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COMPUTER APPLICATIONS IN QUANTITATIVE EPR SPECTROSCOPY OF METALLOPROTEINS

BY

L. Paul Rosenberg
B.S. Bridgewater State College, 1977

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Chemistry

May 1985

This dissertation has been examined and approved.

Dissertation director, N. Dennis Chasteen
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Clarence L. Grant, Professor of Chemistry

Richard L. Kaufmann, Professor of Physics

May 3, 1985
Date

This work is dedicated to the memory of my parents.

Hazel F. Rosenberg
September 8, 1909 - July 30, 1979

Herbert N. Rosenberg
January 27, 1911 - July 30, 1954

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ABSTRACT

COMPUTER APPLICATIONS IN QUANTITATIVE EPR
SPECTROSCOPY OF METALLOPROTEINS

by

L. Paul Rosenberg

University of New Hampshire, May, 1985

This dissertation examines the use of computers in quantitative EPR spectroscopy. The computers used ranged from hand held calculators to large main frame systems. Applications discussed are protein assay calculations, an EPR minicomputer interface and software system and the modification of an existing EPR simulation program to include corrections for strains in the $\underline{g}$ and $\underline{A}$ tensors. The modification permits more accurate linewidth simulation for lines with large M_I values. The computer interface and software allows for the collection of EPR spectra, which can then be stored, scaled, added, subtracted (for comparison) and double integrated. The program enhances weak signals by signal averaging. Double integration was used to assist in the study of early iron binding in horse spleen apoferritin. Iron(II) was added to apoferritin followed by oxidation by a variety of methods. In all cases an iron(III) EPR signal was observed at $\underline{g}' = 4.3$ which was attributed to mononuclear Fe(III) bound to the protein; this signal increased until 0.5 equivalent per subunit of added iron. In another experiment increasing amounts of Tb(III)

were added to apoferritin solutions. Subsequent addition of 0.5 equivalent of iron(II) per subunit resulted in an Fe(III) signal that decreased as a function of added Tb(III). It was also found that ultracentrifugation of commercial ferritin yields a light, low iron content, fraction which showed a majority of the iron signal intensity relative to the heavy, iron rich, fraction. These results suggest that iron core starts to form at an initial binding site that lies between two adjacent subunits resulting in a 0.5 equivalent of binding site per subunit and that this site also serves as the nucleus of core formation within the ferritin molecule. As the core grows beyond 0.5 equivalents per subunit more of the mononuclear sites are converted into growing core. At 0.5 equivalents per subunit double integration shows that only 20% of the added iron is EPR active suggesting a majority of the added iron is present as polymeric iron (core) species.

CHAPTER I

INTRODUCTION

Prior to the late sixties the use of general purpose computers on-line with chemical instrumentation was quite limited. The use of special purpose time averaging devices (called computers at that time) were common in selected applications. These devices were useful but very limited in their capabilities and could only be used with one or two types of equipment. An example of such unit was the Varian C-1024 time averaging computer. This device would collect data from either Varian NMR or EPR spectrometers and sum repeated spectral scans to enhance weak signals, but the C-1024 could do little else.

Computer technology has rapidly advanced since the early seventies and with that advance has come a precipitous drop in the price of computer hardware. The size of computer hardware has also decreased with the decreasing price and both effects can be ascribed to the development of MOS/LSI (Metal Oxide Semiconductor / Large Scale Integration) miniaturization technology. For example, computer mainframes, not systems, that cost about a quarter of a million in 1955 had dropped in price to five thousand by 1975 without loss of computational power (1). See Table I. This trend has continued into the eighties.

This rapid advance placed a very powerful tool at the

Table I. Evolution of computer technology.

Technology	Computer Cost	Number of Components
Vacuum Tube	$250K	10,000's
Transistors	$100K	1,000's
Integrated Circuits	$12K	100's
LSI	$5K	10's

From reference 1.

disposal of the scientific community and by the late seventies it had become apparent that research could be considerably enhanced by interfacing older existing equipment to a laboratory data system. By 1978 most new instruments were already marketed with microprocessors to enhance capability and usefulness to the researcher. An example is the Cary 219 spectrophotometer which brought wavelength scanning, cell selection and slit adjustment under microcomputer control.

Computer assisted EPR spectroscopy can be very useful in solving problems in metallobiochemistry. Very often one wishes to determine the quantitative amounts of EPR active species present and the use of a computer greatly facilitates this determination. Also in metal systems species are encountered that produce simple broad lines, such as iron systems, or very complex spectra such as in vanadyl systems. The computer greatly assists us in securing the maximum amount of information present in these diverse systems.

Goals

The object of this research was to develop computer methods to assist in quantitative electron paramagnetic resonance (EPR) spectroscopic studies of metalloprotein systems. The project developed in several stages. First, the computer hardware had to be selected from the many options available (discussed later in this chapter). Secondly, software was developed for EPR studies. Finally, the computerized EPR spectrometer was used to study the early

iron binding in equine spleen ferritin. In addition, a computer program for simulating EPR powder spectra of paramagnetic metal species was modified to include the modeling of microinhomogeneities in the g and A tensors, a situation common in biological materials.

In the course of this research automated techniques ranging from hand held programmable calculators to large scale time-share computer systems were used to advance our understanding of the metalloprotein ferritin. In this chapter, the reasons for selection of the computer system are presented along with the features of the system. I will also introduce the other types of computer tools used in this project.

Computer Selection

In selecting a computer we had several goals. First, the computer had to be able to collect data from the instrument. In EPR, the analog (continuously varying) voltage signal has to be converted to a digital (coded format) signal. This binary representation of the data can then be stored and then manipulated in a high level language such as BASIC or Fortran. It was also necessary that the data be stored on a permanent medium such as magnetic tape or disk. Software was needed to scale, shift and subtract spectra, to pinpoint spectral features, to time average spectra for weak signal enhancement, and to perform other calculations such as integrations, Fourier transformations, etc..

With these goals in mind four options were open to the

Chemistry Department in early 1978. The first option was to purchase a microprocessor and interface it to departmental equipment. This option would be least expensive in initial cash outlay (about one thousand dollars) but would have required extensive effort in low level programming and in digital electronics hardware design projects. It was determined that this option was too expensive in man-hours of development time. Our goal was for the computer interface to facilitate research and not be a research project unto itself.

A second option was the purchase of a turn key system. Such a system is designed to be dedicated to a certain instrument and is purchased preprogrammed with all major functions that a user might need. These systems generally allow the user to program special functions if such requirements arise. Varian marketed such a system for use with their EPR equipment but the system price was about twenty-five thousand dollars and very limited in capability. Such a system could not have been used on other equipment. This, in conjunction with the high cost, limited our pursuit of this option.

A third option considered was an IBM (International Business Machines) device coupler (model 7406). This instrument would allow one to attach a chemical instrument, such as an EPR, to a time sharing computer system such as UNH's DEC-10 system. In such a system the large computer system sees the lab equipment as a terminal. Details of the

function of this device are published elsewhere (2). The purchase of this instrument was proposed to the Chemistry Department in October 1978 and with departmental approval the campus computer services group was contacted. This group strongly advised against using such a system and suggested that we investigate a stand alone lab system such as the newly released DEC (Digital Equipment Corporation) MINC-11. The funds were secured and this system was purchased.

The DEC MINC-11

The DEC MINC-11, although more expensive than the IBM device coupler, was a more versatile and capable system. It contained a microprocessor based computer which could serve the department as a stand alone computer system as well as a data collection and manipulation device. At the time of purchase it was pointed out that this unit could be readily attached to many departmental instruments (EPR, stopped flow spectrophotometer, Cary 219 spectrophotometer and Mossbauer spectrometer just to mention a few), and could process the data collected from these sources. As a stand alone system the MINC could also be used for word processing and could also be used for jobs that had previously been run on the large campus time sharing system. Examples would be statistical treatment of data or small scale simulations.

The high level language that was first available on the MINC was an enhanced version of BASIC that had lab functions built into the language. An optional Fortran enhancement

was purchased later. The Fortran package included MACRO assembly language.

The MINC was purchased with the following standard features: The system had an LSI-11/2 microprocessor (a miniaturized version of the popular PDP-11/03 system) with 64 kilobytes of on-line random access memory (a byte is an eight bit computer word that corresponds to one character). Our unit also had four RS-232 serial data communications ports, and a dual drive RX/02 eight inch floppy disk system capable of on-line data storage of one megabyte of information. The system was also supplied with a VT105 plotting video terminal. This terminal was able to plot with moderate resolution and also allowed for manual selection of position along the x-axis of a plotted spectrum.

Optional equipment initially purchased enabled the system to communicate with lab equipment. An analog-to-digital (A/D) converter module and a digital-to-analog (D/A) converter module with associated clock module allowed for data collection from equipment (such as the EPR) and data output to recorders or plotters respectively.

The A/D converter was a 12 bit device and therefore had a possible precision of 1 part in 4096. This precision was suitable for our application. The maximum rate that this unit could operate was stated to be 23000 conversions per second; however, software considerations forced by the high level language only allowed a rate of 8000 conversions per second. A note should be made here that this rate is for

data collection into main memory. If a vast amount of data were to be collected, i.e. more than a full workspace, then data would have to be passed onto the disk and this transfer operation would slow the rate considerably. The dynamic input range for the A/D converter was from +5.12 volts to -5.12 volts. For our application, the EPR interface, the y-axis analog signal provided an output of ±0.50 volts. This mismatch in voltage levels prevented the use the full A/D precision. A preamplifier module was later purchased that allowed for closer matching of the output voltage of the EPR and the input voltage into the MINC. Even with this closer match the input into the MINC and the concurrent spectral plot on the EPR chart still could not be simultaneously optimized. The preamplifier module also allowed for several program controlled gain settings and also allowed for resistance and current inputs. These latter input modes were not used in my work but afford many potential applications.

The key feature of the MINC that made it so powerful was the high level computer language. The system was purchased with an enhanced version of BASIC (Beginners All purpose Symbolic Instructional Code). The useful features of this language will be discussed below. Our system was later upgraded with Fortran (an abbreviation for FORmula TRANslation) and since this system was not extensively used at first it will not be discussed.

The BASIC package was designed for ease of use. The

manuals provided with the system were well written and in a tutorial format that guided the user through several self instruction steps. For example, one user was able to progress from a novice level to writing functional data collection programs in about one week of part time use (3). The operating system for the BASIC system was written to appear as an integral part of the BASIC language and is a subset of the RT-11 operating system available on many DEC small computer systems. An operating system is the program that allows for such background tasks as printing and copying files, reading disk file directories, keeping track of computer time, calling programs from disk to be run and for creating, editing and deleting files. A review of lab operating systems can be found in Analytical Chemistry (4) and Scientific American (5).

