Lawrence Berkeley National Laboratory
Recent Work

Title

ESTIMATION OF PROTEIN QUANTITY AND QUALITY BY X-RAY PHOTOELECTRON SPECTROSCOPY

Permalink

https://escholarship.org/uc/item/4sj5n5xn

Authors

Klein, Melvin P.
Kramer, Leo N.

Publication Date

1970-06-10

eScholarship.org Powered by the California Digital Library
University of California

Presented at the FAO/IEAE Symposium
on "Plant Protein Resources: Their
Improvement through Application of
Nuclear Techniques," Vienna, June 8-12, 1970

UCRL-19899
Preprint

c. 2

RECEIVED
LAWRENCE
RADIATION LABORATORY

AUG 19 1970

LIBRARY AND
DOCUMENTS SECTION

ESTIMATION OF PROTEIN QUANTITY AND QUALITY BY X-RAY PHOTOELECTRON SPECTROSCOPY

Melvin P. Klein and Leo N. Kramer

June 10, 1970

AEC Contract No. W-7405-eng-48

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 5545

LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

UCRL-19899
c.2

46

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

Estimation of Protein Quantity and Quality by X-Ray Photoelectron Spectroscopy*

Melvin P. Klein and Leo N. Kramer

Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, University of California, Berkeley, California 94720

(Abstract)

X-ray photoelectron spectroscopy offers a method of high potential for estimating 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 photo-ejected. 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, $E_{K.E.} = h\nu - B.E.$ 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. Preliminary experiments

---

*Work performed under the auspices of the U. S. Atomic Energy Commission.

have demonstrated: 1) Total protein may be estimated by quantitative determination of the nitrogen peak; 2) in proteins rich in lysine and arginine the amide nitrogen may be distinguished from amino nitrogen; and 3) the sulfur content may be estimated 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. Concomitantly, some improvement in spectral resolution may be anticipated which should permit better distinction between amino and amide nitrogen and perhaps the distinction between cystine, cysteine, and methionine.

Nuclear magnetic resonance spectroscopy is discussed briefly, and it is concluded that this method offers slight potential for determining protein quantity and quality in grains.

Introduction

A convenient, rapid, economical, and non-destructive method for determining the quantity and quality of grain proteins is desired for mass screening operations. In this paper we present very preliminary results of a new method which can now satisfy some of these requirements and offers the potential of satisfying all of them.

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,

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

where $E_{K.E.}$ 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.[1]

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.[2,3] 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.[2,4]

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 make our own way completely in 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 free amino nitrogen as a measure of lysine and arginine 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.[5]

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 $SO_4^=$ and that it most probably entered the sample during ammonium sulfate precipitation of the protein.[6]

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.[7] The cytochrome C spectrum is similar to that of hemoglobin but shows a higher sulfur content. It also contains $SO_4^=$. 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 possibly arises from nitrogens bonded to iron. The low energy sulfur shoulder definitely arises from sulfur bonded to iron and is characteristic of such bonding.[5]

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. This sample was prepared by pulverizing the entire bean. We do not interpret the high energy shoulder.

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. $\frac{\text{Area N1S}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{9.4} \times \frac{14}{\text{Weight of compound}} = \% \text{ N}$

Table I gives the quantitative estimates for five 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 is different from that used as the calibration standard. For the seeds, the experimental weight percent nitrogen, determined from the photoelectron spectra, was converted to percent protein by assuming a nitrogen:protein weight ratio of 0.15. Two values are shown for the kidney bean: The value of 19.3 percent assumes that all of the nitrogen signal of Fig. 4 arises from protein. The value of 15.3 includes only the fraction of the nitrogen line lying at lower energies.

Conclusions

The experiments reported in this paper have taken place during a period of less than six weeks and as such are sketchy at best. They are to be considered a preliminary feasibility study. The results do indicate that the method can be developed to: 1) provide a means for measuring the total protein content by nitrogen analysis, 2) estimate the lysine and arginine content under favorable conditions by distinguishing between the amide and amino nitrogens, and 3) estimate the sulfur content.

Since we have so few quantitative data, the reproducibility and standard deviations are yet to be determined. We have not investigated

any effects of sample geometry. If it is deemed worthy of further development as a mass screening technique, it will be necessary either to excise a small quantity of endosperm for analysis or remove a fraction of the hull to expose the endosperm.

