crystalline chlorophyll a; a similar result was also obtained with photosynthetic bacterial chromophores.¹² These spectra are shown in Figure 7. However, the origin of these Mg spectra from such a complex, ill-defined system is not unequivocal (i.e., the spectra from free chlorophyll, bound chlorophyll, other Mg species, or all three).



XBL7010-5467

Figure 5. X-ray photoelectron spectra of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ before and after dehydration. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ yielded similar spectra in frozen solution and frozen solid.



XBL717-5259

Figure 6. X-ray photoelectron spectra of crystalline chlorophyll *a*.

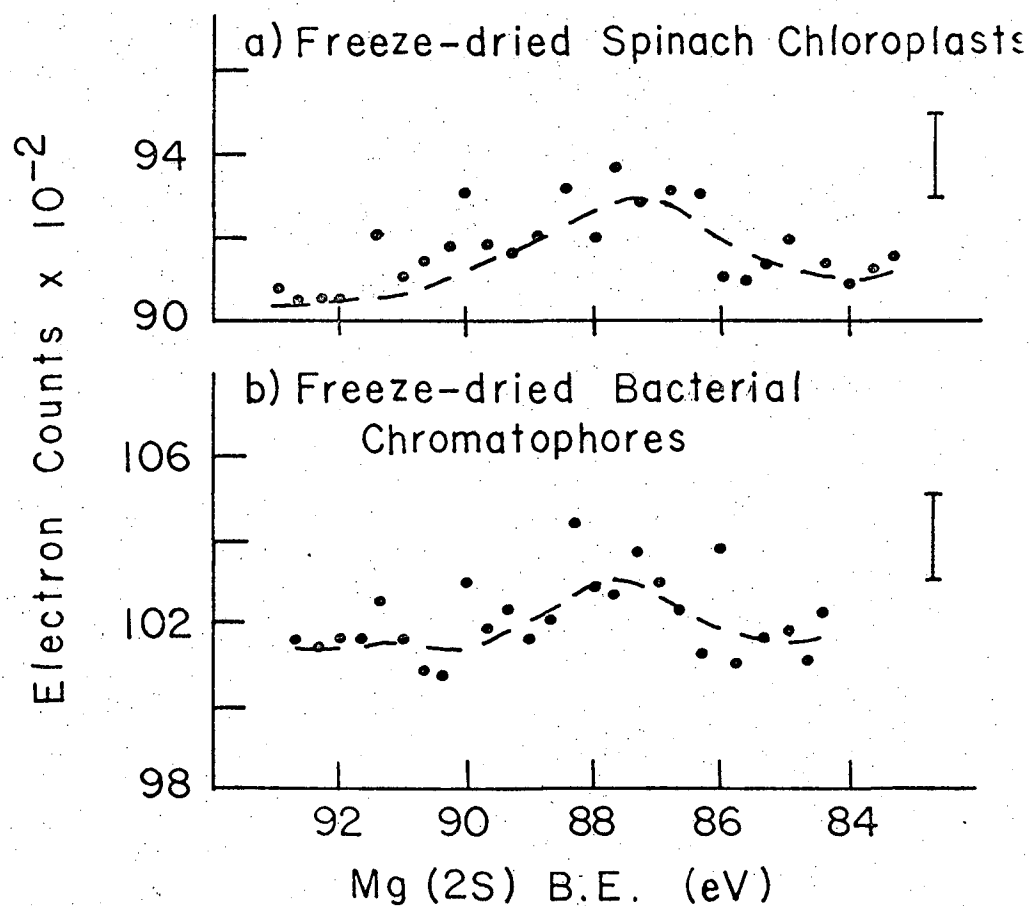
Table I
Calculated Charges and Experimental B.E.'s of
Representative Magnesium Complexes

Molecule	Calculated Atomic Charge		Relative B.E. (eV)
	(S ² config.)	(SP config.)	
Mg(H ₂ O) ₆ ²⁺	+1.94	+1.86	89.0
Mg phthalocyanine	+1.54	+1.10	87.5
Mg (metal)	0 (defined)		85.5

In order to obtain information from a more definitive system, it was decided to investigate subchloroplast particles which could still carry on the photosynthetic process. Such a small chloroplast particle obtained with Triton X-100 has been described by Vernon et al.⁵ Chloroplasts were again prepared by the method of Jensen and Bassham,⁷ and the photo-reactive subchloroplast particles, designated P-D10, were isolated by the method of Vernon. This particle contains 50 chlorophylls:1 cytochrome b_6 :1 cytochrome f :0.5 P700.

A frozen suspension of P-D10 particles exhibited a noisy, broad photoelectron spectrum of magnesium 2S electrons; this is shown in Figure 8(a). Further attempts to improve upon this ambiguous spectrum were unsuccessful due to reasons noted earlier. No unequivocal interpretation of this spectrum can be made, except that the center of the spectral peak occurs near 88 eV, which is relatively close to that of crystalline chlorophyll a. This spectrum may correspond to a combination of different magnesium ions, other than that in chlorophyll.

In order to obtain a more unambiguous magnesium spectrum, P-D10 particles were again prepared as before, washed with distilled water to remove any loosely bound magnesium, and then freeze-dried. Figure 8(b) shows the magnesium photoelectron spectrum of these particles, time-averaged over a period of 20 hours. Although the spectrum is again somewhat noisy, the peak shape and position can be ascertained. The peak occurs at about 87.6 eV, and has a half-width of approximately 2.1 eV, both of which correspond to the magnesium



XBL7010-5468

Figure 7. (a) X-ray photoelectron spectrum of Mg2S electrons from freeze-dried spinach chloroplasts.

(b) X-ray photoelectron spectrum of Mg2S electrons from freeze-dried photosynthetic bacterial chromatophores.

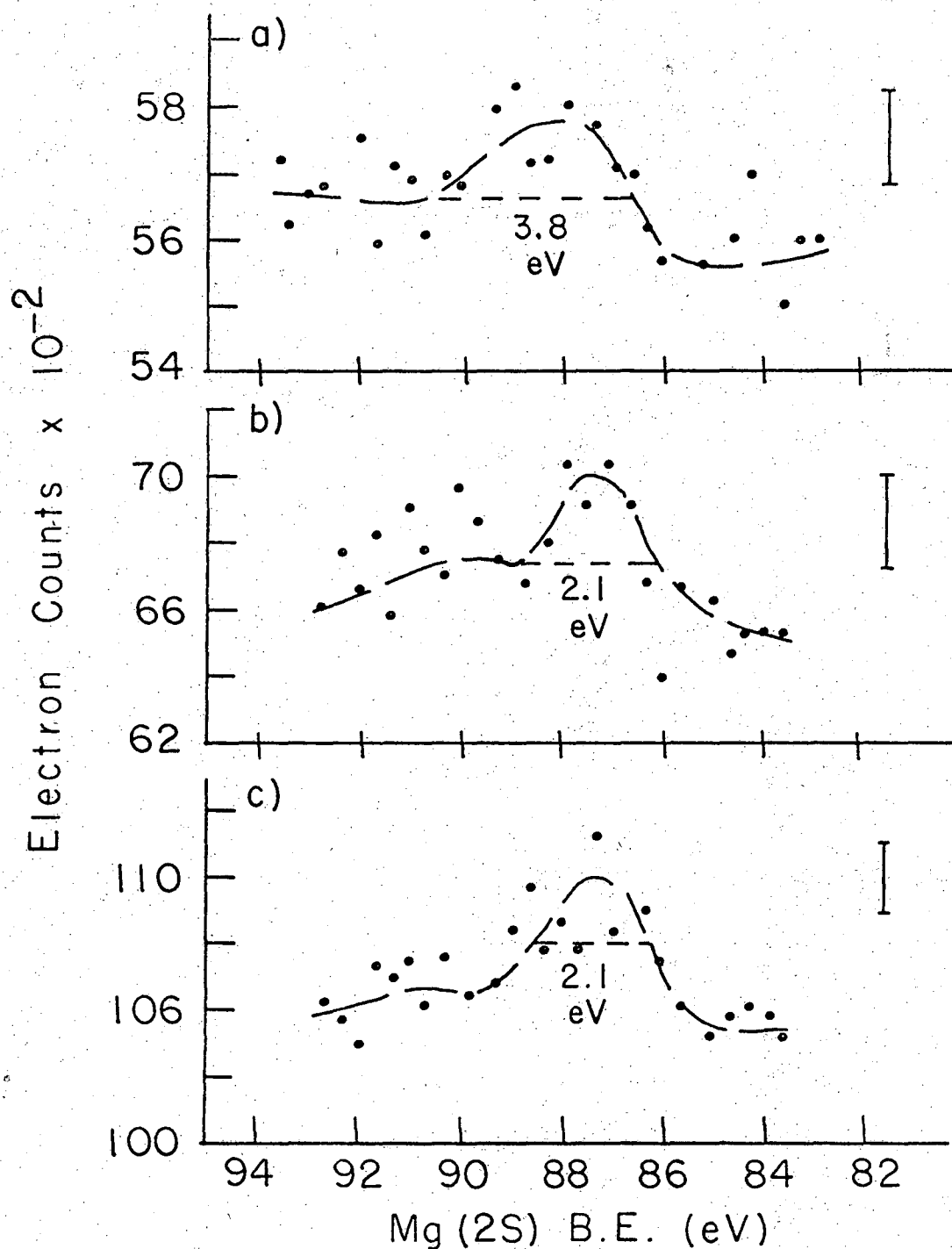


Figure 8. (a) X-ray photoelectron spectrum of a frozen suspension of P-D10 particles. (b) X-ray photoelectron spectrum of freeze-dried P-D10 particles after washing with distilled water. (c) X-ray photoelectron spectrum of illuminated P-D10 particles corresponding to Fig. 7(b).