The powerful feature of this version of BASIC was built in functions for lab data manipulations such as collection of data, and the plotting of this data on the computer terminal screen. These special commands made data collection from different types of devices very easy. The Fortran system does not have such conveniences built in and required that the user be much more expert in computer use. The operating system used was the full version of RT-11 and is therefore very similar to other DEC operating systems such as DEC TOPS-10, DEC VAX-VMS and DEC RSX-11. There was no semiautomatic graphics package nor any easy way to communicate with connected lab equipment. Although a prime selling

point of the upgrade was the speed of communication with devices via the A/D converter, this procedure was actually slower. This was in spite of the fact that program execution was in fact faster. Documentation for the Fortran upgrade was also much less helpful in finding needed information, if the information was indeed present.

Subsequent to my work, Fortran enhancements were acquired to allow for by-passing these difficulties. This allowed for programming of the data acquisition system in Fortran so that this language's speed could be utilized.

Other Computer Applications

Another aspect of my research was to use a large time sharing system to modify a preexisting EPR powder pattern simulation program. This program was resident on the UNH time sharing DEC-10 system. This application took advantage of the high computational power available on a large mainframe system. The simulation program was too large for the DEC-MINC system and the lab computer system was too slow to do effective simulations of this complexity.

However, recent advances have further increased the power of lab systems. Currently the IBM PC with custom software can approach the DEC-10 in speed and available memory for approximately six thousand dollars (6,74).

A final example of computational power in the lab is in the use of hand held calculators. These units are now available with BASIC and many have hard copy printers. Such devices can be used for the solution of many calculations

that would be time consuming otherwise. Examples might be in the generation of theoretical titration curves or the calculation of protein assay results. An example of such an assay program is presented in appendix B.

Thesis Overview

The greatest advantage gained from the use of this data system for my research was the double integration of EPR signal intensity. From this we were better able to understand the initial iron binding in horse spleen apoferritin. The use of computer generated EPR difference spectra using this system has been reported elsewhere (7). Details of the importance of double integration in quantitative EPR work are covered in chapter II of this work. Results of the ferritin experiment are the subject of chapter III.

The modification of the EPR powder spectrum simulation program is covered in chapter IV and a listing of the modified computer program is given in appendix C.

In appendix A a full listing and description of the software developed for the MINC to assist EPR spectroscopy is presented.

CHAPTER II

QUANTITATIVE EPR SPECTROSCOPY

EPR has proven to be a very useful technique for probing the nature of paramagnetic metal centers in proteins and other systems that possess EPR active metal ions. The many fertile areas in which EPR has been used to gain insight into protein structure and function have been reviewed (8, 9). In addition to native metal systems, Chasteen and coworkers have shown that the oxycation vanadyl, VO^{2+}, can be used as a probe in systems in which the native metal is either EPR inactive or its spectrum gives little information (an example is the iron binding site in human serum transferrin) (10).

In this chapter an overview of the applications of quantitative EPR in biochemistry will be presented along with the potential problems in doing such work. The use of EPR quantitatively has not been as popular as qualitative EPR. Several years ago, quantitative EPR was used to examine Mn^{2+} in water by Guilbault et al. (11). These authors used the peak-to-peak height of one peak in the six line first-derivative EPR spectrum as means of measuring the free ion concentration in solution. They reported an analytical linear range of 10^{-6}M to 10^{-3}M for the aquo Mn^{2+} species with a precision of $\pm$ 0.4% and an accuracy of $\pm$ 2%. This system was quite simple and free of inter-

ferences. Later, Guilbault and Mersel investigated potential interferences from other metals and from a variety of anions in solution (12). They noted that the introduction of chelators, such as ethylenediaminetetraacetic acid (EDTA), led to a breakdown of the method of peak-to-peak heights. It was found that chelators cause line broadening and an associated reduction in the peak-to-peak amplitude.

In addition to measuring Mn^{2+}, Guilbault also developed elaborate schemes to determine Fe^{3+}, Cr^{3+}, VO^{2+}, Cu^{2+} and Gd^{3+} in the same aqueous solution. These methods required several wet chemical steps to block interferences in order to determine concentrations by peak-to-peak amplitude measurements (13). Methods were also developed using masking agents such as ethylenediamine to quantitatively determine several metal ions in the same solution (14). Moyer and McCarthy studied the effects of factors such as instrumental, environmental and procedural techniques on quantitative measurements (15).

Subsequent to this early work, quantitative EPR methods were used for a few specific problems. Vanadium in proteins was studied by Fitzgerald and Chasteen(16). A method for the determination of the concentration of paramagnetic metals in marine environmental samples was developed by Burgess et al. (17) and EPR active metals in mollusk shells and extrapallial fluids were studied by Blanchard and Chasteen (18,19) and Misogianes and Chasteen (20), respectively.

Quantitative EPR spectroscopy was also used to deter-

mine the stoichiometry of binding of VO^{2+} to human serum transferrin (21) and to conalbumin (22). Concentration of the EPR active species was determined by using peak-to-peak heights. Such approximations are generally valid because there is not a significant change in peak widths or shapes during the experiment. Significant problems arise when comparing signals of different shape and type.

In the late 70's investigators began carefully examining the sources of error in quantitative EPR work. Warren and Fitzgerald studied both instrumental and sample variances (23). Goldberg further studied the effects of instrumental parameters on experimental precision and accuracy (24,25).

Typical EPR spectra are observed as first derivative signals. To quantify the area of these peaks it is necessary to integrate the signal twice. The first integration produces an absorbance peak and the second integration yields the area under this absorbance peak. In Figure 1, typical first derivative spectra of Gaussian and and Lorentzian lines are presented. It should be noted that the Lorentzian line possesses much broader wings than the Gaussian line. The ramifications of this difference will be discussed in greater detail later in this chapter.

Instrumental Considerations in Quantifying EPR Signals

The principal advantage gained by interfacing the Varian EPR spectrometers to a computer was the ability to

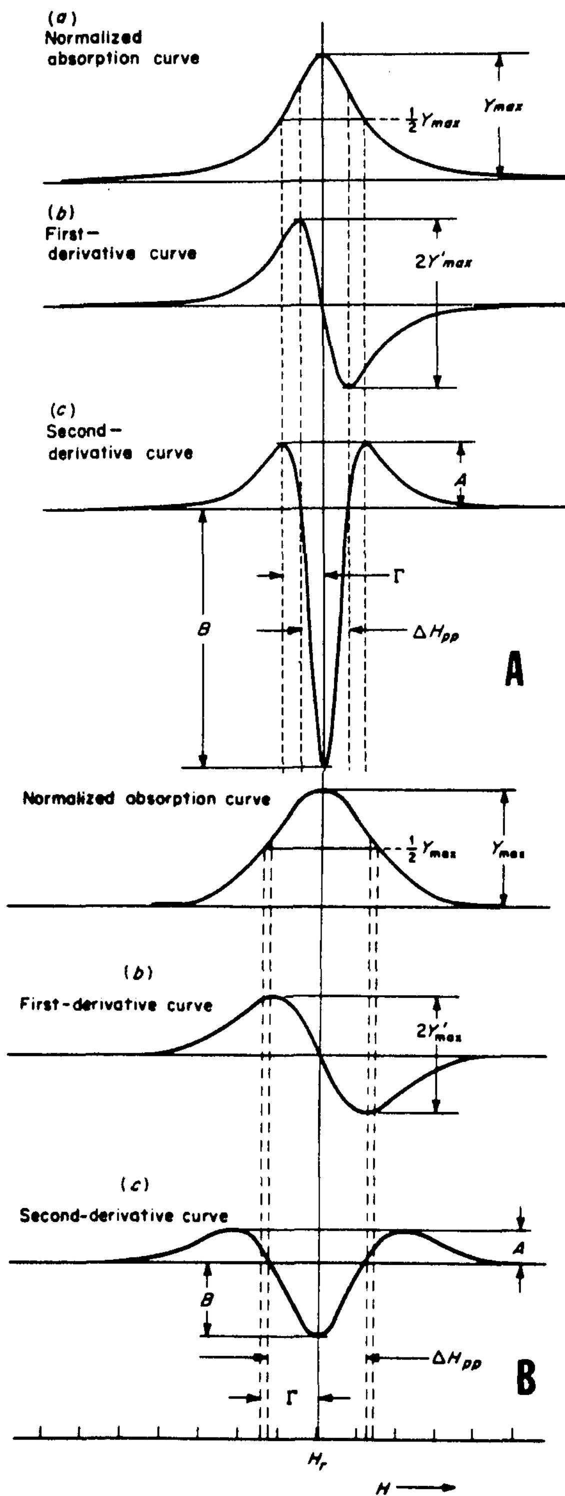


Figure 1. Absorbance, first and second derivative spectra for the Lorentzian (A) and Gaussian (B) lineshapes. Definitions for $\underline{Y}_{max}$, $\underline{Y}'_{max}$, $\underline{\Delta H}_{pp}$ and $\underline{\Gamma}$ (half width at half height) are presented. Figure from reference 27.

quantify EPR signals. As in NMR, the measured signal is proportional to the number of "spins" present in the sample; however, many other factors must be considered when attempting to determine absolute amounts of paramagnetic species present.

These factors were discussed by Warren and Fitzgerald (23) and Goldberg (24,25) in great depth and will be overviewed here. The standard deviation associated with the error in a signal can be viewed as the square root of the sum of all possible variances. The two major potential areas of error are those attributable to the instrument and those attributable to the variability in the sample itself. These errors can occur either in magnetic field position (x-axis) or in peak size (y-axis). Sources of error will be categorized by the axes in which the effect is observed.

Instrumental errors that affect the magnetic field are the: 1) field set, which affects the peak position; and 2) scan range, which could result in errors in assignment of peak width. This could also lead to errors in determining the integrated area under the curve. 3) Modulation amplitude has a direct effect on the resulting peak size; however, if this parameter is too large, a shift in peak position can also result. 4) Incorrect modulation frequency can also lead to erroneous results, so care must be taken to ensure that this parameter is properly set if it is under operator control.

There are several operator controls on the microwave bridge that could affect the EPR results. They include the power setting, cavity iris setting (detector current) and AFC control (frequency adjust). Peak height is related as the square-root of power and therefore great care must be taken to keep this parameter constant from run to run. Goldberg therefore recommends that a power meter be used if very accurate measurements are required. Cavity iris adjustment should not be a problem when working with E-line EPR spectrometers but could cause problems with older models. It is also important that the frequency of the klystron remain constant throughout an experiment as a change in frequency will cause a shift in the observed peak position.

Receiver gain is also an important parameter since the size of the recorded signal is controlled by this adjustment. It should be noted that this control is simply a voltage divider network that is made up of resistors each with about one percent tolerance. We expect therefore deviation between the actual gain observed and the instrument setting. Goldberg discusses this problem in detail and suggests two possible solutions. The first is to calibrate the voltage divider circuit by collecting EPR spectra on a fixed sample at various gains. With this calibration data the operator can correct for the deviations present in this control. The second method is to rebuild the circuit so that the gain given is in fact the value set on the dial.