It should also be pointed out that the dose rate of X-irradiation received by the samples in the present instrument is about 10^3 rads/h and that the experiments reported here occupied several hours per sample.

New instrumentation currently under development promises to reduce the time per sample by nearly 100-fold, which would have the dual advantage of increasing the number of samples which could be analyzed and of reducing the radiation dosage. This new instrumentation also anticipates some improvement in spectral resolution which may assist in distinguishing amino from amide nitrogen and possibly distinguishing cystine from cysteine and methionine.

Appendix

It is natural to compare this method of protein analysis with others which have been either tested or proposed. Since both neutron activation and fast proton reactions have been reported to this panel in this and previous meetings, they will not be discussed further in this paper.[8,9,10] The one other method which has been given some consideration is that of nuclear magnetic resonance (NMR). Although NMR of nitrogen might on superficial grounds appear a hopeful method, in our opinion it offers relatively little promise for the reasons which follow.

The nuclear magnetogyric ratio of N^{14}, the abundant isotope, is only some 7 percent of that of the proton, which immediately tells us that the inherent sensitivity will be extremely low. A more serious feature, however, is that the nuclear spin, I, is unity and that the nucleus possesses a relatively large quadrupole moment. This quadrupole moment will interact with any electric field gradients which result from the chemical bonding to the nitrogen. Such field gradients occur unless the symmetry of the ligands surrounding the nitrogen is extremely high as occurs in gaseous NH_3 or the NH_4 ion in acidic solution. In virtually all other situations, including those of interest here, the symmetry will be reduced. The consequences of these quadrupolar interactions are to split the NMR line into a doublet whose spacing depends on both the magnitude of the interaction energy and on the direction of the bonds with respect to the magnetic field direction. The results may broaden the NMR lines so that their intensity virtually vanishes. These considerations have been supported by many observations and indeed are the principal reasons why N^{14} NMR has remained a virtually undeveloped research tool.[11]

Similar considerations apply to two other elements, sulfur and oxygen, which are of interest. Magnetic resonance may be observed only for the isotopes S^{33} and O^{17}, both of which naturally occur in low abundance and possess nuclear quadrupole moments. NMR of C^{13} has developed markedly in recent years and is now at the stage where it is making significant contributions to problems such as the solution conformation of small molecules

and proteins of modest sizes. The natural abundance of C^{13} is 1.1 percent, which limits the sensitivity. Little work has been done on the NMR of this nuclide in solids, which is the state of protein in grain.

The use of NMR of hydrogen for the estimation of the oil content of oil-rich grains has been brought to a very useful state by a group in Yugoslavia directed by Professor Blincs. This favorable situation occurs because the oil is highly mobile and thus the proton NMR lines are very narrow, yield large signals, and may be rather easily quantified.

In summary, our experience in the field of NMR leads us to believe that this method of analysis has a low potential as a method of mass screening for protein quantity and quality. The underlying physical basis for this assertion stems from the fact that the transition energies are small in comparison with kT at other than very low temperatures. In quantitative terms the transition energies in NMR are of the order of 10^{-8} - 10^{-9} eV. By contrast, the energies involved in the X-ray photoelectron method are of the order of 10^{3} eV whereas the nuclear methods are concerned with energies in the range of 10^{7} eV.

Melvin P. Klein and Leo N. Kramer

TABLE I

QUANTITATIVE DETERMINATION OF NITROGEN AND SULFUR CONTENT

Compound	Element	Experimental Weight %	Calculated Weight %
Cysteine* (1)	Nitrogen	8.2	8.5
	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

Seed	Element	Experimental Weight %	Calculated Protein %
Barker barley seed	Nitrogen	1.9	12.7
Dark red kidney bean	Nitrogen	2.9 (2.3)	19.3 (15.3)