XBL7010-5469

spectrum of chlorophyll a.

On the assumption that the chlorophyll molecules are still in a relatively "natural" environment in these freeze-dried P-D10 particles, we decided to investigate the effect of light upon biologically active chlorophyll magnesium. It has been determined that when using a chlorophyll concentration of 50 $\mu\text{gms/ml}$ in a 0.3 cm cell width, the optimum activation flux for 668 λ light (in order to carry on light induced photosynthetic processes) is 3×10^{-9} einsteins/ $\text{cm}^2\text{-sec}$.¹⁰ Estimating a chlorophyll a monolayer concentration at 10×10^{13} molecules/ cm^2 , then 2×10^{15} molecules/ cm^2 would be present in 20 monolayers of 10 $\text{\AA}$ thickness each. This would correspond to a distance of 200 $\text{\AA}$ from which photoelectrons would be emitted without significant energy loss (as is suggested by Siegbahn³). This quantity of chlorophyll molecules represents the maximum quantity of chlorophyll molecules absorbing light in the photoelectron experiment. By using a calibrated flux meter, the incandescent light to be used to illuminate the P-D10 particles had a flux of approximately 15 nanoeinsteins- $\text{cm}^2\text{-sec}^{-1}$ when passed through a 670 glass filter. This flux is much greater than is necessary to activate all the chlorophylls that may contribute to the photoelectron spectrum.

The Mg (2S) photoelectron spectrum of freeze-dried P-D10 particles while illuminated by the incandescent light noted above is shown in Figure 8(c); a similar spectrum of the frozen suspension of P-D10 particles was too ambiguous to

analyze. Once again the spectrum is noisy, but the peak appears to occur at about 87.6 eV and has a half-width of approximately 2.1 eV; this corresponds to the B.E. of the magnesium of P-D10 particle without light. The lack of spectral clarity makes comparison between the P-D10 spectra difficult and the only definite observation is that there does not appear to be any significant difference between the Mg (2S) spectrum of freeze dried P-D10 particles without or with illumination.

The observation that the Mg (2S) photoelectron spectra of the P-D10 particles does not differ appreciably from that of crystalline chlorophyll a indicates that the chemical environment about the chlorophyll magnesium does not differ significantly between isolated chlorophyll and that which is still in its original biological environment. This chemical environment of chlorophyll magnesium appears to be one of appreciable covalent character, and one would expect any change in the electronic environment of the chlorophyll molecule to have some type of effect upon the magnesium atom (although probably minor). However, the question as to whether light has any effect upon the magnesium atom in biologically active chlorophyll has not been unequivocally answered by these experiments. Even if the chlorophyll's biological activity in the freeze-dried P-D10 particles was still intact, any effect upon the magnesium atom due to illumination may be so small that detection would be impossible by this method (especially in view of the poor

spectral clarity). Consequently, the only definite conclusion available is that the electronic environment of the chlorophyll magnesium in the P-D10 particles (taken as a whole without differentiating between "reaction center" atoms) does not appreciably change upon illumination of the particles.

REFERENCES - SECTION VI

1. L. P. Vernon and G. R. Seely, "The Chlorophylls," Academic Press, New York, N.Y. (1966).
2. O. V. S. Heath, "The Physiological Aspects of Photosynthesis," Stanford University Press, Stanford, Calif. (1969).
3. K. Siegbahn, et al., "ESCA-Atomic, Molecular and Solid State Structures Studied by Means of Electron Spectroscopy," Almqvist and Wiksellsboktryckeri AB, Uppsala, Sweden (1967).
4. F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," 2nd ed., Interscience Publishers, New York, N.Y. (1966), p. 431.
5. L. N. Kramer and M. P. Klein, J. Chem. Phys., 51(8), 3618 (1969).
6. L. N. Kramer and M. P. Klein, submitted to J. Chem. Phys.
7. R. G. Jensen and J. A. Bassham, Proc. Nat. Acad. Sci., 56(4), 1095 (1966).
8. L. N. Kramer and M. P. Klein, J. Chem. Phys., 51(8), 3620 (1969).
9. L. P. Vernon, E. R. Shaw, and B. Ke, J. Biol. Chem., 241(17) 4101 (1966).
10. A. Sun, private communication.
11. K. Siegbahn, C. Nordling, and J. M. Hollander, UCRL-10023 (1962).
12. The bacterial chromatophores were generously provided by Dr. Hans Steffen.

Section VIII. Grain Protein Quantity and Quality:

A Rapid Non-destructive Means of
Evaluating Cereal Grain is Provided by
X-ray Photoelectron Spectroscopy.

Introduction

The increasing world population has been accompanied by demands to maintain a commensurate food supply. The cereal grains, legumes and oil seeds provide the major protein sources in the less developed countries. It was thus natural to seek methods to improve the yields of these crops. The successes of Borlaug and his collaborators in these endeavors, acknowledged recently by the award of the Nobel Peace Prize, have led to the so-called "green revolution" (1). It is desirable that increased yields be accompanied by concomitant increases in the quantity of protein per grain, by the quality of the protein as measured by the content of essential amino acids and by the digestibility of the protein. This last property can be evaluated only by feeding trials; it lies outside our area of competence and will not be discussed further.

In order to assess the relative nutritional values of the massive number of seeds involved in any plant protein improvement program, it would be desirable for the agronomist and plant breeder to have available a convenient, rapid, economical, and non-destructive method for determining the quantity and quality of grain protein (2). We present some results of a new method which can now satisfy some of these requirements and offers the potential of satisfying them all.

Method

When a sample is irradiated with light of sufficient energy, electrons may be ejected and their kinetic energy is given by the well

known Einstein relation,

$$\text{K.E.} = h\nu - \text{B.E.}$$

where K.E. is the kinetic energy of the electrons, h is Planck's constant, ν is the frequency of the light quantum, and B.E. is the binding energy of the electrons in the sample. If the photon energy is increased sufficiently, by using X-rays, electrons may be ejected from the inner or core levels of the constituent atoms of the sample. Since photon or X-ray energies are known to high accuracy, the binding energies may be determined with precision by measurement of the kinetic energies. The measurements may be performed with a variety of devices, but most commonly magnetic and electrostatic deflection analyzers are employed. The overwhelming majority of the energy levels of the elements across the periodic table have been determined in this manner. Although the complementary method of X-ray emission contains the same information, the former method, called X-ray photoelectron spectroscopy, offers so many direct advantages that we will confine the present discussion thereto.

The method is outlined schematically in Fig. 1. In this figure are sketched the discrete energy levels of a particular atom in a compound as well as those levels which are a collective property of the compound as a whole and make up its valence band or molecular orbitals. An X-ray photon is shown lifting an electron from the 2s level of this atom into the continuum of energies or, equivalently, removing it to infinity. At this point the electron enters the spectrometer wherein it is brought to a focus and impinges on a detector when the electron has the correct energy (more rigorously the correct momentum). The

energy range is scanned by varying the strength of the magnetic field and thus a spectrum is traced out by recording the number of electrons reaching the detector at each value of magnetic field. This method was developed and brought to its present state of refinement by the group at the Institute of Physics, University of Uppsala, Sweden (3).

Since all atoms are constructed in the same manner, it is possible to photoeject their electrons, and by a suitable choice of exciting X-ray energy and particular atomic level one can distinguish among the different constituent atoms of the sample. It is apparent, then, that calibration against a sample of known elementary composition will permit a qualitative and quantitative analysis of the unknown sample. The absolute sensitivity of the method is very high although it is not especially suitable for detecting small amounts of one element in the presence of a large excess of other elements.

More recently it has been shown that a given element in different chemical configurations exhibits chemical shifts of its binding energies (4,5). These chemical shifts thus extend the utility of the method so that not only the total quantity of a given element may be determined, but also the type or types of chemical bonding situation in which the atom is situated. The origins and theoretical foundations for these chemical shifts are rather well understood (4,6,7).

Experimental Design

Since virtually all of the previous work in this field has been directed toward precision measurements of the energies of the photoelectrons, we were obliged to devise techniques to permit the quantitative

determinations of the relative numbers of relevant atoms contained in the samples. We were further interested in establishing the feasibility of measuring not only total protein by nitrogen, but also of assessing the amino nitrogen as a measure of lysine, arginine and histidine and the sulfur as a measure of cysteine, cystine, and methionine. Our principal research endeavors prior to our introduction to the present topic are related to iron and sulfur and the non-heme iron-sulfur proteins known as the ferredoxins. Accordingly, we had some prior knowledge of sulfur spectra but no current nitrogen data were pertinent to the present problem (8).

The first task undertaken was the observation of the nitrogen and sulfur spectra of relevant amino acids, simple peptides, and known proteins. Fig. 2 shows spectra of three such compounds. The cystine spectra show lines from both nitrogen and sulfur. The designations N1S and S2P indicate that the electrons arise from the 1S level of nitrogen and the 2P level of sulfur, respectively. The di-peptide, L-isoleucyl-L-alanine, exhibits a nitrogen peak considerably broader than that of the cystine. This peak may be decomposed into two peaks of width equal to the cystine peak; one component is of the same energy as the cystine peak and we conclude that it corresponds to the amino nitrogen. The other peak occurs at lower binding energy and is attributed to the amide nitrogen formed during the peptide bonding. The lower lines originate from a sample of hemoglobin. The nitrogen peak occurs at the position of lower binding energy and is therefore the amide nitrogen. The sulfur peak is shown magnified by 50-fold and exhibits an increasing shoulder at higher binding energies. We

believe this shoulder arises from sulfur as $\text{SO}_4^{=}$ and that it most probably entered the sample during ammonium sulfate precipitation of the protein (9).