Several factors should also be kept in mind when working with the spectra recorded on chart paper. The scan rate is important since peaks that are scanned too rapidly will be reduced in size. One can insure that this does not happen by making runs at several scan rates over the sharpest peak in a spectrum and then selecting the fastest scan time that does not result in peak size reduction. The time constant control must also be optimized in conjunction with the scan rate. (The time constant control is a resistor / capacitor network that will allow a low impedance path to ground for the higher frequency noise components.) Very weak signals will be accompanied by relatively large amounts of noise and therefore require longer time constants to filter out this noise. At higher time constants the pen response is much slower (due to charging of the RC network) and therefore longer scan times are required to obtain full instrument response. As in any analytical method there is inherent inaccuracy in reading the results from the chart output. This, of course, could lead to both errors in the determination of the X and Y positions of a spectrum.

Significant errors can arise from problems with the sample itself. The first problem of this kind is reproducible cavity filling. Great care must be taken when placing samples into the microwave cavity as the pen response is directly related the amount of analyte in the active region of the cavity. The most reproducible situation is when the cavity is full on each run. EPR tubes

filled to a depth of 3 cm accomplish this. Other important factors are orientation angle and cell positioning as each affects cavity tuning. Care should be taken to insure that the flat cell or Dewar insert is placed in the cavity in as reproducible manner as possible.

Cavity temperature also is an important factor since the EPR response depends on the Boltzmann distribution between spin states. The signal amplitude normally has a 1/T dependence. Generally when working with the Dewar insert this temperature should be fairly constant but when using the variable temperature unit care must be taken to see that the temperature remains reproducible from run to run.

The signal strength must be above the limit of detection but below the deviation from linearity with concentration. The linear range generally covers three to four decades in concentration. If the upper limit of linearity is exceeded, generally above 1 mM concentration, then one must dilute the sample to bring it into the linear range.

Lastly one should not neglect any error associated with wet chemical steps such as volumetric dilution, filtration and weighing. Such error should be small with careful lab work, but when working with the small volumes typical of protein studies, small absolute error can lead to rather substantial relative errors.

EPR Signals

The first derivative of the resonance absorbance signal is a consequence of using a modulated magnetic field with concur-

rent phase sensitive detection as noted earlier. This detection method increases the EPR detection limit by increasing the obtainable signal-to-noise ratio. A full discussion of this method can be found either in Poole (26) or in Wertz and Bolton (27). In some applications second and even third derivatives are presented; these higher order derivatives assist in the interpretation of spectral fine structure. For a discussion of third derivatives see Halpern and Phillips (28).

EPR lines usually appear as either Lorentzian or Gaussian shapes (Fig. 1). Lorentzian lines are usually observed in systems in solution. This shape can be derived from the Bloch equations, the equations that relate magnetization along the three principal axes to the $\underline{T}_1$ (spin-lattice) and $\underline{T}_2$ (spin-spin) relaxation times. This derivation results in equation 1 for absorption (see section 11-3 of reference 29).

$$g(\omega) = \frac{T_2}{\pi} \left(1 + T_2^2(\omega + \omega_0)^2\right)^{-1} \quad (1)$$

where $g(\omega)$ is the signal strength at frequency ω, $\underline{T}_2$ is the spin-spin relaxation time and ω_0 is the Larmor frequency in radians per second. Equation 1 can be also expressed in terms of field and linewidths as in equation 2 (35).

$$Y = Y_{max} \frac{\Gamma^2}{\Gamma^2 + (H - H_r)^2} \quad (2)$$

where $\underline{Y}$ is the signal intensity at field position $\underline{H}$, $\underline{Y}_{max}$ is the maximum signal intensity (i.e. when $\underline{H} = \underline{H}_r$) and $\underline{H}_r$ is the field strength at the center of the resonance. Γ(gamma) is the half width of the absorbance peak at the $\underline{Y}_{max}/2$ position (Fig. 1). This equation can be differentiated and an expression for the customarily observed first derivative line generated (equation 3).

$$Y' = -Y_{max} \frac{\Gamma^2}{[\, \Gamma^2 + (H - H_r)^2 \,]^2} \qquad (3)$$

Here $\underline{Y}'$ is the amplitude of the derivative signal at magnetic field position $\underline{H}$. These lineshape equations are quite useful and can be used to simulate simple EPR spectra.

Gaussian lines generally arise with solid state samples. Pake attributes the Gaussian line to "a random distribution of resonance frequencies for noninteracting dipoles" (30). On the other hand, McMillan points out that Gaussian lines arise from systems in which there is broadening due to spin-lattice interactions and dipolar spin-spin interactions whereas Lorentzian lines result when exchange interactions can occur (31). Wertz and Bolton state that Gaussian lineshapes arise when there is a "superposition of many components" (27) and states that such lines are inhomogeneously broadened. They define this term as the case where each paramagnetic center is subjected to a slightly different magnetic field and the observed full resonance line is the

summation of these individual components (27).

The equation presented by Carrington (29) for the Gaussian line in frequency units is presented as equation 4.

$$g(\omega) = \frac{T_2}{2\pi^{-1/2}} \exp\left(-0.5\, T_2^2 \,(\omega - \omega_0)^2\right) \quad (4)$$

Here all the variables have been previously defined for the Lorentzian lineshape. Wertz and Bolton present a normalized equation for this system in magnetic field units (equation 5).

$$Y = Y_{max} \exp[(-\ln 2)(\Gamma^{-2})(H - H_r)^2] \quad (5)$$

Differentiation leads to an expression for the first derivative Gaussian line (equation 6).

$$Y' = Y_{max}(2\ln 2)(\Gamma^{-2})(H - H_r) \exp[(-\ln 2)(\Gamma^{-2})(H - H_r)^2] \quad (6)$$

It is possible to derive equations for higher order lines should one wish to study such systems.

Quantitative Analysis

Peak heights of the first derivative line can be used as the analytical response when the linewidth is invariant with concentration or the standards and unknown are the same chemical species. An example of such a system might be a paramagnetic ion in solution without complexing agent present. Standards could be prepared by placing known amounts

of paramagnetic ion into solution and then preparing a standard curve. If matrix effects are present, the method of standard additions can be used. Such an approach was used by Guilbault and Lubrano (11) and by Fitzgerald and Chasteen (16). However, if a system is studied in which peak width varies, it is important that this effect be accounted for. Peak area must then be determined.

Brinkman and Freiser (32) were the first to take linewidth into account; however, these workers did not account for differences in lineshape. Poole has shown that area can be easily determined for a line that varies in both linewidth and lineshape by using equation 7.

$$A = kY'\Delta H^2_{pp} \qquad (7)$$

Here A is the area under the absorption curve, Y' is the full peak height and ΔH_{pp} the peak-to-peak width of the first derivative line (Fig. 1). The constant k is a function of line shape. For a Lorentzian line the value of k is $(2/3)^{1/2}/2$ and for Gaussian lines has a value of $(\mathrm{pi})e^{1/2}/2$.

Poole also shows that from a regular absorption curve area can be calculated from equation 8.

$$A = kY\Delta H_{1/2} \qquad (8)$$

Where $\Delta H_{1/2}$ ($=2\Gamma$) is the full width of the peak at

half height and *Y* is the peak height of the absorption signal. The variables are also illustrated in Figure 1. The variable *k* once again depends on line shape and varies between pi/2 for Lorentzian lines and $0.5(pi)^{1/2}/(\ln 2)^{1/2}$ for Gaussian lines.

These equations can only be applied when a limiting case lineshape (Lorentzian and Gaussian) is encountered. In many cases neither extreme is the case and the line falls somewhere in between the two extremes. In these instances it is necessary to carry out a full double integration of the first derivative absorption line to determine the proper number of spins present in the sample. In the present work this approach was taken. It provided a general method for any system without prior knowledge of the line shape of the EPR signal.

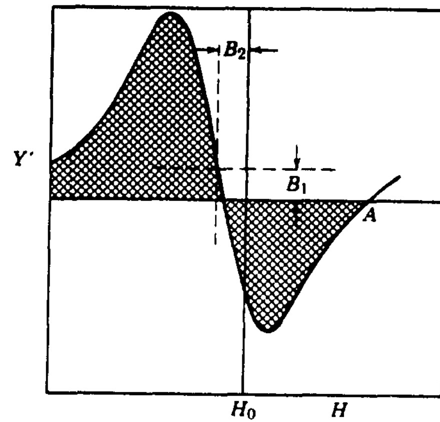
The method of double integration employed two sequential summations of the arrays in which the spectrum is stored. In this method a sloping baseline in the first derivative spectrum is corrected linearly to ensure that the wings have a zero *Y* value in the integer arrays. It is most important that this correction be also done after the first summation since even a minor displacement from the baseline is integrated into the resulting absorption peak or second integral. An example of this is shown by Poole in Figure 2 in which the *x*-axis base line is offset by a constant amount represented by *Bl*. The second integration is taken as the summation of the resulting absorption curve

and the results are presented as a sum.

Lorentzian lineshapes tend to have considerable intensity in the wings. This presents a problem because significant spin concentration information can be lost in the baseline. Gaussian lines converge on the baseline much more rapidly and therefore pose little problem. Goldberg developed an equation to calculate a correction factor when integrating over a fixed spectral interval. The amount of information lost in the wings can be very large. For example, double integration of a simple Lorentzian line with limits between points where the signal amplitude is 0.5% of the peak maximum results in 22% of the spin concentration information being lost (24).

Computer Experiments

In the early stage of this work, calculations suffered from rather large imprecision. When a single Lorentzian line was simulated and noise was superimposed upon the line (signal-to-noise ratio of 33.), typical results of the double integrations of a series of these 'identical' spectra lead to a relative standard deviation (standard deviation divided by the mean) of 0.26. Noise was added to the spectrum by taking the calculated Lorentzian spectral points and adding random numbers that varied equally about zero. The magnitude of the noise was varied by program input. Careful investigation of this problem revealed that the noise led to error in the baseline correction routine of the double integration program. Table II shows the results from



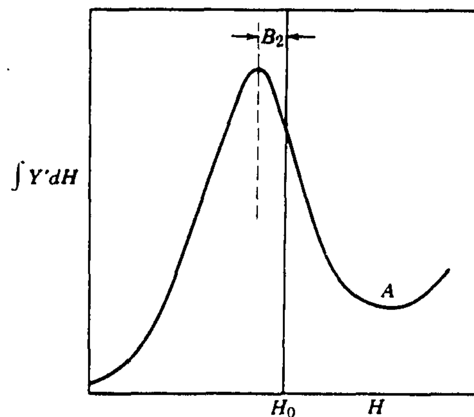


Figure 2. Resulting absorption line when an improper baseline is selected for integration. Offset of baseline is represented by B1 in the figure. B2 is the displacement of the spectrum from the center position which will not lead to problems in double integration applications. Figure from reference 26.

another computer experiment in which the *y*-axis baseline was shifted in increments of one unit and eliminating the baseline correction routine normally employed in the double integration program. A very large effect is observed. It is important to note that the range of *y*-axes values in the spectrum collected from an instrument is 4096 (from -2048 to +2047, the A/D converter dynamic range) and therefore a shift in baseline of a single unit is not a very large relative error. The reason such a large effect on the double integral is observed (Table II) is that the offset error is introduced twice in the calculation and is compounded by the summation process. This exercise shows that the noise present in the wings of a spectrum might introduce a small baseline bias leading to a relatively large scatter in the resultant double integral.