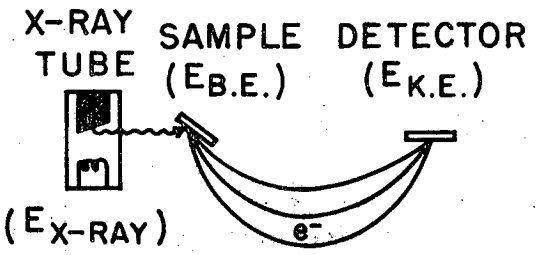
FIGURE CAPTIONS

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

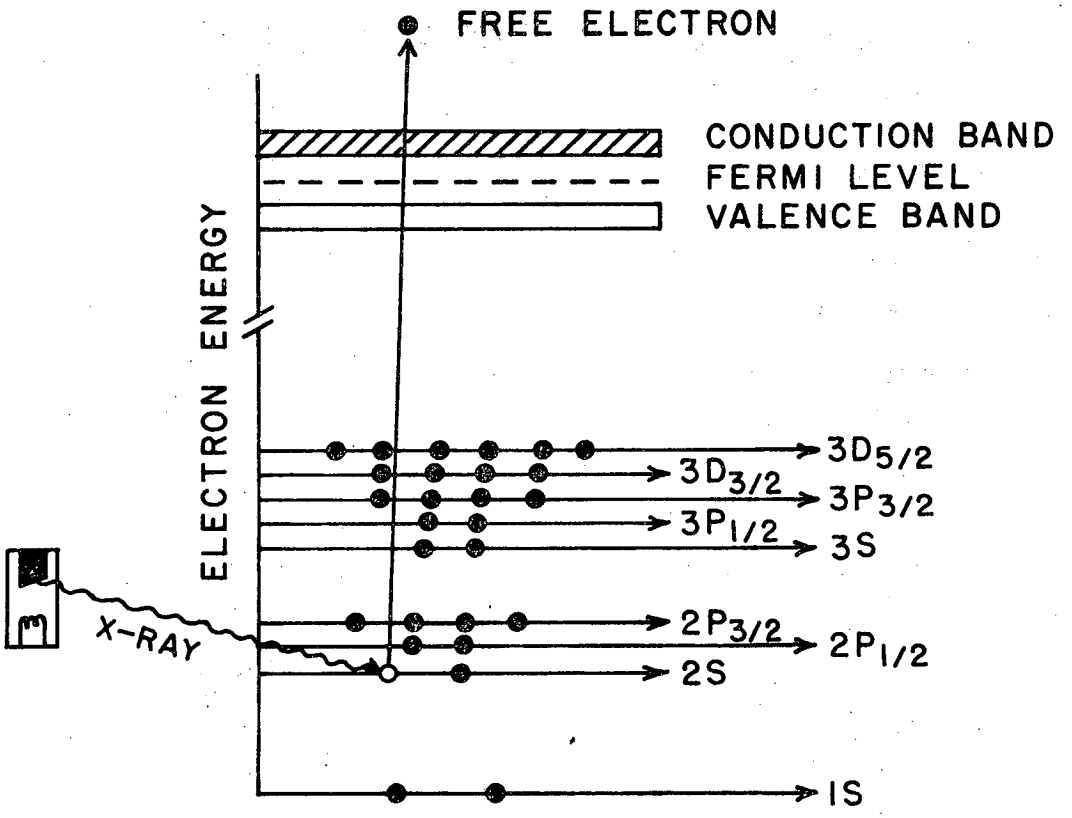
Figure 2. X-ray photoelectron spectra of three compounds showing nitrogen and sulfur lines. The dipeptide nitrogen line is decomposed into two components of equal width. One component is isoenergetic with the cystine nitrogen and is assigned to the amino nitrogen. The other component, isoenergetic with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