Figure 3 shows the nitrogen and sulfur spectra from three protein samples, hemoglobin (repeated for continuity), equine cytochrome C, and the apo-ferredoxin and its native form from Clostridium pasteurianum. This latter protein has a molecular weight of 6000 and contains 8 iron atoms, 8 moles of cysteine, and 6-8 moles of acid labile sulfur (10). The cytochrome C spectrum is similar to that of hemoglobin but shows a higher sulfur content. It also contains $\text{SO}_4^{=}$. The apo-ferredoxin spectra are qualitatively similar to both the heme-proteins, except for the larger quantity of sulfur. The native ferredoxin spectra are significantly different from the heme-proteins; both lines are broader and exhibit rather obvious shoulders. The nitrogen shoulder at higher binding energy probably arises from nitrogen entering as NH_4^+ during ammonium sulfate precipitation of the protein. The lowenergy sulfur shoulder definitely arises from sulfur bonded to iron and is characteristic of such bonding (8).

Figure 4 shows the nitrogen spectra of Barker barley seed and of a dark red kidney bean. The barley spectrum is very similar to the other proteins; the kidney bean spectrum is considerably broader and exhibits a high energy shoulder.

Figure 5 shows the nitrogen spectrum of a light red kidney bean and the curve has been decomposed into two components by computer. The major peak, at lower binding energy, represents the amide nitrogen, while the smaller peak at higher binding energy represents the amino nitrogen. Figure 6 shows the sulfur region of this same sample

Figure 7 shows the nitrogen spectrum of a sample of ramona wheat where again the experimental data have been fit by two peaks corresponding to the amide and amino nitrogen. Figure 8 shows the sulfur region of the wheat sample.

All of the foregoing spectra were excited by Mg K α radiation at 1252 eV and analyzed in the Berkeley iron-free spectrometer (11).

Sample Preparation

The nature of photoelectron spectroscopy imposes constraints on the form of the samples. Since the kinetic energy of the photoelectrons is very low, usually less than 1.5 keV, they must be analyzed in vacuo to reduce scattering. Also, because of their low energy, they are able to penetrate a distance of only a few tens to a few hundreds of angstroms in solid material. Thus only those electrons originating within this distance from the surface of the sample escape elastically and give rise to discrete photoelectron lines. Only the surface of a given sample is of use.

Accordingly, for these studies the samples were prepared as powders by grinding in a ball mill. For the quantitative determinations the biochemicals were mixed with NaCl and then pulverized together. The amino acids, di-peptides, hemoglobin, and equine cytochrome C were obtained from commercial sources. The ferredoxin was prepared in the laboratory of Professor J. C. Rabinowitz in Berkeley. The seed samples were obtained from the seed station of the University of California, Davis, California.

Quantitative Determinations

The basic relative sensitivity for nitrogen and sulfur was obtained from a sample of cystine mixed with NaCl. The areas under the N1S, S2P, and Na2S peaks were integrated by computer and were treated as follows:

Sulfur

1. $\frac{\text{Area S2P}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{\text{Moles of compound}} = 14/1 \text{ atom equivalent}$
2. $\frac{\text{Weight of compound} \times \% \text{ S}}{32} = \text{Moles of compound}$
3. $\frac{\text{Area S2P}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{14} \times \frac{32}{\text{Weight of compound}} = \% \text{ S}$

Nitrogen

4. $\frac{\text{Area N1S}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{\text{Moles of compound}} = 9.4/1 \text{ atom equivalent}$
5. $\frac{\text{Weight of compound} \times \% \text{ N}}{14} = \text{Moles of compound}$
6. $\frac{\text{Area N1S}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{9.4} \times \frac{14}{\text{Weight of compound}} = \% \text{ N}$

These sensitivity factors are specific for our instrument and for the particular sample geometry and method of data analysis.

Table I gives the quantitative values for several samples determined by this method. The two cysteine samples were prepared independently and are shown as an indication of the reproducibility of the method. The cystine sample was different from that used as the calibration standard. For the seeds, the experimental weight percent nitrogen, determined from the photoelectron spectral amide nitrogen peak, was converted to percent protein by assuming a nitrogen:protein weight ratio of 0.15. Table I includes both nitrogen and sulfur analyses by both X-ray photoelectron spectroscopy

and by conventional wet chemical analysis. Except for the light red kidney bean, the results are comparable. The apparent discrepancy for the light red kidney bean sample may be attributed to the fact that this material is extremely hard and may not have been milled adequately to produce a sufficiently fine powder.

Also shown in Table I is the distribution of protein between the embryo and the total seed for the light red kidney bean. To ascertain the validity of the hypothesis that the component of the nitrogen photoelectron line ascribed to the amino nitrogen did in fact correspond to the quantity of basic residues, an amino acid analysis was performed. The results, derived from such an analysis on the total acid hydrolysate, are given in Table II wherein the fraction of the basic residues is shown to be 17.4%. This value equals that derived from the photoelectron data thus substantiating the assumption.

Conclusions

This report demonstrates that the quantity and quality of grain protein may be determined by X-ray photoelectron spectroscopy. The quantity of protein may be determined by quantitation of the amide nitrogen photoelectron line. Two measures of the quality of the protein are available: 1) The basic amino acids including lysine, arginine, and histidine may be determined by quantitation of the amino nitrogen photoelectrons, and 2) the sulfur-containing amino acids, cystine, cysteine, and methionine, may be quantitated by measuring the sulfur photoelectron line, although the sulfur measurements are not yet perfected.

The quantity of material required to perform the measurements need

be only a few milligrams. Indeed, the active volume giving rise to the photoelectron lines is probably no greater than a few tens of micrograms of material of unit density. Thus, it is feasible to excise a small section of a seed for the analysis while retaining the remainder of the seed for future planting. We performed one such experiment and were able to bring the bulk of the seed to germination.

The experiments reported here were designed to test the feasibility of the method to measure protein quality and quantity. Thus, care was taken to measure the entire nitrogen and sulfur regions of the photoelectron spectrum. The times required for these nitrogen determinations were approximately 1 hour per sample. Such a large period of time clearly limits the utility of this method for mass screening operations. Were it satisfactory to perform only a total nitrogen--and thus total protein--analysis on a large number of samples, one could dispense with the high spectral resolution and measure only the integrated nitrogen intensity. Such a measurement could be accomplished in about 2 minutes per sample. This estimate is for the spectrometer observation time alone and does not include the time required for sample preparation. Since the seed material is analyzed in its native state, aside from the pulverizing operation, the time consuming preparative steps requisite to and employed in purely chemical methods are eliminated, as are the separate and independent procedures required for each element of interest. Newer instrumentation promises to offer a significant further saving in time.

Summary

X-ray photoelectron spectroscopy offers a method of high potential for determining the quantity and quality of grain protein. In this method a small quantity of material is irradiated with X-rays from a suitable target material and electrons from all constituent atoms may be photoejected. The kinetic energy of these photoemitted electrons is equal to the photon energy of the incident X-rays minus the binding energy of the level from which the electrons are emitted, $K.E. = h\nu - B.E.$ This kinetic energy, and hence the binding energy, are determined by a magnetic or electrostatic momentum analyzer, and are characteristic of the atom and level whence the electrons originated. All elements across the periodic table may be investigated. Calibration against compounds of known elementary composition permits a quantitative determination of the amount of each atomic species contained in the sample. The binding energies exhibit chemical shifts which permit a distinction between a given element in different chemical groupings. These experiments have demonstrated: 1) Total protein may be evaluated by quantitative determination of the amide nitrogen peak; 2) amino nitrogen may be distinguished from amide nitrogen, thus providing a measure of the basic amino acids lysine, arginine and histidine; and 3) the sulfur content may be determined by observing the sulfur photoelectrons.

Instrumentation currently under development promises to reduce the time per sample from hours to minutes and thus render possible large-scale screening.

References

1. L. R. Brown, Science **170**, 518 (1970).
2. This requirement was discussed at two symposia devoted to the topic of grain protein improvement. a) New Approaches to Breeding for Improved Plant Protein (Proc. Panel Röstänga, 1968), IAEA, Vienna (1969), and b) Improving Plant Protein by Nuclear Techniques (Proc. Panel Vienna, 1970), IAEA, Vienna (1970).
3. S. B. M. Hagstrom, C. Nordling, K. Siegbahn, in Alpha-, Beta- and Gamma-ray Spectroscopy (K. Siegbahn, Ed.), Vol. 1 (North-Holland, Amsterdam, 1968), p. 845.
4. Siegbahn, et al.. ESCA, Atomic, Molecular and Solid State Structure Studied by Means of Electron Spectroscopy (Almquist and Wiksells, Uppsala, 1967).
5. C. S. Fadley, S. B. M. Hagstrom, J. M. Hollander, M. P. Klein, D. A. Shirley, Science **157**, 1571 (1967).
6. C. S. Fadley, S. B. M. Hagstrom, M. P. Klein, D. A. Shirley, J. Chem. Phys. **48**, 3779 (1968).
7. L. N. Kramer and M. P. Klein, Chem. Phys. Letters **8**, 183 (1971).
8. L. N. Kramer and M. P. Klein, J. Chem. Phys. **51**, 3618 (1969).
9. L. N. Kramer and M. P. Klein, unpublished results.
10. R. M. Malkin and J. C. Rabinowitz, Ann. Rev. Biochem. **36**, 113 (1967).
11. K. Siegbahn, C. Nordling, J. M. Hollander, Lawrence Radiation Laboratory Report UCRL-10023 (1962).