The lack of precision in the first version of the program was aggravated because spectra were stored as integers and the first and second integration is scaled to a range of values that could be plotted automatically by the MINC software on the VT105 terminal. This caused rounding errors and resulted in a loss in the precision of the calculation. When such scaling is circumvented, the reproducibility improves to give a relative standard deviation of 0.13, about one half the value with automatic scaling. The results are summarized in Table III. A subsequent version of the program utilized floating point calculations for the double integration which rectified the precision problem encountered

Table II. Effect of having spectrum offset from a zero baseline. For this computer experiment the spectra were simulated with of $2Y'_{max}$ of 129, peak width (Γ) of 100 and no noise (equation 3). Normal program baseline correction is aborted and the range of integration was 40 times Γ.

Baseline offset	Double integral x 10^{-6}
0	2.5
1	4.5
2	12.9
3	21.3
4	29.7
5	38.1

with integer calculations.

It should be pointed out that the noise folded into the spectrum is very severe for this computer experiment and represents a worst case example. In fact typical peak-to-peak height for the first derivative is 129 units while typical values for the standard deviation of the noise would be 3.3 units resulting in a signal-to-noise ratio of 39. (Signal-to-noise is defined as the peak-to-peak height divided by the standard deviation of the baseline noise.) Table IV shows the effect of noise on the relative standard deviation of the double integrals for a series of spectra simulated with different amounts of noise present. When our signal-to-noise is as high as 387 the relative standard deviation has become a very reasonable 0.012.

These calculations highlight the need for baseline correction. The data obtained in Table II were not corrected for a misplaced baseline. However, when baseline correction was accomplished prior to the first and second integrations satisfactory results were obtained (Table III). Linear baseline correction is done by taking the first and last ten data point and determining the baseline slope and displacement from the center of the y-axis (zero position). The spectral array is then offset to correct for the slope and displacement. When baseline correction is carried out baselines offset by single integer amounts, up to five, resulted in a second integrations with less than a 0.001 relative error (absolute error divided by true value).

Table III. Comparison of the results when data is scaled to allow for automatic plotting. Spectra were simulated with $2Y'_{max}$ = 129, line width (Γ) = 100 and a noise factor of 10 giving a signal to noise ratio of 39. Linear baseline correction used the first and last ten data points to determine baseline slope and displacement. Range of integration was 40 times Γ.

Double integration value without noise is equal to 2.56×10^6

	No scaling	Scaling
Number of runs	11	11
Mean value of the double integration	2.42×10^6	2.18×10^6
Relative Standard Deviation	0.13	0.26
Percent error from the true value	5.4%	14.8%

Notes:

Relative standard deviation is the absolute standard deviation divided by the mean.

The signal-to-noise ratio is determined by dividing the peak-to-peak amplitude by the standard deviation of the noise in the baseline.

Table IV. Precision of double integration as a function of added noise. The line was simulated with $2Y'_{max}$ = 129 and a peak width (Γ) of 100 with baseline correction. The noise factor is the amplitude of the random signal superimposed onto the simulated line. The relative standard deviation is a measure of the scatter of the results about the true value of the double integration.

Noise factor	Typical S/N	Relative Standard Deviation	Number of Simulations
10	12.9	0.13	12
5	25.8	0.028	6
1	129	0.012	8

A series of spectra was simulated to test the relationship between $\underline{Y}_{max}$ on the value of the double integral. We expect these to be directly related from equation 7. This equation predicts that the peak height should be proportional to the double integral when the line shape and width remain constant. The results of the computer experiment summarized in Table V verifies this expectation. A regression analysis carried out using Minitab (33) gives a least-squares line with a slope of 245 and an intercept of -0.035×10^6. The correlation coefficient squared for this line 0.998.

Isotropic and Anisotropic Spectra

Two limiting cases are encountered when doing EPR studies on metal protein solutions, namely the motionally narrow limit and the rigid limit. These two cases are usually observed for spectra obtained with room temperature and frozen solution samples respectively. In the room temperature case, the EPR active species undergoes rapid reorientation on the EPR time scale; in the resulting spectrum the magnetic anisotropy is averaged to zero leading to an isotropic spectrum. Figure 3 shows the spectrum for the isotropic system $VO(H_2O)_5^{2+}$ in room temperature solution. The observed $\underline{g}$ value is the average value of $\underline{g}$ for the three principal axes of the molecule. The observed hyperfine couplings, $\underline{A}$, is also an average of three principal values. An eight line EPR spectrum is observed because of the hyperfine coupling of the free electron to the vanadium

Table V. Comparison of resulting double integral from different values of $\underline{Y}_{max}$. Spectra simulated with a peak width of 100 and a noise factor of 5 and using baseline correction.

Y_{max}	Double Integral x 10^{-6}
1000	0.27
2000	0.43
3000	0.67
5000	1.19
7000	1.63
9000	2.13
11000	2.72

m = 245 and b = -0.035×10^{6}. r^2 = .998

nucleus of spin 7/2. Theory predicts that each transition should be of equal probability (29) and therefore of equal intensity. At first glance this does not appear to be the case since the peak-to-peak amplitude decreases upon moving away from the center of the spectrum. However, the second integral of this spectrum shows that each component of the eight line multiplet has equal intensity.

Care should be exercised when making judgments about the concentrations from the apparent peak heights. Figure 4 shows two first derivative EPR signals which might lead to an incorrect conclusion as to the relative concentrations represented. Although, the amplitude of the spectrum on the left is smaller, it corresponds to a spin concentration six times that of the spectrum on the right.

The second limiting case is observed with frozen solutions at nitrogen temperature (77 K) where rotational averaging of the molecular anisotropy is absent. The analyte in not free to tumble nor is it present in an ordered solid state structure. In this case the spectrum displays a "powder pattern" envelope of lines. In Figure 5 the spectrum of the pentaaquovanadyl complex, which has axial molecular symmetry, is shown. The spectrum is essentially composed of two superimposed spectra. One of these spectra is due to those principal magnetic tensor components parallel to the vanadium oxygen double bond and the other perpendicular to this axis. Each directional component has a corresponding $\underline{g}$ value (the $\underline{g}$ value is measured from the "center" of

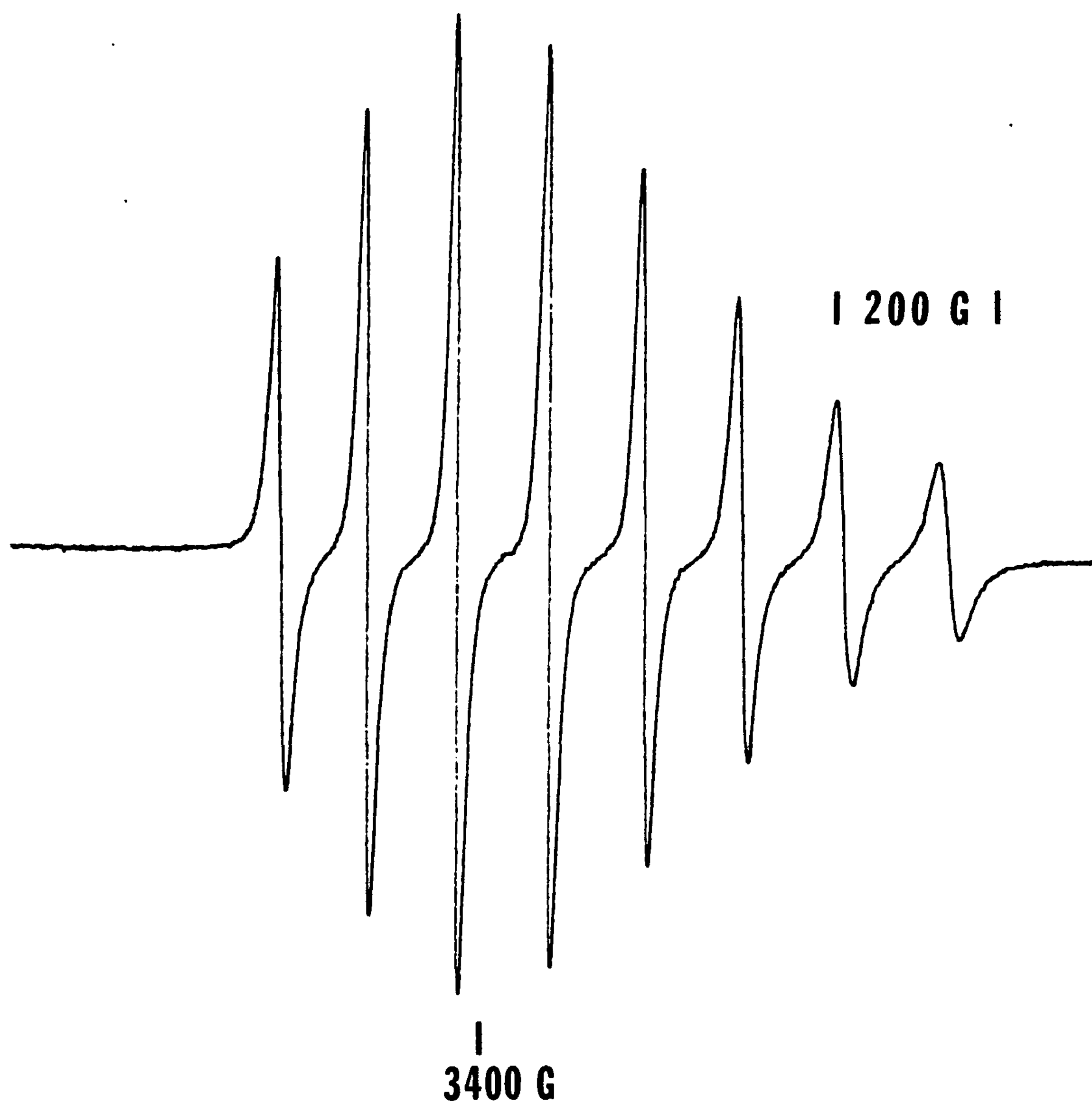


Figure 3. Spectrum of pentaaquo vanadyl at room temperature. Spectral center is at 3400 Gauss with a scan range of 2000 Gauss. The eight lines are due to the hyperfine coupling to the spin 7/2 vanadium nucleus. This spectrum was collected and plotted using the developed computer system.