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

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

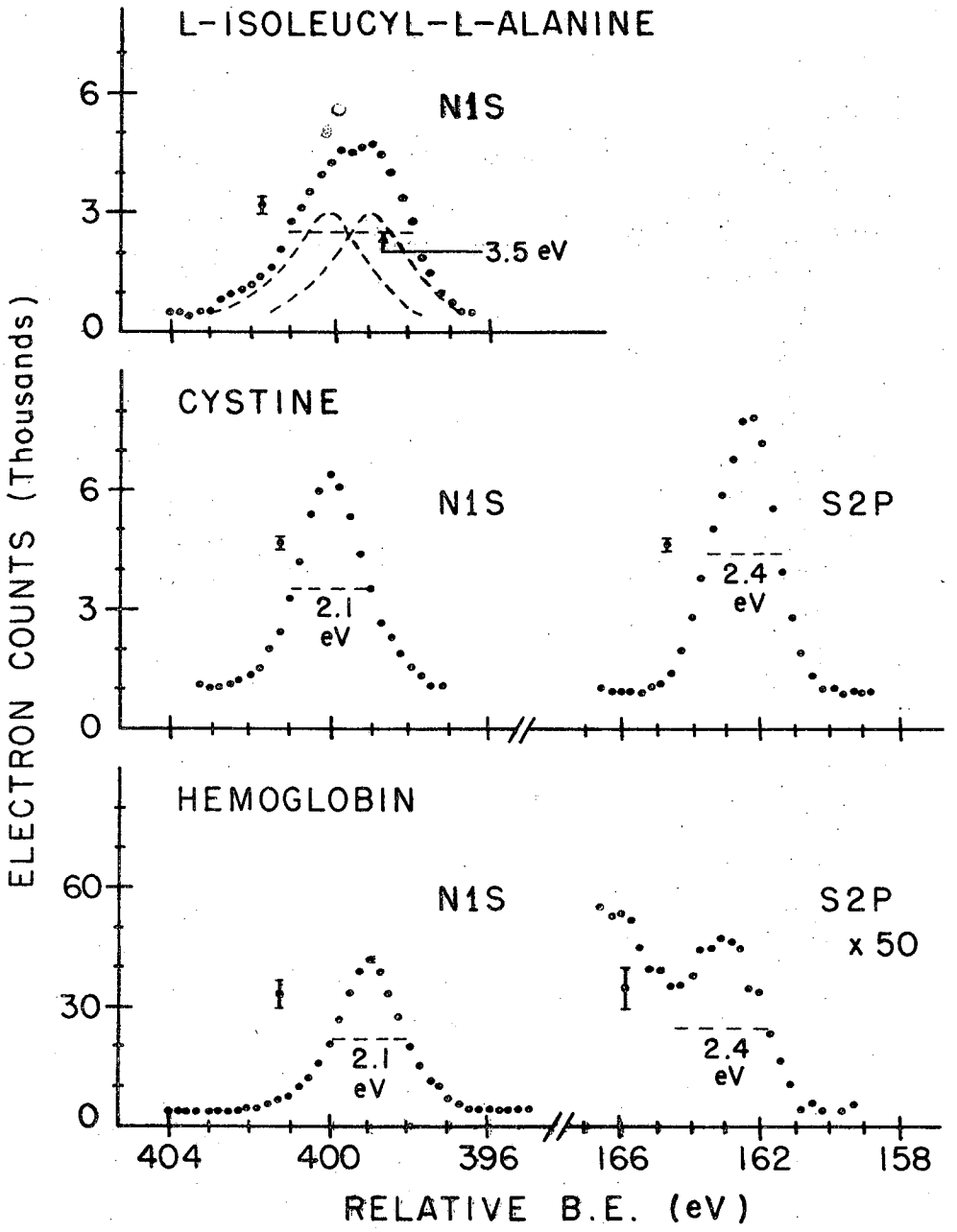


$$E_{B.E.} = E_{X\text{-}RAY} - E_{K.E.}$$
(Known) (Measured)