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TABLE I
QUANTITATIVE DETERMINATION OF NITROGEN AND SULFUR CONTENT

<u>Compound</u>	<u>Element</u>	<u>Experimental Weight %</u>	<u>Calculated Weight %</u>
Cysteine* (1)	Nitrogen	8.2	8.2
	Sulfur	18.0	19.3
Cysteine* (2)	Nitrogen	8.5	8.5
	Sulfur	20.0	19.3
Cystine (1)	Nitrogen	13.0	11.7
	Sulfur	26.3	26.7

*Cysteine-hydrochloride-
monohydrate

ELEMENTAL ANALYSIS

<u>Seed</u>	<u>% N(XPS)</u>	<u>% N(anal)</u>	<u>% S(XPS)</u>	<u>% S(anal)</u>
Barker Barley	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2	.05 $\pm$.02	.01 $\pm$.01
Ramona Wheat	1.6 $\pm$ 0.2	1.7 $\pm$ 0.2	.05 $\pm$.02	.06 $\pm$.03
Rapida Oats	2.2 $\pm$ 0.2	1.9 $\pm$ 0.2	.1 $\pm$.03	.02 $\pm$.02
Light Red Kidney Bean	3.2 $\pm$ 0.3	4.1 $\pm$ 0.3	.08 $\pm$.04	.13 $\pm$.06

SEED PROTEIN DISTRIBUTION (LIGHT RED KIDNEY BEAN)

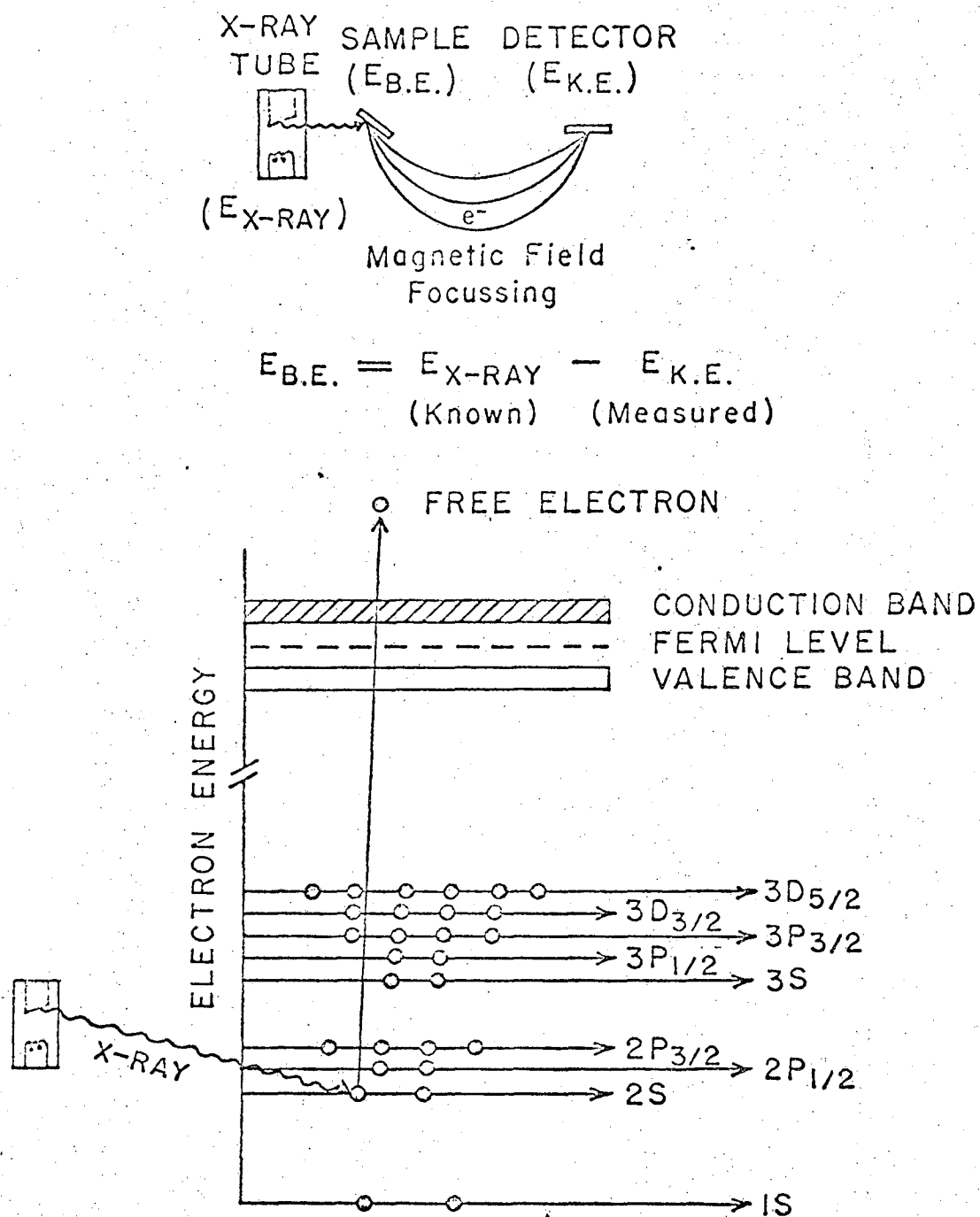
<u>Sample</u>	<u>% N(XPS)</u>	<u>% N(anal)</u>	<u>% Basic A.A.(XPS)</u>	<u>% Basic A.A.(anal)</u>
Endoplasm	3.1 $\pm$ 0.3	4.0 $\pm$ 0.3	17 $\pm$ 5	17.4
Embryo and Endoplasm	3.1 $\pm$ 0.3	4.2 $\pm$ 0.3	18 $\pm$ 5	

TABLE II
AMINO ACID ANALYSIS - LIGHT RED KIDNEY BEAN

<u>Amino Acid</u>	<u>Quantity (μmole)</u>	<u>Amino Acid</u>	<u>Quantity (μmole)</u>
Aspartic Acid	.163	Allo-isoleucine	.007
Threonine	.030	Isoleucine	.060
Serine	.016	Leucine	.109
Glutamic Acid	.203	Tyrosine	.011
Proline and Cystine	.063	Phenylalanine	.057
Glycine	.099	NH ₃	.369
Alanine	.087	Basic Amino Acids	
Cystine	.001	Lysine	.093
Valine	.086	Histidine	.039
Methionine	.005	Arginine	.078

Total A.A. content = 1.207 μ mole

$$\% \text{ Basic A.A.} = \frac{.210}{1.207} \times 100 = \underline{17.4\%}$$



XBL 705-5218

Figure 1. Energetics and analysis of X-ray photoelectrons.