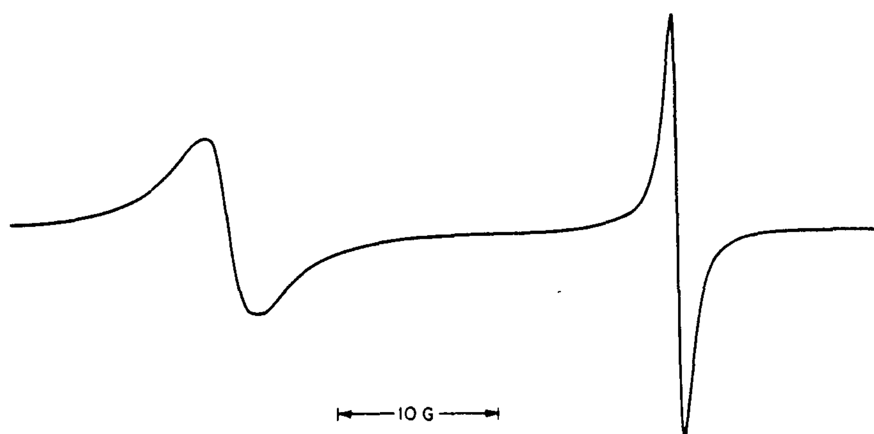


Figure 4. An EPR spectrum showing two peaks of different amplitude and width. The left peak represents a system with six times the number of spins as the right peak. (Figure from reference 27.)

the spectrum) and A value. The hyperfine coupling constant, A, is determined from the distance between neighboring lines. Analysis of vanadyl EPR spectra can be found in Chasteen (10) and Holyk (34).

Quantification of EPR spectra of frozen samples represents a somewhat special situation. Double integration can be used but once again such rigorous treatment is usually not required. Peak-to-peak heights multiplied by peak widths squared on well resolved perpendicular lines can be used. Since it has been shown that the outlying parallel lines are true absorbance peaks (35), the area under these peaks can be calculated by triangulation, planimetry or cut and weigh methods. The standards used to compare intensities should have as close a line shape to the unknown as possible. Templeton and Chasteen have utilized this method to verify that all VO^{2+} added to a fulvic acid system was in fact EPR active (36).

One aim of the present research was to employ a laboratory computer with the Varian EPR spectrometers and to develop software for quantitative work. Many transition metal protein complexes do not readily lend themselves to the approximations discussed above. The hardware/software system which was developed allowed double integrations of digitized spectra to be done automatically. Manual methods of double integration are presented in Alger (37). However, these manual methods are laborious, time consuming and prone to error.

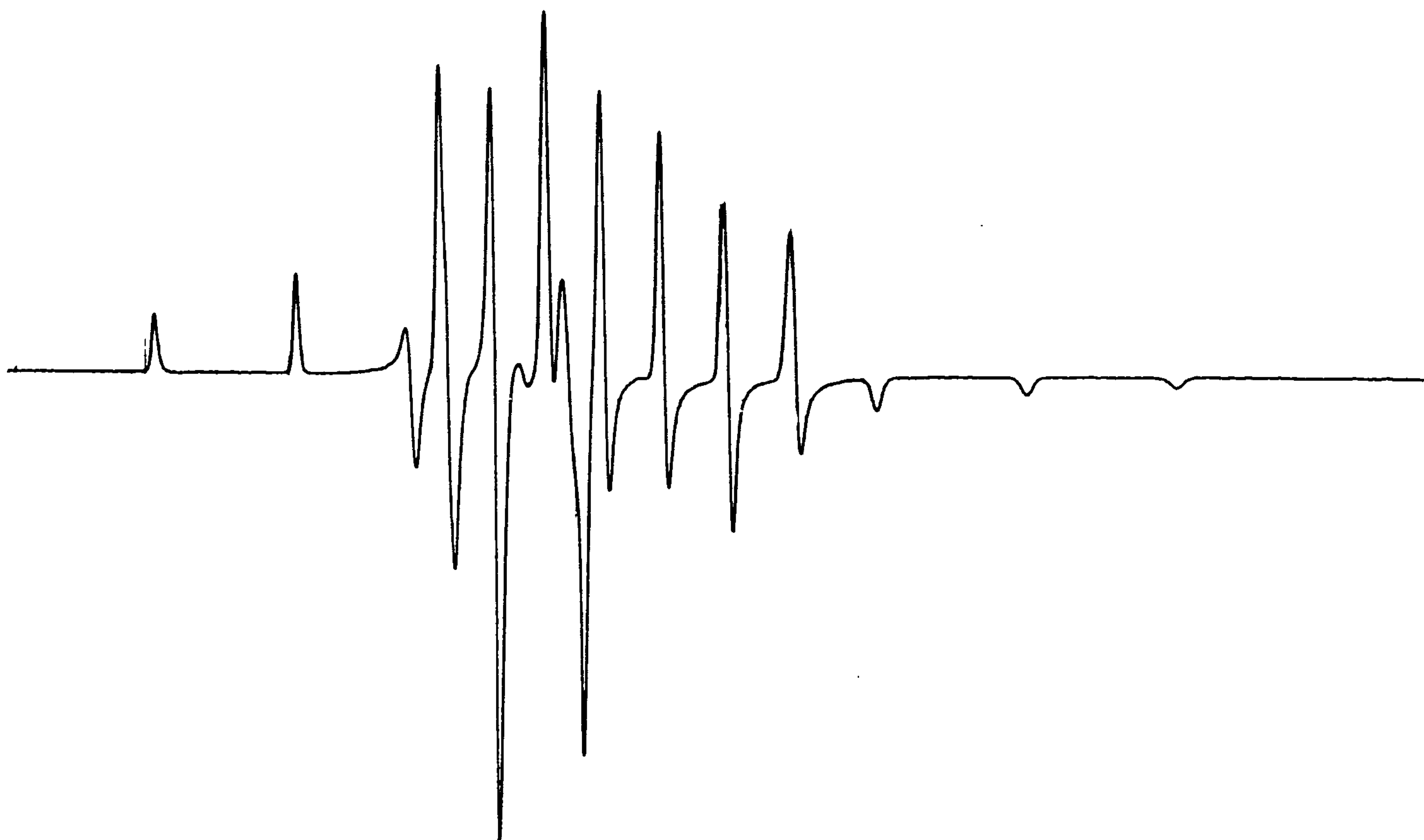


Figure 5. The spectrum of petaaquovanadyl (2+) at liquid nitrogen temperature (77 K). For this experiment a field set of 3400 G, a scan range of 2000 G with 0.2 G modulation at 100 kHz was used. The microwave power was set to 2 mW at 9.090 GHz and the receiver gain was 250. The scan time was 29 minutes with a time constant of 0.3 sec.

Example of Program Integration

The first derivative spectrum of pentaaquovanadyl ion in aqueous room temperature solution is shown in Figure 6A. Figure 6B and 6C are the program generated first and second integrals. Note that the steps in Figure 6C are of equal size indicating that equal intensity is associated with each line. The software also provides numerical output and allows for integration over user selected spectral intervals.

Future Work

As mentioned before, the results of a concentration determination depend on the lineshape. Lorentzian lines, commonly observed with liquids, cannot be completely integrated due to intensity in the wings. Goldberg's correction factor, however, works only for pure Lorentzian lines. He points out that accurate area determinations can be made only if one knows whether the lineshape is pure Lorentzian or Gaussian. It would be useful to develop a method of determining $\underline{k}$ (equation 7) for lineshapes that fall between the Lorentzian and Gaussian and to determine correction factors that could be used for broad lines that are not completely Lorentzian. Poole (26) presents a table that compares ratios of various measurable parameters for EPR lines of these two lineshape extremes. An excerpt of this table is presented here as Table VI. Spectra could be simulated for intermediate cases and relationships of shape parameters, such as in Table VI, determined for such lines. These could in turn be used to develop correction factors

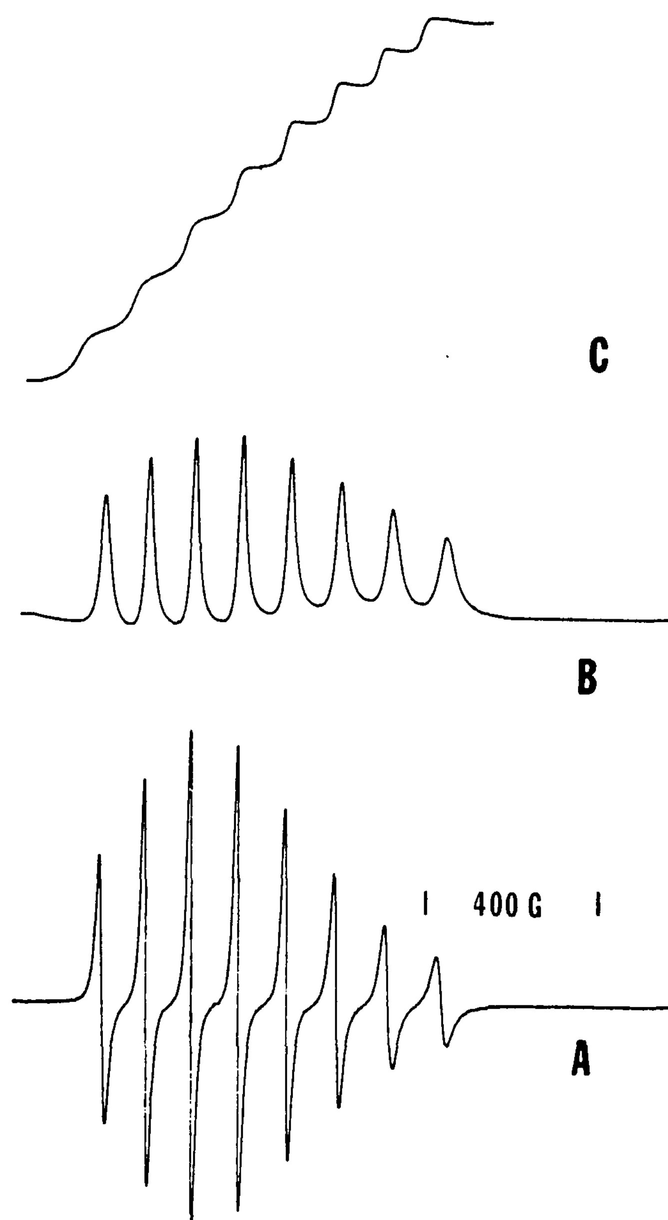


Figure 6. Spectra of pentaaquovanadyl ion at room temperature. Spectral center at 3200 G with a scan range of 2000 G. The eight lines are due of the hyperfine coupling to the spin 7/2 vanadium nucleus. Figure 6A is the computer collected first derivative spectrum. Figure 6B is the first integral (absorption) spectrum of this ion. Figure 6C is the second integral of figure 6A. Note the similarity of this spectrum to an integration one might see for an NMR spectrum.

for determining areas of experimental lines from peak heights and linewidths or for correction factors for double integrations of very broad lines. Such corrections would be useful, however, only for spectra consisting of relatively few lines.

Table VI. Relationships of measurable shape parameters compared for Gaussian and Lorentzian lineshapes, from reference 26.