XBL705-5218

Fig. 1



XBL705-5216

Fig. 2

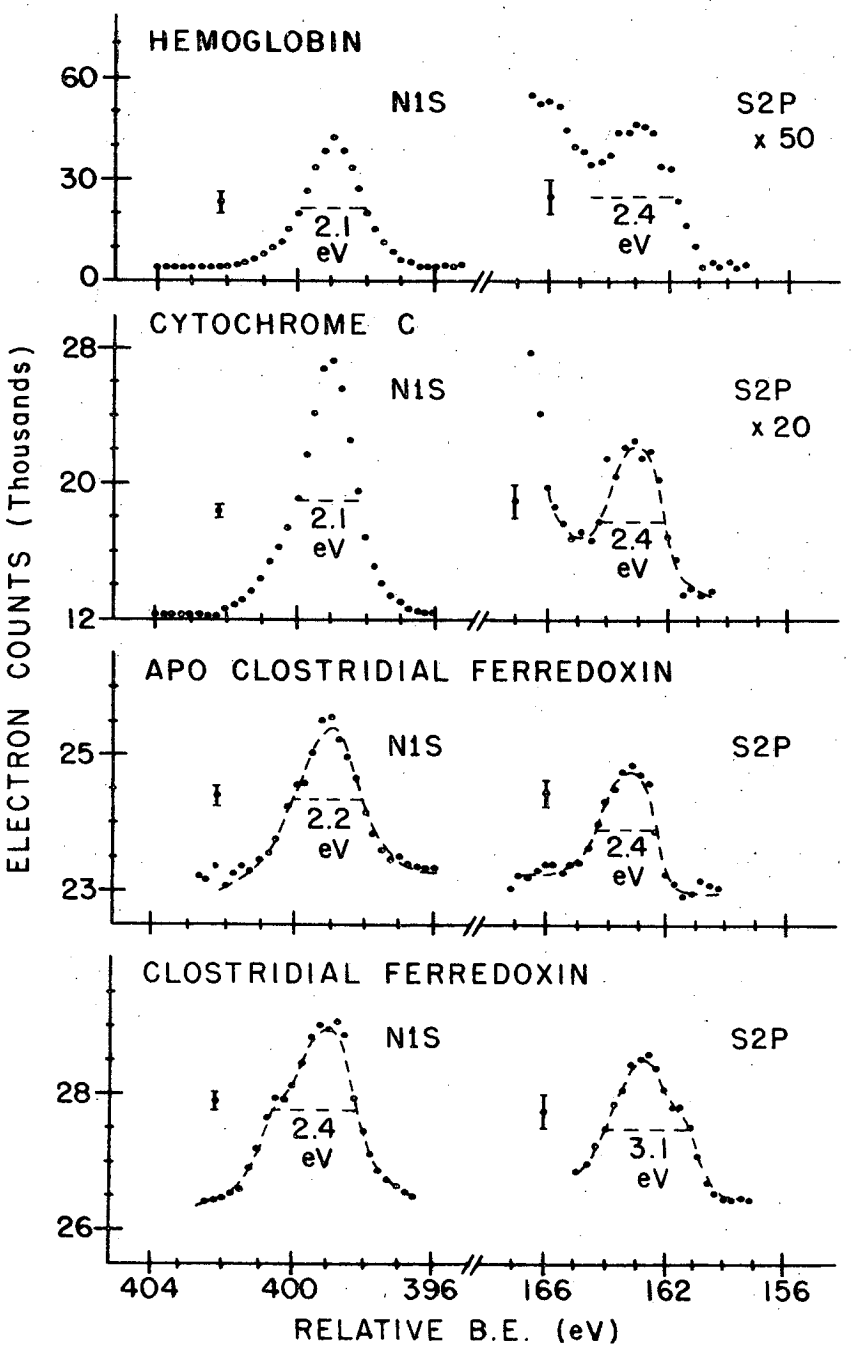

HEMOGLOBIN
N1S
S2P
x 50
2.1 eV
2.4 eV
CYTOCHROME C
N1S
S2P
x 20
2.1 eV
2.4 eV
APO CLOSTRIDIAL FERREDOXIN
N1S
S2P
2.2 eV
2.4 eV
CLOSTRIDIAL FERREDOXIN
N1S
S2P
2.4 eV
3.1 eV
ELECTRON COUNTS (Thousands)
RELATIVE B.E. (eV)
404
400
396
166
162
156
XBL705-5219