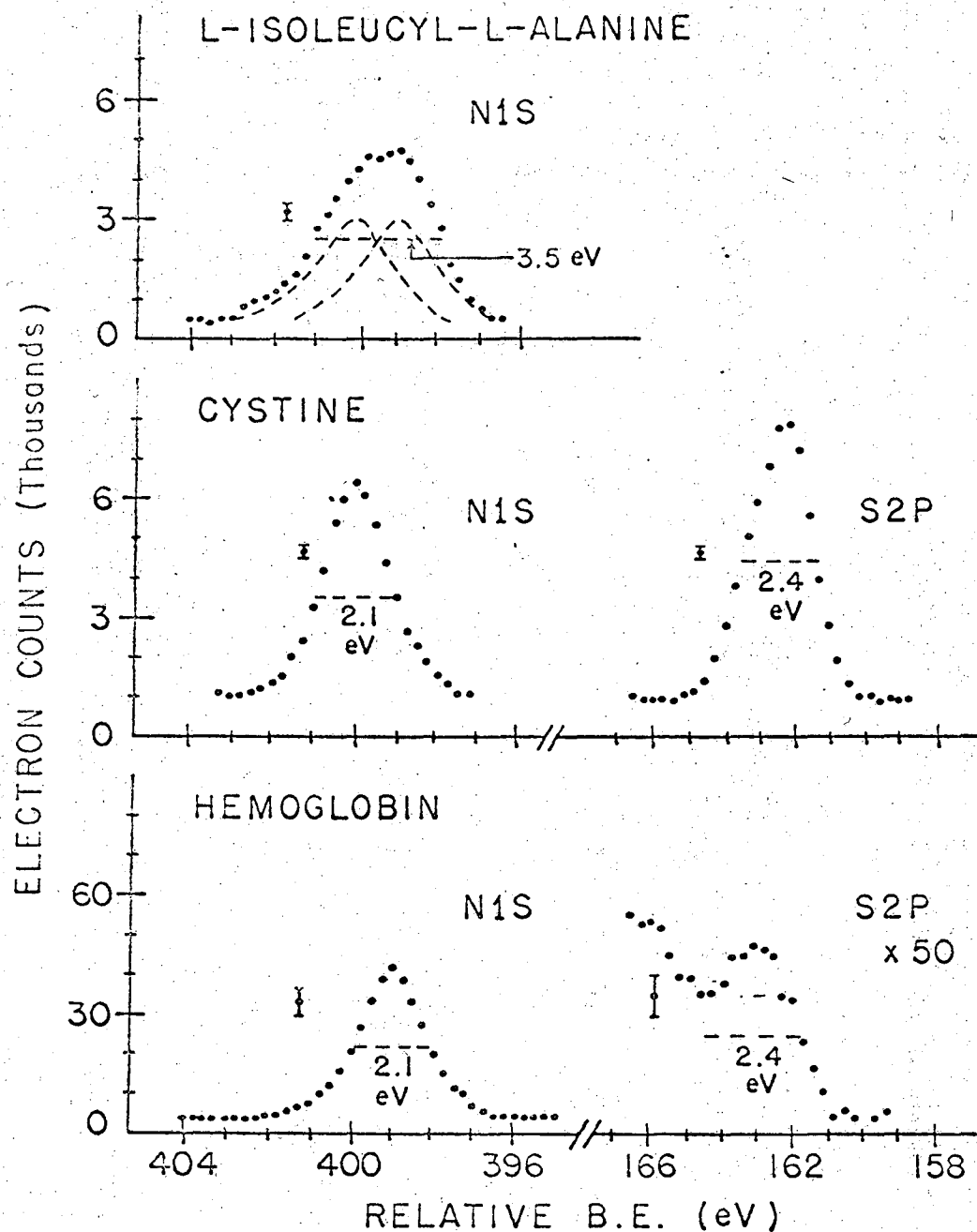
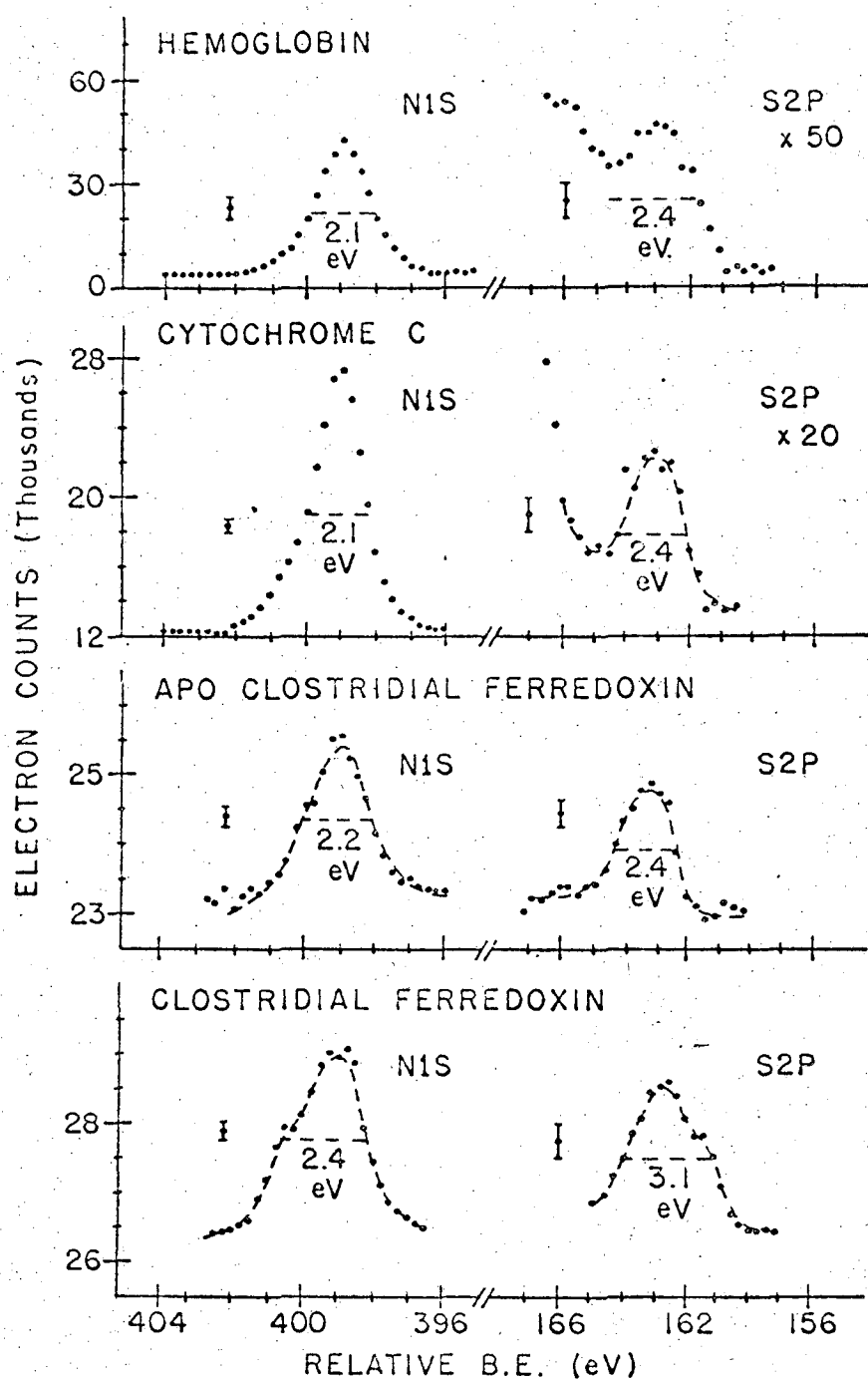


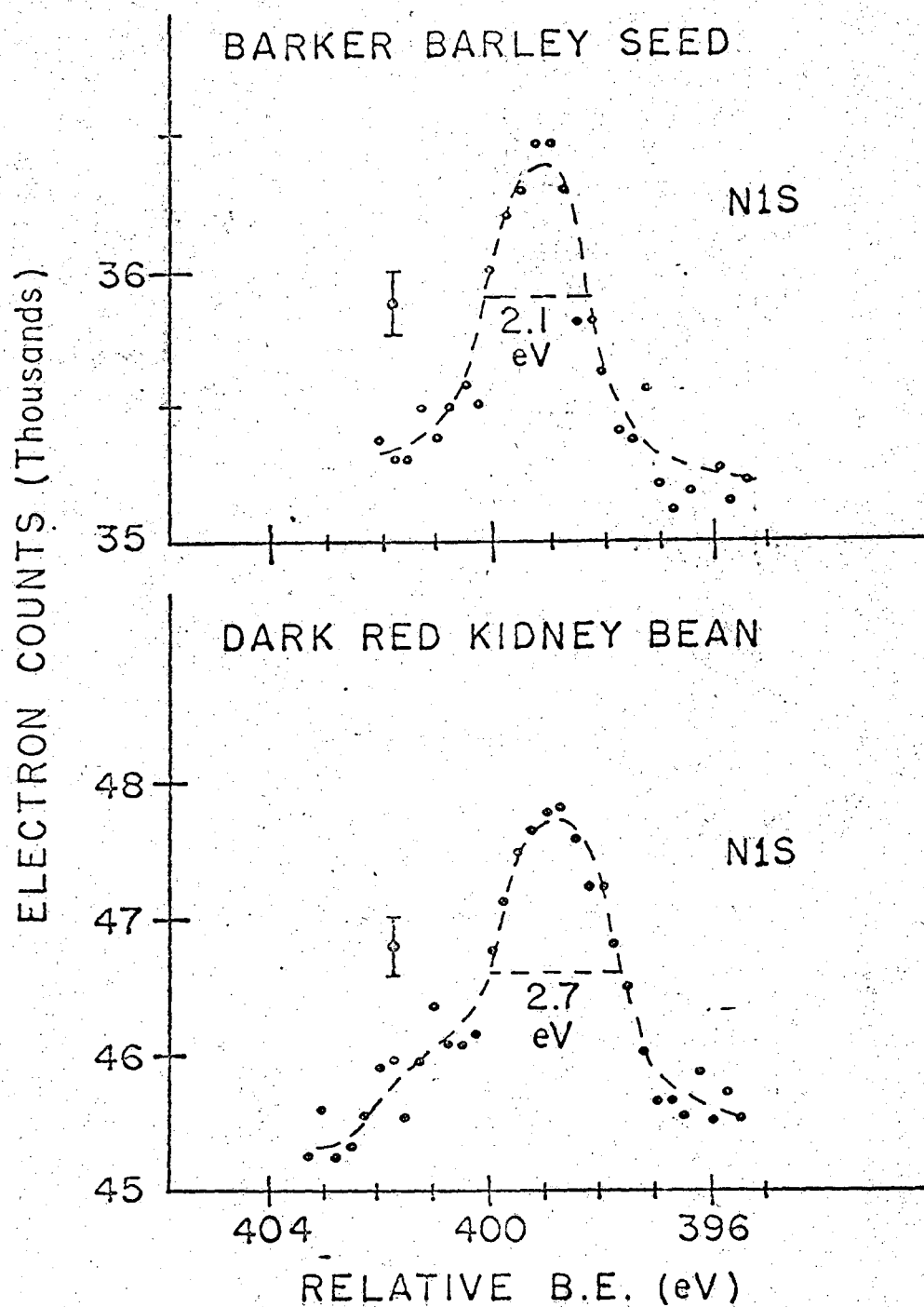
Figure 2. X-ray photoelectron spectra of three compounds showing nitrogen and sulfur lines. The dipeptide nitrogen line is decomposed into two components of equal width. One component is isoenergetic with the cystine nitrogen and is assigned to the amino nitrogen. The other component, isoenergetic with the hemoglobin, is assigned to the amide nitrogen.

XBL705-5216



XBL705-5219

Figure 3. Nitrogen and sulfur X-ray photoelectron spectra of three proteins.



XBL 705-5217

Figure 4. Nitrogen X-ray photoelectron spectra of two seeds.