Parameters	Gaussian	Lorentzian
$\Delta H_{1/2}/\Delta H_{pp}$	$(2\ln 2)^{1/2}=1.1776$	$3^{1/2}=1.7321$
$y_m/(y'_m \Delta H_{pp})$	$e^{1/2}/2=0.8244$	$4/3=1.3333$
$A/(y_m \Delta H_{1/2})$	$0.5(pi/\ln 2)^{1/2}=1.0643$	$pi/2=1.5708$
$A/(y'_m \Delta H^2_{pp})$	$0.5(pi*e/2)^{1/2}=1.0332$	$2pi/3^{1/2}=3.6276$

$e=2.7183$
$pi=3.1416$
$\ln 2=0.69315$

$\Delta H_{1/2}$ Half width at half height of the absorption line.

ΔH_{pp} Peak to peak line width of first derivative line.

y Absorption peak height

y' One half the peak to peak amplitude for the first derivative line.

A Area under the absorption peak.

CHAPTER III

EARLY IRON BINDING IN HORSE SPLEEN APOFERRITIN

Introduction

Iron has several important physiological functions. Some of the major human iron containing proteins are summarized in Table VII. Hemoglobin and myoglobin are the proteins of oxygen transport and storage respectively and together contain about 67% of the four grams of iron present in the adult male (38). Iron storage is the next most abundant iron pool where 25% of the total body iron is equally distributed between ferritin and hemosiderin. The remaining iron is found among the iron transport protein transferrin and the various enzymes that require iron for activity. These latter proteins include cytochromes, catalase, peroxidase, cytochrome c, oxidases, flavoprotein dehydrogenases and oxigenases, all of which have iron as an integral part of their active sites.

The body is very efficient at maintaining the proper level of iron. Daily absorption is low, about 1 mg/day, for normal people. Average dietary intake of iron for an adult male is approximately 18 mg of which only about 6% is taken up through digestion, the remainder being excreted. Good sources of bioavailable dietary iron include red meats (high in hemoglobin), fish and animal muscle and liver. Unfortu-

Table VII. Major Iron Proteins and their function. Reference 47.

Protein	Iron valence	Function
Hemoglobin	Fe^{2+}	Oxygen transport
Myoglobin	Fe^{2+}	Oxygen storage
Transferrin	Fe^{3+}	Iron transport
Ferritin	Fe^{3+}	Iron storage
Homosiderin	Fe^{3+}	Iron storage
Catalase	Fe^{2+}	Metabolism of H_2O_2
Cytochrome c	Fe^{2+}/Fe^{3+}	Terminal oxidation
Peroxidase	Fe^{2+}/Fe^{3+}	Metabolism of H_2O_2
Cytochrome and oxidase	Fe^{2+}/Fe^{3+}	Terminal oxidation

nately the iron found in plants is not usually efficiently absorbed. Iron is lost through bleeding, including menstruation, and pregnancy. Other rare conditions can also lead to iron loss. For these individuals the potential for deficiency is greater; therefore, such people require greater amounts of dietary iron to maintain needed levels (46).

Figure 7 shows the major pathways for internal iron exchange as presented by Hellman and Finch in 1974 (39). A more detailed scheme was given by Cavill et al. in 1981 (40). This diagram shows the transport of iron by transferrin between the gut, the extravascular system, the erythroid marrow, and the reticuloendothelial cells where red blood cell catabolism takes place (41). It also shows the exchange of iron by transferrin with the parenchymal tissues. In this diagram the major areas of iron storage are in the reticuloendothelial stores and in the parenchyma of various organs, i.e. the liver, spleen etc..

Iron

The importance of iron in biology is of no surprise considering the abundance of this element on earth. Iron is a major component in the earth's core and the fourth most abundant element in the crust, following oxygen, silicon, and aluminum (42,43). Composition of meteorites suggest that iron is abundant throughout our solar system. Table VIII shows the isotopic distribution of the element (44).

Iron is known to exist in several oxidation states (-II, 0, I, II, III,IV,V); however, only oxidation states II

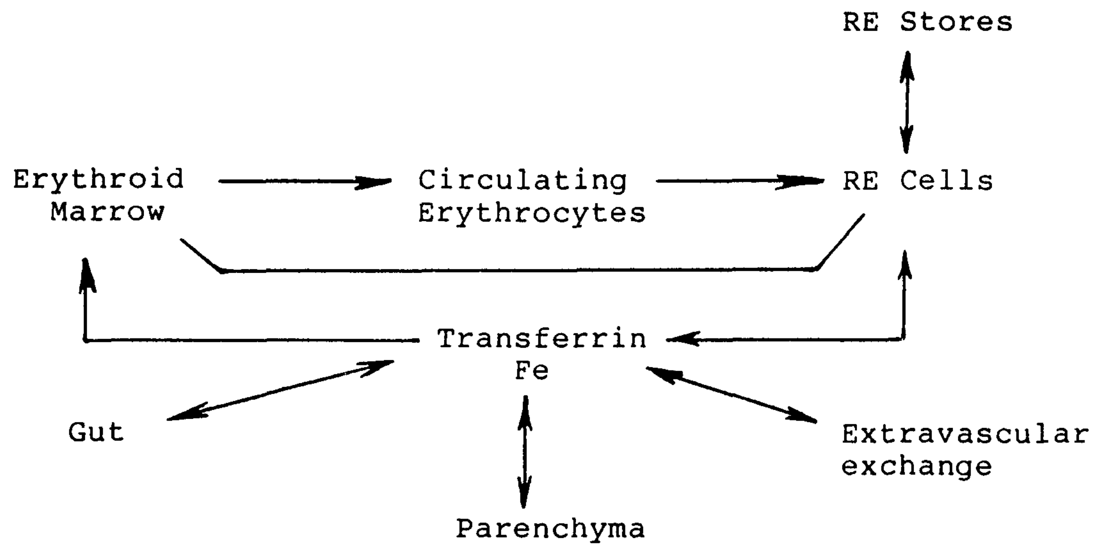


Figure 7. Internal iron exchange. RE, reticuloendothelial cells collect iron as the result of catabolism of red cells. Adapted from reference 39.

Table VIII. Iron Isotopes. (References 44, 45, 46.)

Mass number	Percent abundance
54	5.82%
56	91.66%
57	2.19%
58	0.33%

Iron radio isotopes 52, 55, 59 are used for biochemical research.

and III are stable in aqueous solution and therefore important in biology. The ease with which these oxidation states are interconverted is probably responsible for the ubiquitous presence of this metal in organisms from bacteria to higher plants and animals (47).

Bates (48) has reviewed the chemical properties of Fe^{3+} and Fe^{2+}, pointing out the different behaviour of these ions in solution, (Table IX). It should be noted that Fe^{3+} is very unstable, having a tendency to hydrolyze and form complex polymers in aqueous solutions. This factor has important implications in the biochemistry of iron and how the element is stored within ferritin.

It is very unlikely that Fe^{3+} is transported from one biomolecule (i.e. transferrin) without an intervening reduction or chelation step. The nature of these transport steps are not well understood. A review of the cellular process of iron uptake was recently presented in a paper by Dautry-Varsat and Lodish (49). Bates, however, proposes several possible chemical schemes in his paper for the effective removal of iron from transferrin (48). Also noteworthy is the fact that iron, in either oxidation state, can exist in high or low spin forms. In the spectrochemical series (see Figure 8) iron, in both states, changes from high spin to low spin in the vicinity of bipyridyl and o-phenanthroline. The heme porphyrin also falls in this transition region and it is suggested that this high/low spin switch is important in the function of hemoglobin (50).

Table IX. Comparison of properties of the common exidation staes of iron. Reference 50.

Property	Oxidation state	
	II	III
ionic radius	8.3 pm	6.7 pm
electron configuration	d^6	d^5
spin state (ligand dependent)	high or low	high or low
preferred ligands	soft bases i.e. nitrogen, sulfur	hard bases i.e. oxygen, water, carbonate, PO_4^{3-}, $RCOO^-$
pKa's (aquo ion)	9.5	2.2, 2.3
polymerization tendency	low	high
maximum concentration of aquo ion at pH 7	0.1M	1.0×10^{-18}M

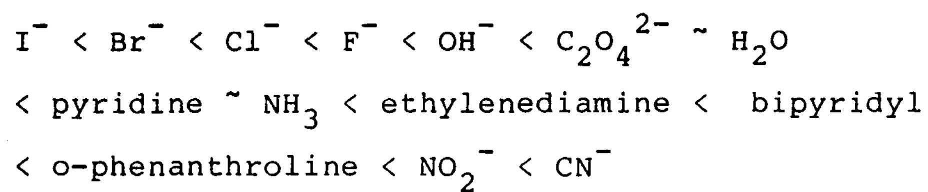


Figure 8. Spectrochemical series of common ligands. Strong ligands result in low spin complexes with iron. Reference 50.

It is interesting to note that both oxidation states are important in biochemisty. For example, Fe^{2+} is the active form in hemoglobin and myoglobin but Fe^{3+} is the oxidation state in ferritin, transferrin and hemosiderin. Other iron containing proteins use the interconversion of the iron oxidation states as an integral part of their function, for example the cytochromes which catalyze the oxidation of substrates with a concurrent reduction of molecular oxygen (51).

Proteins of Iron Metabolism

It is important to control the oxidation of iron. If free Fe(II) air oxidizes to Fe(III), potentially dangerous species can result. These include peroxide, superoxide and hydroxyl radical, which can inflict damage to cells. To avoid these problems molecules have evolved to mediate the transfer and use of iron throughout the body (52). The proteins of iron metabolism are transferrin and ferritin. In addition there is another iron storage protein, hemosiderin.

Transferrin has been reviewed by Chasteen (53) and by Aisen and Listowski (52). The transferrins are the group of proteins responsible for iron transport and are found in the chordates but not generally in the lower life forms. Martin et al. recently reported a transferrin type molecule in the crab, Cancer magester (54). Transferrins are found in the serum, milk, tears, leukocytes and egg white and usually bear a name that indicates its source.

Currently there is significant research investigating the iron release and iron binding mechanisms of transferrin in that these processes might also be important in ferritin function. Bates (48) proposed several mechanisms for this process at the Fifth International Conference on Proteins of Iron Storage and Transport.

The most important iron storage protein is ferritin. The protein is widely distributed in nature and is found in both plants and animals as well as bacteria and fungi. Ferritin is a hollow sphere of protein made up of 24 similar subunits of approximate molecular weight 18,500. The total molecular weight of the protein is about 450,000. The sphere has an outside diameter of 1200 to 1300 pm with an inner core size of 750 pm (52). The molecule has 432 symmetry and has six channels along the four-fold axes that allow access into the interior cavity. These channels are hour glass shaped with a diameter of 140 pm on the exterior protein surface narrowing to 70 pm and then increasing to 140 pm on the inside protein surface (55). There are also eight channels located on the 3-fold axis. Iron is stored within the inner cavity. This cavity can hold up to 4500 Fe(III) atoms in the form of a ferric oxyhydroxide microcrystalline particle. This particle also has a small amount of phosphate at its surface (55). Specifics of iron uptake by ferritin will be discussed in depth later.