Fig. 3

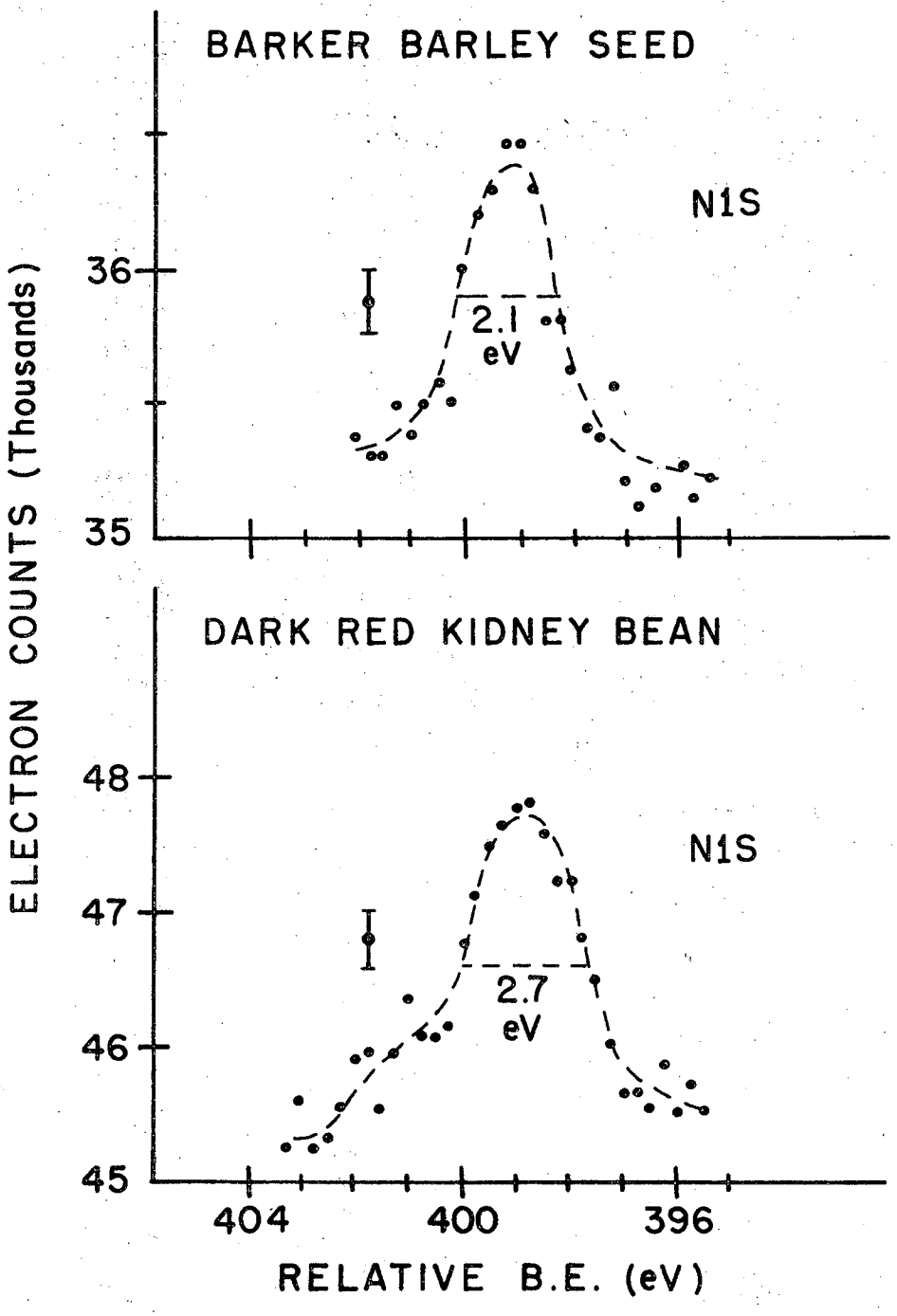

BARKER BARLEY SEED
N1S
2.1
eV
36
35
DARK RED KIDNEY BEAN
N1S
2.7
eV
48
47
46
45
ELECTRON COUNTS (Thousands)
404
400
396
RELATIVE B.E. (eV)
XBL 705-5217

Fig. 4

REFERENCES

[1] HAGSTRÖM, S., NORDLING, C., SIEGBAHN, K., in "Alpha- Beta- and Gamma-Ray Spectroscopy (SIEGBAHN, K., Ed.), Vol. 1, p. 845, North-Holland, Amsterdam (1968).

[2] SIEGBAHN, et al., "ESCA, Atomic, Molecular and Solid State Structure Studied by Means of Electron Spectroscopy", Almquist and Wiksells, Uppsala (1967).

[3] FADLEY, C. S., HAGSTRÖM, S. B. M., HOLLANDER, J. M., KLEIN, M. P., SHIRLEY, D. A., Science 157 (1967) 1571.

[4] FADLEY, C. S., HAGSTRÖM, S. B. M., KLEIN, M. P., SHIRLEY, D. A., J. Chem. Phys. 48 (1968) 3779.

[5] KRAMER, L. N., KLEIN, M. P., J. Chem. Phys. 51 (1969) 3618.

[6] KRAMER, L. N., KLEIN, M. P., Unpublished results.

[7] MALKIN, R. M., RABINOWITZ, J. C., Ann. Rev. Biochem. 36 (1967) 113.

[8] KOSTA, L., RAVNIK, V., DUMANOVIC, J., in "New Approaches to Breeding for Improved Plant Protein", IEAE, Vienna, 1969, p. 161.

[9] JOHANSSON, A., LARSSON, B., TIBELL, G., EHRENBERG, L., ibid., p. 169.

[10] LUSE, R. A., this meeting.

[11] HOLDER, B. E., KLEIN, M. P., J. Chem. Phys. 23 (1955) 1956.

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720