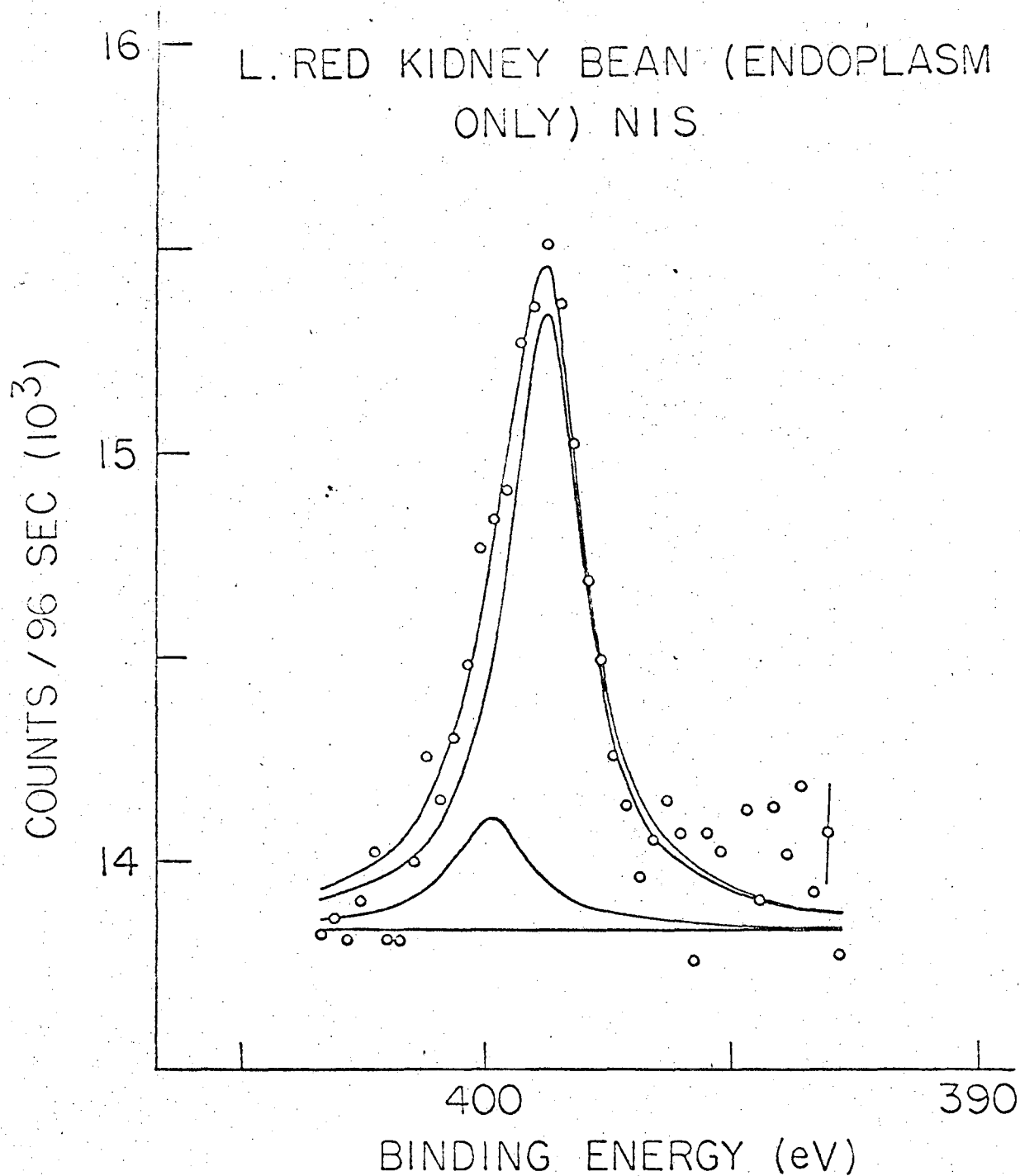
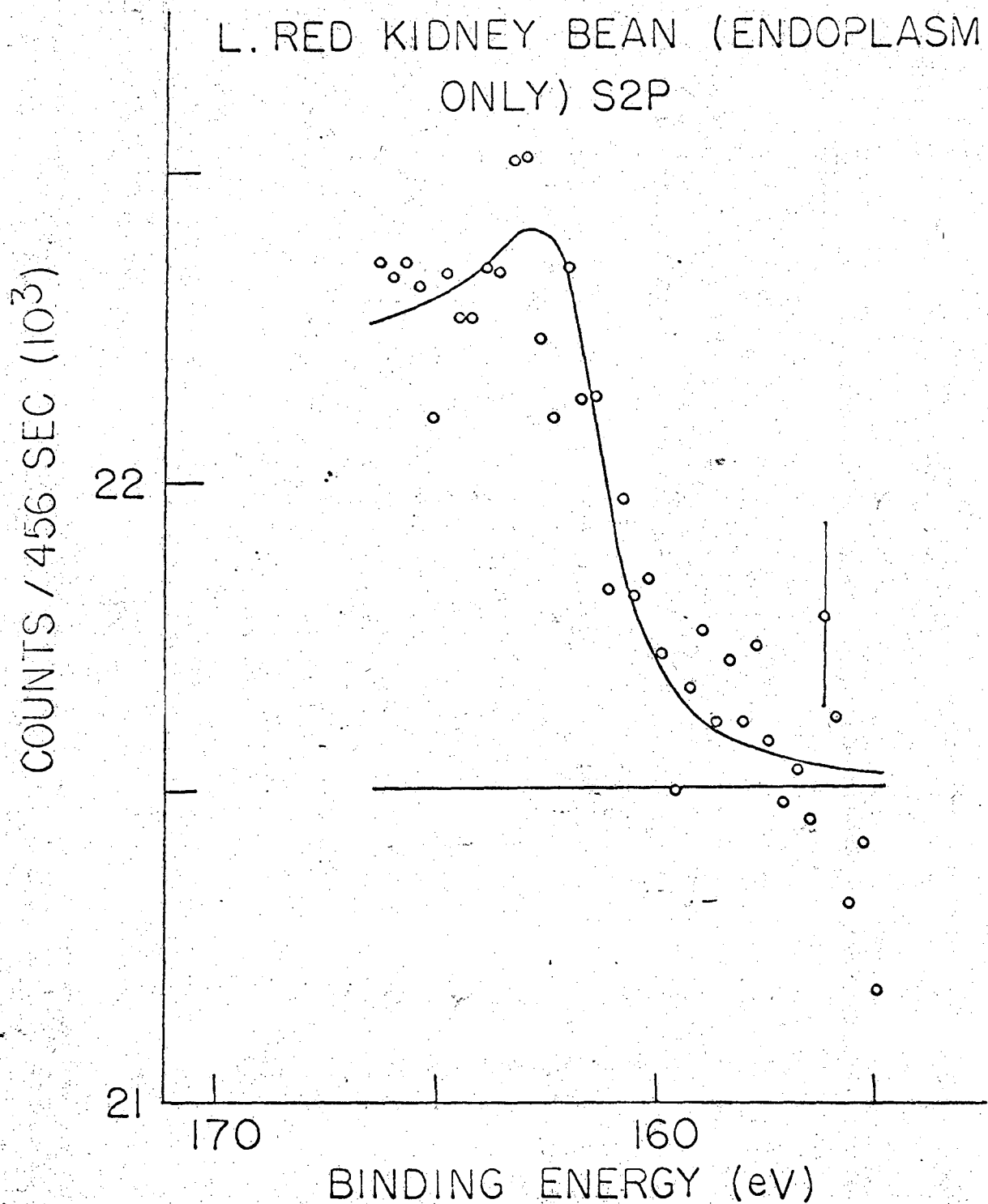
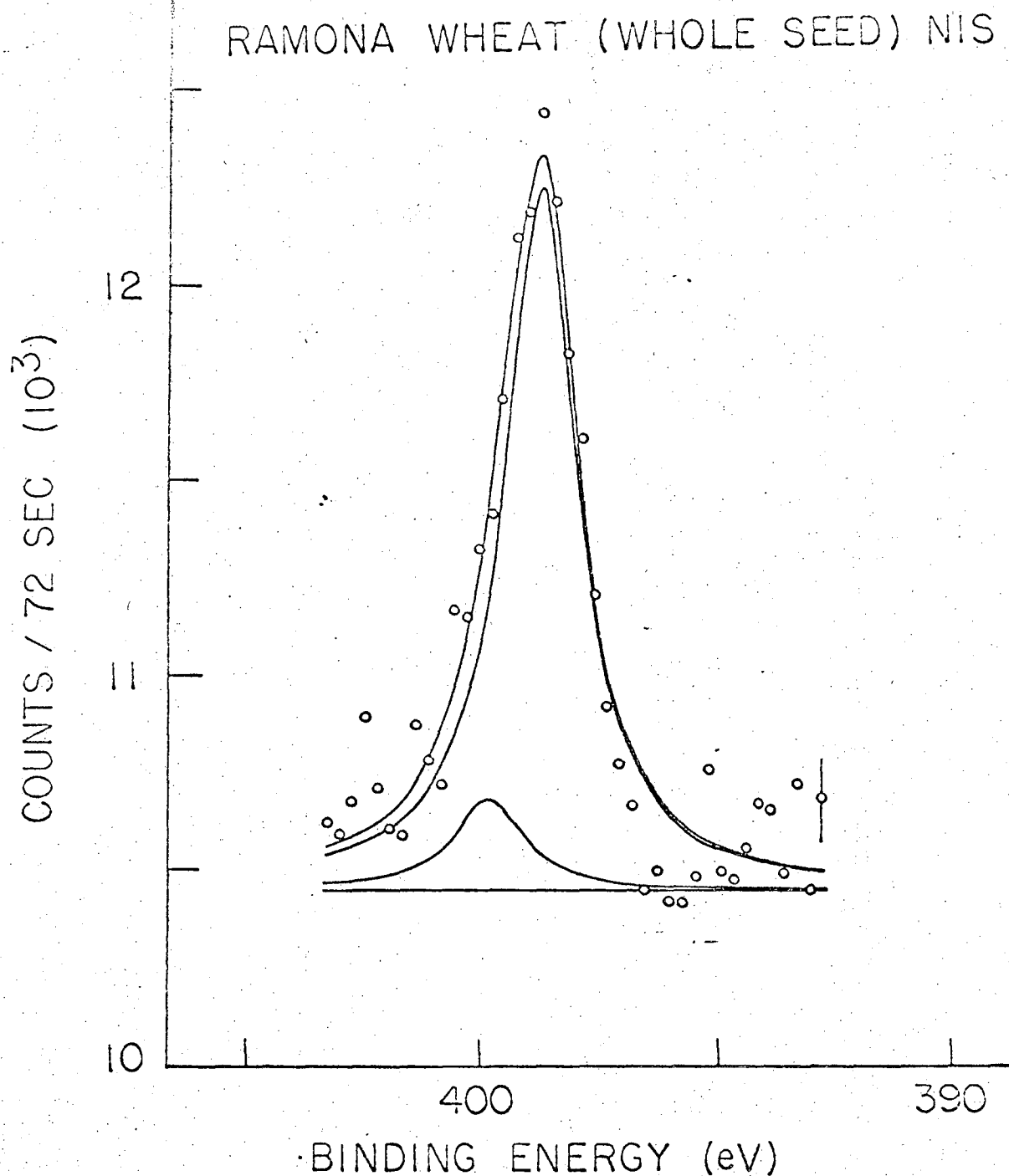