The other iron storage protein, hemosiderin, is not well defined and contains protein and inorganic iron in

various proportions and may be derived from degraded ferritin (52). It has also been proposed that this protein is aggregates of ferritin molecules of various composition (52). It has been suggested that hemosiderin formation starts with apoferritin synthesis followed by iron loading. The iron causes a change in the surface of the protein and this change leads to aggregation of ferritin molecules. These aggregates then lose protein and precipitation of the residue results (56). Hemosiderin to ferritin ratios tend to be very high in individuals in which iron overload is a clinical problem. The hemosiderin tends to be highly concentrated in the parenchymal cells of many organs (57, 58). It has been shown that hemosiderin can serve as a source of iron (55) in certain organisms.

Ferritin Function

Many key questions remain concerning the mechanisms of ferritin function. The details of the process of iron deposition within this unusual protein are unknown. Also, an important process of interest is the reverse action, the release of iron from ferritin. Two models have been proposed for the formation of ferritin. The first of these proposes that the apoferritin shell is initially formed and then iron is added sequentially to that shell. A second model, proposed by Pape et al. (59) has the iron micelle formed first which is subsequently surrounded by protein subunits. Experimentally it has been shown that iron crystallites form more rapidly when apoferritin is present which

suggests that the biomolecule plays an active role in their formation. However, the Pape model might be applicable to the formation of ferritin from crystallites from which the protein shell has been catabolized. There is no literature that addresses this question.

There have been two mechanisms proposed for the first model. The first of these was advanced by Crichton and Roman in 1978 (Figure 9)(60). In their mechanism two Fe(II) ions are bound to adjacent subunits with molecular oxygen forming a bridge between them. Reduction of the oxygen takes place forming a peroxo bridge between the now oxidized irons. Subsequent hydrolysis results in the formation of two ferric oxyhydroxide species which are then displaced to a nucleation site within the molecule by incoming Fe(II). This model states that all the Fe(II) must be oxidized at this site on the protein.

This model has several problems. The first is the implication that O_2 is the only oxidant. This is not the case because $KIO_3/Na_2S_2O_3$ can be used as an oxidant in the formation of ferritin (55). It, also implies that there are two types of protein sites present, one for oxidation and another for nucleation of core formation. For this model to be correct, core formation should be inhibited by blocking the Fe(II) site with other metal ions; however, experiments have shown that once established core continues to grow even in the presence of metal ion inhibitors of initiation (55).

The second mechanism, proposed by Macara et al. in

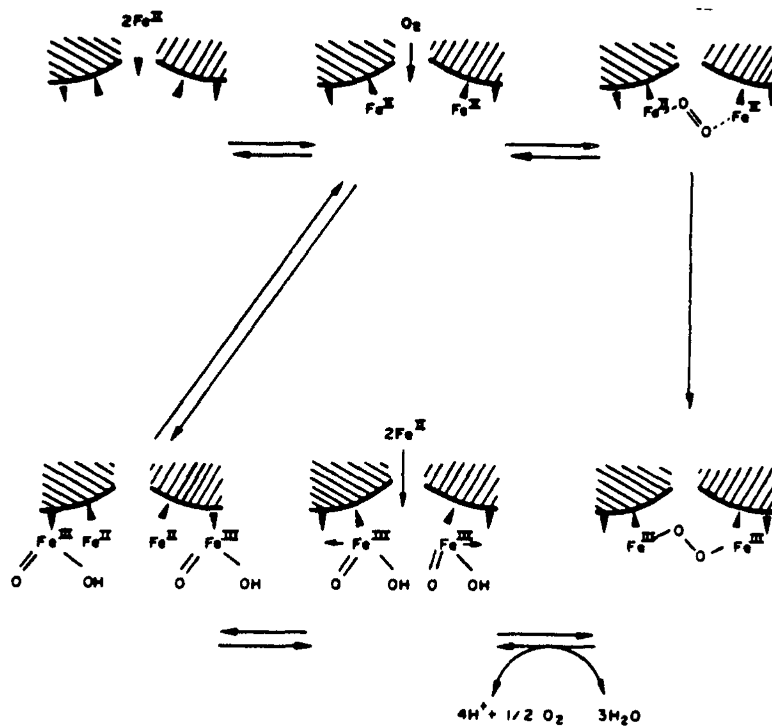


Figure 9. Mechanism of ferritin formation with specific sites for Fe(II) oxidation and different sites for Fe(III) deposition. Reference 60.

1972 (61) simply states that once the Fe(II) is oxidized, it serves as a nucleation site for further Fe(II) oxidation whereupon the microcrystallite continues to grow at the initial site. Oxo and hydroxy bridges from coordinated water form and these serve as high affinity sites for further iron oxidation and deposition.

How iron is mobilized is also an important question. In *vitro* this is done by the addition of reductants and chelators that reduce and chelate the iron. Reductants that have been used for this purpose include dithionite, thioglycolate and other thiols (62). In *vivo* reduced flavins such as $FADH_2$, $FMNH_2$ or riboflavin could serve as reducing agents. These species are also small enough to penetrate the channels of the ferritin shell (63).

The goal of this study was to study initial iron binding in horse spleen apoferritin. The high spin EPR active Fe(III) resulting from the addition of Fe(II) to apoferritin followed by oxidation was monitored by EPR. The results provide some insight into the mechanism of iron deposition and help substantiate the proposal of Macara et al. (61).

Experimental

Horse spleen ferritin, cadmium free, was purchased from Miles Laboratories. The remaining chemicals were purchased from the sources indicated. Water was taken from the Environmental Engineering departments Nanopure system or by further ion exchange of Chemistry department house distilled water.

Apoferritin was prepared by dialyzing 5 mL commercial ferritin (100 mg/mL) in a 23 mm wide Spectrapor 1 dialysis tube at 4° C. (Molecular weight cutoff 6000 to 8000.) The tubing was boiled in 0.5 M sodium bicarbonate ($NaHCO_3$) followed by rinsing with distilled water. Dialysis was against 3 liters of 1% thioglycolic acid (TGA) (Aldrich) that was adjusted to pH 6.7 with aqueous ammonia (NH_3) (Fisher). The dialysis bath was changed after eight hours to 3 L of 0.1% TGA which was also 0.15 M in biological grade Sodium Chloride (NaCl) (Fisher). This solution was adjusted to pH 7.21 by adding aqueous ammonia. The dialysis solution was changed two more times with fresh solution of this composition. Upon this last change of the dialysis solution, no additional blue coloration was observed in the dialysis tube - solution interface. One final equilibration using only 0.15 M NaCl was carried out. The pH of the resulting apoferritin solution was 7.11. When additional iron removal was required, the ferritin solution was taken through several added steps. One milliliter of the apoferritin solution was placed in a 3 mL Amicon ultrafiltration cell fitted with a prewashed PM30 membrane. The solution was ultrafiltered with 1% TGA at pH 6.8 in 0.15M NaCl until no additional purple color was observed in the ultrafiltrate. The resulting solution was then washed with 100 mL of 0.15M NaCl solution using a 1 L Amicon reservoir.

In the assay of apoferritin for residual iron content the protein samples were diluted with water by factors of 4,

10 and 20 and then analyzed using atomic absorption spectrometry on a Varian Techtron Model AA-3 modified with Model AA-5 electronics at 248.3 nm.. Iron standards were made using ferrous ammonium sulfate (Baker). The working curve was in the range of 0 to 10 ppm. Typical error in the data points on the standard curve was approximately $\pm 3\%$ RSD. Scaling of the results of the unknowns to account for dilution yielded precise results. The typical amounts of iron that remained after this procedure was less than 0.04 equivalents per subunit of ferritin.

Commercial ferritin was studied by ultracentrifugation in the following manner. One milliliter of commercial ferritin was layered onto a pad of four ml of a 30% w/v sucrose (Baker) solution in three centrifuge tubes. These tubes were ultracentrifuged using a Beckman L2-65B Ultracentrifuge using a SW-65TI head at 48500 revolutions per minute. This resulted in a force of $1 \times 10^6 \times g$. Both the heavy and light fractions were collected for further analysis by atomic absorption and EPR spectroscopies.

The various electron paramagnetic resonance (EPR) experiments were carried out on either a Varian E-4 or a Varian E-9 Spectrometers. All measurements were conducted at X-band frequency (9 GHz). The data was collected and manipulated using the MINC laboratory computer (Digital Equipment Corporation). Ultraviolet and visible spectroscopy was carried out on the Varian Cary 219 spectrophotometer and fixed wavelength assays on a Bausch & Lomb Model

505 spectrophotometer.

EPR titrations of ferritin with iron(II) were carried out under a variety of conditions which are outlined below. In the first experiment iron(II) was introduced as 15 mM ferrous ammonium sulfate (Baker) using a Hamilton Microliter Syringe to 450 microliters of 1.0 mM apoferritin to which 50 microliters of 1M Hepes buffer, pH 7.28, had been added. The titration was carried out in an uncapped (to allow for air oxidation) one milliliter Pierce reactivial with vigorous stirring using a magnetic stir plate and a micro stir bar. 300 microliters of this solution was transferred to a 3 mm quartz EPR tub using a 500 microliter Hamilton syringe equipped with a 1 foot teflon needle. The tube was then sealed with a rubber serum stopper. This solution was slowly frozen in a liquid nitrogen filled dewar. This frozen solution was then quickly transferred to the liquid nitrogen dewar insert which remained undisturbed in the EPR resonant cavity throughout the experiment. Bumping in the dewar insert was minimized by inserting a Kimwipe tissue at the top of the dewar insert to hold the EPR tube in place. After the spectrum was obtained the sample was removed from the insert and allowed to thaw. This solution was then returned to the reaction vial for further addition of iron(II). After each addition of iron the solution was allowed to stir for five minutes to allow for full air oxidation.

A titration was also carried out at pH 6.0 using the

above procedure. The solution was buffered using MES (2-(N-morpholino)ethanesulfonic acid (Aldrich) which had aqueous ammonia added to set the pH of the buffer.

Subsequent experiments were modified in the manner in which the iron and oxidant were added. In the next experiment iron(II) was added in increasing amounts to a series of 1.0 mM apoferritin solutions. These solutions were 0.3M Hepes buffer at pH 7.0 and placed in tubes sealed with rubber serum stoppers which were rendered anoxic prior to the iron addition by flowing moist nitrogen over the solutions for a period of one hour. After the addition of the iron(II) the solutions were shaken and then opened exposing them to the air for oxidation.

In a final method of iron addition the apoferritin samples were treated as above but in place of air oxidation, the various anoxic protein/iron(II) solutions were oxidized by the addition of potassium iodate (KIO_3, Fisher) and sodium thiosulfate ($Na_2S_2O_3$, Fisher) in a one to four mole ratio. Care was taken to add sufficient but not excessive amount of oxidant to the solutions so that only the added iron(II) would be oxidized and not other oxidizable species in the solution.