Figure 5. Nitrogen X-ray photoelectron spectrum of light red kidney bean decomposed into two components corresponding to the amide nitrogen at lower binding energy and amino nitrogen at higher binding energy. XBL713-5065



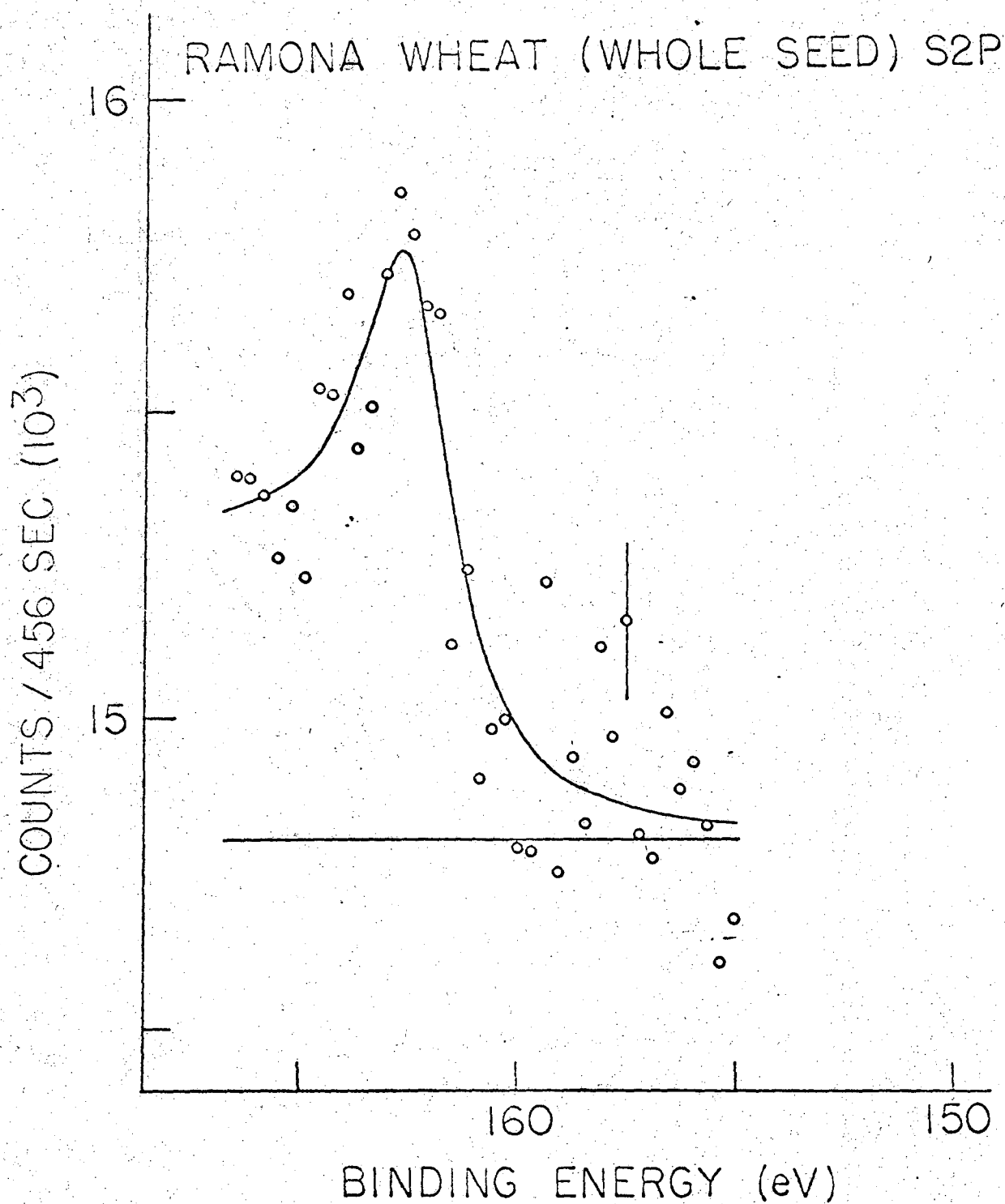
XBL713-5068

Figure 6. Sulfur X-ray photoelectron spectrum of light red kidney bean



XBL713-5069

Figure 7. Nitrogen X-ray photoelectron spectrum of ramona wheat decomposed into two components corresponding to the amide nitrogen at lower binding energy and the amino nitrogen at higher binding energy.



XBL713-5067

Figure 8. Sulfur X-ray photoelectron spectrum of ramona wheat seed.

APPENDIX. APPLICATION OF EXTENDED HUCKEL THEORY TO X-RAY
PHOTOELECTRON SPECTRA OF TRANSITION METAL
COMPLEXES. CORRELATIONS BETWEEN ELECTRON
BINDING ENERGY AND CALCULATED ATOMIC CHARGE IN
IRON AND SULFUR COMPOUNDS

APPLICATION OF EXTENDED HÜCKEL THEORY
TO X-RAY PHOTOELECTRON SPECTRA OF TRANSITION METAL COMPLEXES.
CORRELATIONS BETWEEN ELECTRON BINDING ENERGY
AND CALCULATED ATOMIC CHARGE IN IRON AND SULPHUR COMPOUNDS

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Comparisons of iron and sulphur atomic charges, derived from extended Hückel calculations, with core electron binding energies determined from X-ray photoelectron spectroscopy yield good correlations for neutral molecules. The assumptions and limitations of such correlations are discussed briefly.

Central to the question of the electronic structure of a transition metal complex is its charge distribution. Although the charge is often used as a parameter in the calculation of electronic structure, it has become feasible only recently to measure a quantity directly related to the net atomic charge. Core electron binding energies (B.E.'s), as measured by X-ray photoelectron spectroscopy, can be correlated with atomic charge.

We report such correlations between measured Fe3P electron B.E.'s and, iron atomic charge calculated by means of the extended Hückel MO method. Compounds were chosen to represent a diversity of electronic environments. In particular, our interest in metalloproteins suggested a number of materials containing both iron and sulphur. A similar correlation has been established for the S2P electron B.E.'s in representative sulphur compounds.

Theoretical interpretations of measured X-ray photoelectron chemical shifts have been attempted by other workers with varying degrees of success. Siegbahn et al. [1,2] have investigated simple sulphur, nitrogen, and carbon compounds. Fadley et al. [3] made the first attempt to explain chemical shifts in ionic compounds. Hendrickson et al. [4,5] have carried out semiquantitative calculations on nitrogen and phosphorous compounds. Jolly and Hendrickson [6] have related thermodynamic data to chemical shifts in electron binding ener-

gies. More recent approaches to interpretation of X-ray photoelectron spectra have been presented in several other papers [7-9].

Several assumptions are made when attempting to correlate measured core electronic binding energies with a parameter calculated by means of the extended Hückel formalism. We have assumed that the experimental work function variations among solid samples are negligible. Electronic relaxation effects after removal of a core photoelectron are assumed to be constant, since this method of calculation neglects such effects. (Justification for this assumption is given in a report by Davis et al. [10].) The electrostatic potential contributed by the charge on surrounding ligands has not been included explicitly, but has been assumed to be included implicitly through the modifications of ref. [15]. (Attempts to include a simple point charge modification using a potential varying as q^2/r resulted in no improvement in the correlation.) Finally, we have assumed that the extended Hückel approximations are indeed applicable to the transition metal complexes studied here. A more explicit analysis of the approximations of the extended Hückel formalism and its use in calculating parameters of transition metal complexes has been described elsewhere [11,12].

In our present work both charge and binding energy were calculated using an extended Hückel method formulated by Hoffman [13,14].

Table 1.

Measured and calculated electron binding energies and calculated charges for a diverse series of iron and sulphur compounds. Spectra were produced and analyzed in the Berkeley iron-free electron spectrometer [22]. The widths at half maximum of the Fe3P and S2P photoelectron lines are about 2.6 eV and 2.4 eV, respectively. The relative binding energies are reproducible to about 0.2 eV, and charges are self-consistent to 0.05 charge units. Published synthetic procedures were followed in the preparation of compounds 2, 13, 15, 18 and 25. Compounds 14, 9 and 16 were obtained from Dr. H. H. Wickman, Dr. D. N. Hendrickson and Dr. J. B. Neilands, respectively. All other compounds were obtained from commercial sources. FeS and KFeS₂ gave broad, ill-defined iron photoelectron spectra which were too ambiguous to assign a definite measured binding energy

No.	Molecule	Measured Fe3P B.E. (eV)	Calculated Fe3P B.E. (eV)	Calculated iron charge	Measured S2P B.E. (eV)	Calculated sulphur charge
1	FeF ₆ ³⁻ (K ₃ FeF ₆)	57.7	56.0	+1.81		
2	FeO ₄ ²⁻ (K ₂ FeO ₄)	57.7	56.1	+1.79		
3	Fe(H ₂ O) ₆ ³⁺ (Fe ₂ (SO ₄) ₃ ·(NH ₄) ₂ SO ₄ ·24H ₂ O)	56.6		+1.51		
4	Fe(H ₂ O) ₆ ²⁺ (FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O)	54.2		+0.86		
5	Fe(CN) ₆ ³⁻ (K ₃ Fe(CN) ₆)	55.0		+1.24		
6	Fe(CN) ₆ ⁴⁻ (K ₄ Fe(CN) ₆)	54.0		+1.03		
7	Fe(metal)	52.0	52.4 (def.)	0(def.)		
8	Fe(C ₅ H ₅) ₂	53.7	54.5	+1.00		
9	Fe(C ₅ H ₅) ₂ ⁺ (NO ₂) ₃ C ₆ H ₂ O ⁻	54.9		+1.36		
10	Fe(CO) ₅	54.0	54.7	+1.02		
11	Fe ₂ (CO) ₉	54.6		+1.30		
12	FeS ₂	53.0	53.3	+0.45	161.5	-0.22
13	Fe(S ₂ CNEtEt) ₃	53.5		+0.95	161.5	-0.3
14	FeBr(S ₂ CNEtEt) ₂	54.0	54.1	+0.82	161.4	-0.3
15	Fe(S ₂ C ₆ H ₃ CH ₃) ₂ ·N(n-C ₄ H ₉) ₄ ⁺	53.2		+0.29	161.4	-0.43
16	Ferrichrome A	54.9	55.5	+1.53		
17	Hemin-Cl	54.2		+1.04		
18	Fe ³⁺ Cl Phthalocyanine	54.4		+1.46		
19	SO ₄ ²⁻				167.8	+1.86
20	SO ₃ ²⁻				166.4	+1.40
21	CH ₃ SOCH ₃				165.5	+0.70
22	CH ₃ SSCH ₃				162.7	-0.13
23	CH ₃ SH				162.7	+0.09
24	FeS			+0.43	160.7	-0.43
25	KFeS ₂			+0.35	161.1	-0.46

with modifications [15] to the Coulomb integrals and Slater exponents such that iteration to charge self-consistency could be obtained. This method does not include electron repulsion terms explicitly, and uses empirical parameters for evaluation of the elements of the secular determinant. (The size of the electronic systems considered in this paper renders impractical more sophisticated calculations at the present time.) Coulomb integrals were approximated by Valence

Orbital Ionization Potentials [16] (choosing an electronic configuration of d⁶sp for the iron atom). Off-diagonal elements of the secular determinant were approximated by Cusach's formula [17]. Slater-type orbitals [18] were used for the minimum basis set of wave functions. Charge values, made self-consistent to 0.05 charge units through iteration, were obtained by Mulliken's method of population analysis [19]. Coordinates, bond distances, and bond angles

were obtained from appropriate literature sources, and, in some cases, Quantum Chemistry Program PROXYZ [20] was used to obtain suitable coordinates. Large molecular structures, such as iron phthalocyanine and the dithiocarbamates, were approximated by replacing peripheral carbon substituents with hydrogen atoms in a manner similar to that of Zerner et al. [21].

Table 1 lists the measured and calculated electron binding energies and calculated charges for a diverse series of iron and sulphur compounds. For a representative sampling of iron compounds, the Fe3P B.E.'s were calculated by including the Fe3P electrons in the molecular orbital calculations and using the resulting one electron energies of the three lowest molecular orbitals as the Fe3P binding energies. Comparison of these B.E.'s with the corresponding calculated charge shows a direct correlation between calculated B.E. and calculated charge. From this observation it seems reasonable to expect similar results for attempts to correlate measured electron B.E.'s with either calculated charge or calculated electron B.E.'s when using this extended Hückel method of calculation.

Fig. 1 shows the plot of measured Fe3P B.E. versus calculated charge; the line drawn through

the points is a least squares fit to the data from neutral molecules. From this plot, it is apparent that the data point positions of formally charged ionic complexes depart from those of neutral molecules in a relatively consistent manner. There are two important factors which contribute to this variance with neutral molecule data. One factor is the lattice potential effect, and the other is the overemphasis of covalency in ionic molecules when using the extended Hückel method.

Also shown in fig. 1 is a plot of measured S2P B.E. versus calculated charge. Sulphur d orbitals were not used in these calculations; however, when d orbitals were included for representative sulphur compounds, no significant improvement in the correlation was observed.

It should be noted that if unrestricted *ab initio* calculations (including relaxation effects) were possible for the compounds we have investigated, there would be no reason to expect a *simple* charge versus B.E. correlation (even if there were no solid state effects on the electron binding energy). The observation that our calculations show reasonable correlations between atomic charge and measured B.E. (with the exception of ionic iron complexes) indicates that the assumptions made in establishing these correlations are relatively good. However, the

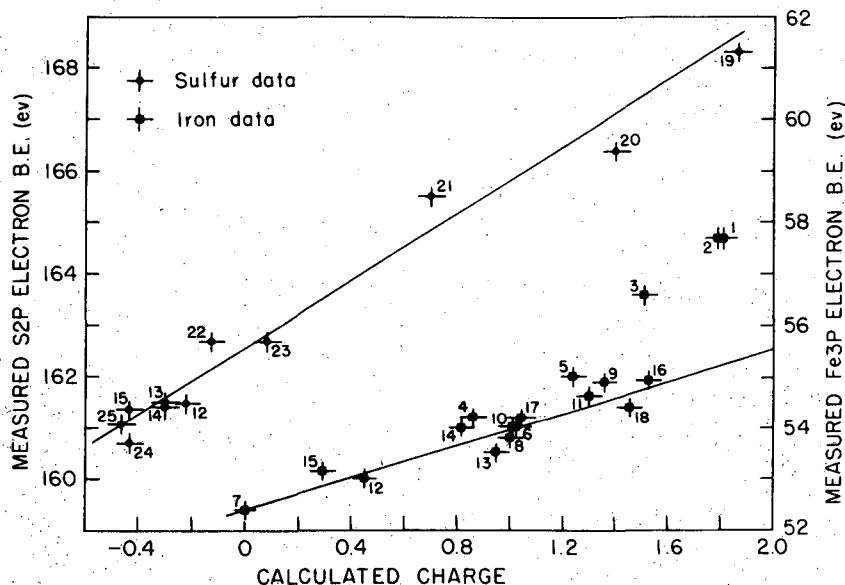


Fig. 1. Plot of measured Fe3P B.E. and S2PB.E. versus calculated charge in iron and sulphur compounds chosen to represent a diversity of electronic environments. The line drawn through the iron data points is a least squares fit to the data from neutral molecules. The numbers associated with the data points correspond to the compounds listed in table 1.

correlations are admittedly somewhat artificial since the simple extended Hückel calculations are insensitive to some important contributions to electron energy, which include relativistic, electron correlation, and relaxation effects. (In fact, the observation that the calculated Fe3P B.E.'s of the neutral iron molecules are consistently higher than the measured B.E.'s is an expected result of this insensitivity.) Nevertheless, until ab initio calculations can be applied easily to large electronic systems, semiquantitative methods, such as used here, may be very useful aids in understanding structural and bonding phenomena.

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REFERENCES

- [1] K. Siegbahn, C. Nordling, A. Fahlman, R. Nordberg, K. Hamrin, J. Hedman, G. Johansson, T. Bergmark, S.-E. Karlsson, I. Lindgren and B. Lindberg, ESCA -atomic, molecular, and solid state structure studied by means of electron spectroscopy (Almquist and Wiksells, Uppsala, 1967).
- [2] K. Siegbahn et al., unpublished results.
- [3] C. S. Fadley, S. B. M. Hagstrom, M. P. Klein and D. A. Shirley, *J. Chem. Phys.* **48** (1968) 3779.
- [4] D. N. Hendrickson, J. M. Hollander and W. L. Jolly, *Inorg. Chem.* **8** (1969) 2642.
- [5] M. Pelavin, D. N. Hendrickson, J. M. Hollander and W. L. Jolly, *J. Phys. Chem.* **74** (1970) 1116.
- [6] W. L. Jolly and D. N. Hendrickson, *J. Am. Chem. Soc.*, to be published.
- [7] H. Basch and L. C. Snyder, *Chem. Phys. Letters* **3** (1969) 333.
- [8] U. Gelius, B. Roos and P. Siegbahn, *Chem. Phys. Letters* **4** (1970) 471.
- [9] M. E. Schwartz, *Chem. Phys. Letters* **6** (1970) 631; **7** (1970) 78.
- [10] D. W. Davis, J. M. Hollander, D. A. Shirley and T. D. Thomas, Lawrence Radiation Laboratory Report UCRL-19515 (1969).
- [11] G. Blyholder and C. A. Coulson, *Theoret. Chim. Acta* **10** (1968) 316.
- [12] H. Basch et al., *J. Chem. Phys.* **44** (1966) 10.
- [13] R. Hoffmann and W. N. Lipscomb, *J. Chem. Phys.* **36** (1962) 2179, 3489.
- [14] R. Hoffmann, *J. Chem. Phys.* **39** (1963) 1397.
- [15] P. C. van der Voorn and R. S. Drago, *J. Am. Chem. Soc.* **88** (1966) 3255.
- [16] H. Basch, A. Viste and H. B. Gray, *Theoret. Chim. Acta* **3** (1965) 458.
- [17] H. Cusachs, *J. Chem. Phys.* **44** (1966) 835.
- [18] J. C. Slater, *Phys. Rev.* **36** (1930) 57.
- [19] R. S. Mulliken, *J. Chem. Phys.* **23** (1955) 1833, 1841, 2338, 2343.
- [20] P. M. Kuznesof, Quantum chemistry program exchange, Indiana University, QCPE 94 (1966).
- [21] M. Zerner et al., *Theoret. Chim. Acta* **6** (1966) 363.
- [22] K. Siegbahn, C. Nordling and J. M. Hollander, Lawrence Radiation Laboratory Report UCRL-10023 (1962).

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