An iron(II)/terbium(III) competition experiment was carried out by adding Tb(III) ($TbCl_3$-$6H_2O$) (Aldrich) in increasing amounts to a series of lmM apoferritin solution. These additions were then followed by the addition of equal amounts of iron(II). A blank (no Tb^{3+} added) was also

run. There was no attempt to deair the samples and the EPR spectra of each sample were run immediately and then again after standing eight hours at room temperature.

Protein assays were accomplished in the following manner. Buffer was placed into acid washed quartz 1.00 cm cuvettes. Small increments of protein were added using a 10 microliter Hamilton syringe. After repeated stirring of the resulting solution by sucking the solution into and then expelling it out of a Pasteur pipet absorbance was determined. Solutions were allowed to clear of bubbles and the solution absorbance was determined. Several more additions were made and absorbances determined. Percent protein was determined from the initial volume in the cuvette, increments of protein added (correcting for change in volume) and from the absorptivity of the protein in a one percent solution (9.6 for ferritin) using a calculator program on either a Texas Instruments SR52 or a TI59 calculator. Molar concentration was calculated using the subunit formula weight of ferritin (18500 D) See appendix B for the calculator program.

Results and Discussion

In this study iron was added to apoferritin in the form of Fe(II) followed by oxidation by a variety of methods. These methods of iron(III) introduction are known to produce the iron crystallites of ferritin core. An EPR signal due to the resulting Fe(III) is shown in Figure 10. This signal appears at $g' = 4.3$ in the resulting X-band EPR spectrum

Figure 10. g'=4.3 X-band EPR signal at 77 K. Conditions: 1.0 mM apoferritin, 0.3M Hepes, pH 7.0. Spectrometer field was set to 1500 G with a scan range of 1000 G. Modulation amplitude was 12.5 G at 100 KHz and the microwave power was 10 mW at 9.192 GHz.

and can be attributed to a high spin Fe(III) monomeric center of low symmetry. The spectrum provides the first direct evidence for the formation of an Fe^{3+} complex with the apoferritin shell. Undoubtedly the apoferritin shell is important for the initiation of core formation.

The intensity of this peak was monitored as a function of added Fe(II) followed by oxidation. The results are plotted in Figure 11. The three different oxidation methods all yield similar curves (Figure 11). Double integrals were evaluated and compared with that of $Fe\text{-}EDTA^{-}$ used as an intensity standard. In the ferritin titration, all added iron is EPR active up to about 0.10 iron per subunit, but subsequent addition results in much of the iron being EPR silent. The EPR silent Fe(III) is probably present as oligomeric or polymeric spheres leading to core formation. It is unlikely that the EPR silent iron is Fe(II) since Fe(III) is the favored oxidation state under the conditions of the experiment and the signal intensity did not change when solutions were allowed to stand for prolonged periods. The iron signal continues to grow with added iron until about 0.5 equivalents per subunit at which point the iron signal intensity starts to drop. At 0.5 equivalents per subunit only 20% of the added iron is EPR active. At higher amounts of added iron this percentage drops dramatically (Figure 12). It is possible that only iron(III) bound to one of the protein binding sites is EPR active with additional iron preferentially adding to the growing crystallite

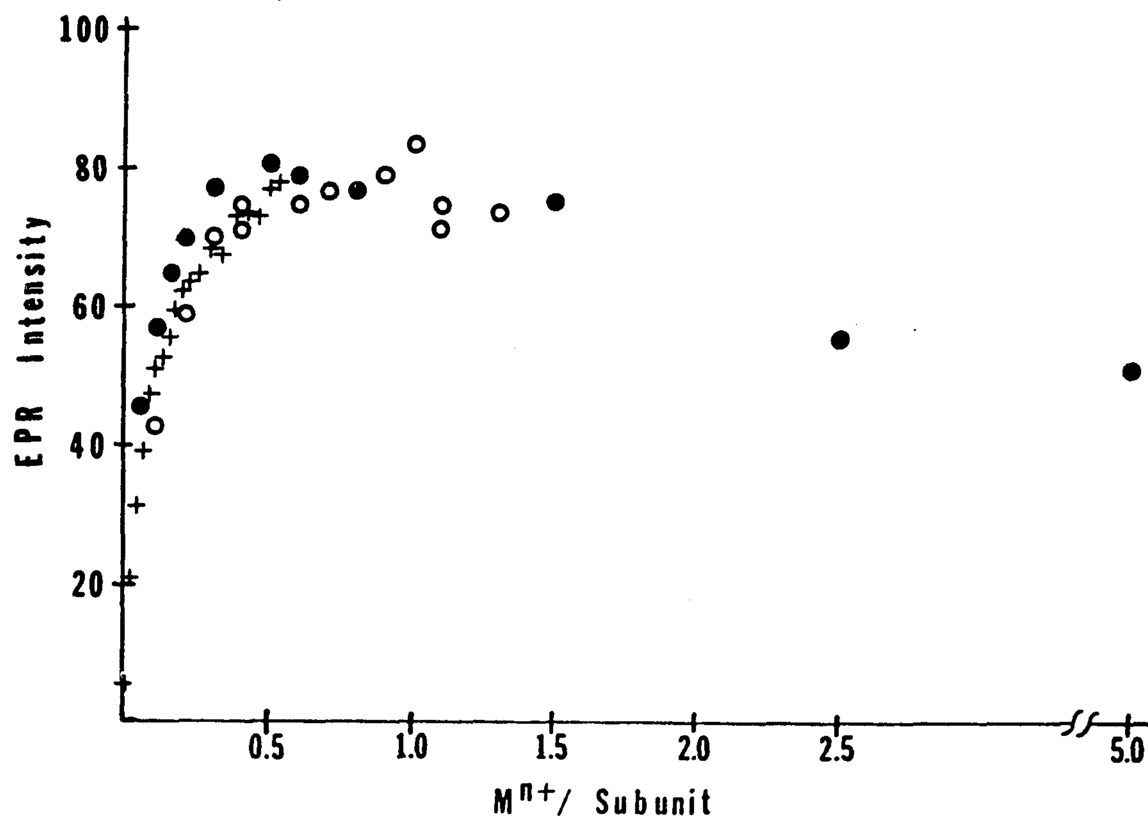


Figure 11. EPR peak-to-peak relative intensity as a function of the Fe^{3+}/subunit ratio. (+) sequential addition of Fe^{2+} to the same sample without prior removal of air. (●) Addition of Fe^{2+} to different anoxic protein samples with subsequent oxidation by $KIO_3/Na_2S_2O_3$. (o) Addition of Fe^{2+} to different anoxic protein with subsequent exposure to air. Reference 67.

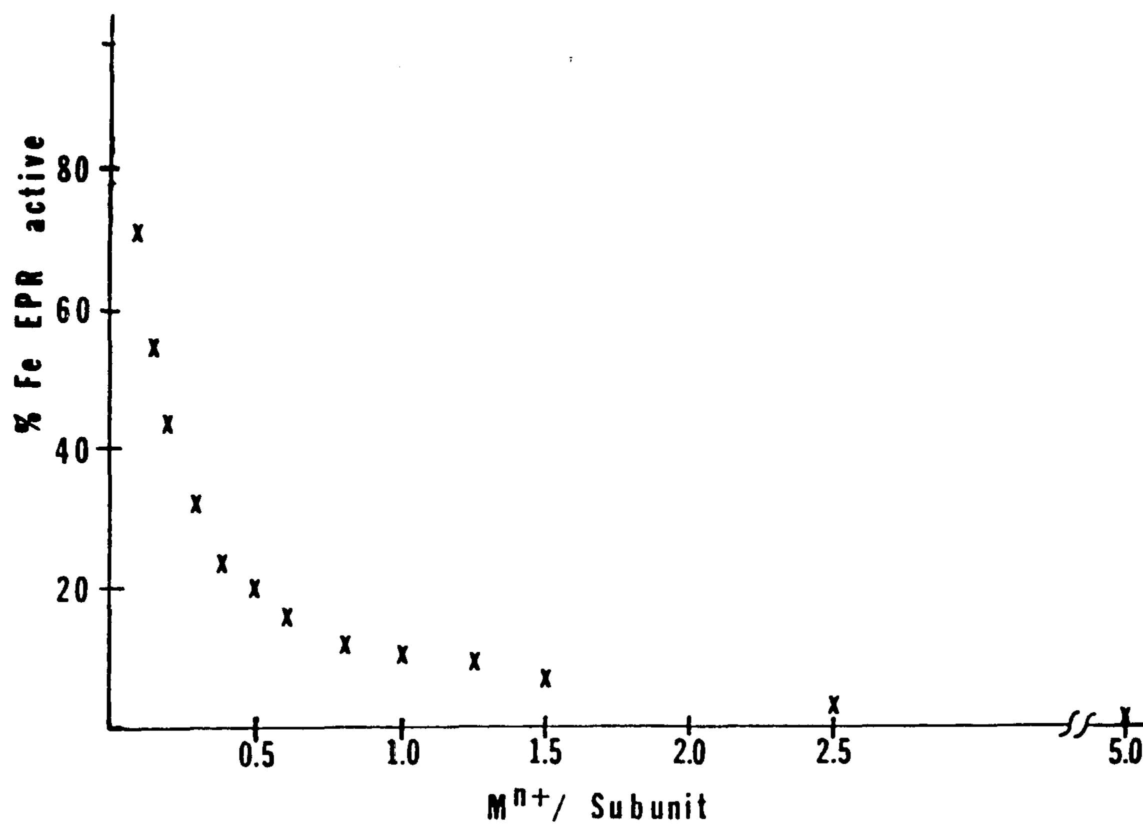


Figure 12. Data from Figure 11 for sequential addition of Fe^{2+} with subsequent $KIO_3/Na_2S_2O_3$ oxidation replotted to show the percentage of the added iron that is still EPR active as the titration progresses.

as suggested by the Macara model (61). The slow decrease in EPR signal serves to highlight the very large affinity of iron for this growing iron core. If added iron were equally likely to bind at a single occupied site, then the iron signal would be expected to decrease much more rapidly than it does beyond a 0.5 equivalents per subunit level (Figure 11).

In another experiment commerical ferritin was separated into light and heavy fractions by centrifugation over a solution of 30% sucrose. The ferritin, which was least dense, suggesting small iron cores, did not migrate into the sucrose and was collected and examined by EPR spectroscopy. The dense, ferritin with larger iron cores, fraction was also collected and examined by EPR. Both fractions exhibited signals at $g' = 4.3$; however, the light fraction exhibited 75% of the total EPR intensity but only contained 6% of the protein. This result shows that ferritin with small crystallites have more mononuclear sites filled than the denser ferritin, indicating that those ferritin molecules have more sites that are not sterically blocked by the large crystallites in the protein cavity.

In a final experiment increasing amounts of Tb(III) were added to a series of apoferritin solutions. Fe(III) was then added at a level of 0.5 equivalents per subunit. The resulting EPR spectra show that the Fe(III) signal at $g' = 4.3$ decreases as a function of the amount of Tb(III) present (Figure 13). The experiment suggests that the

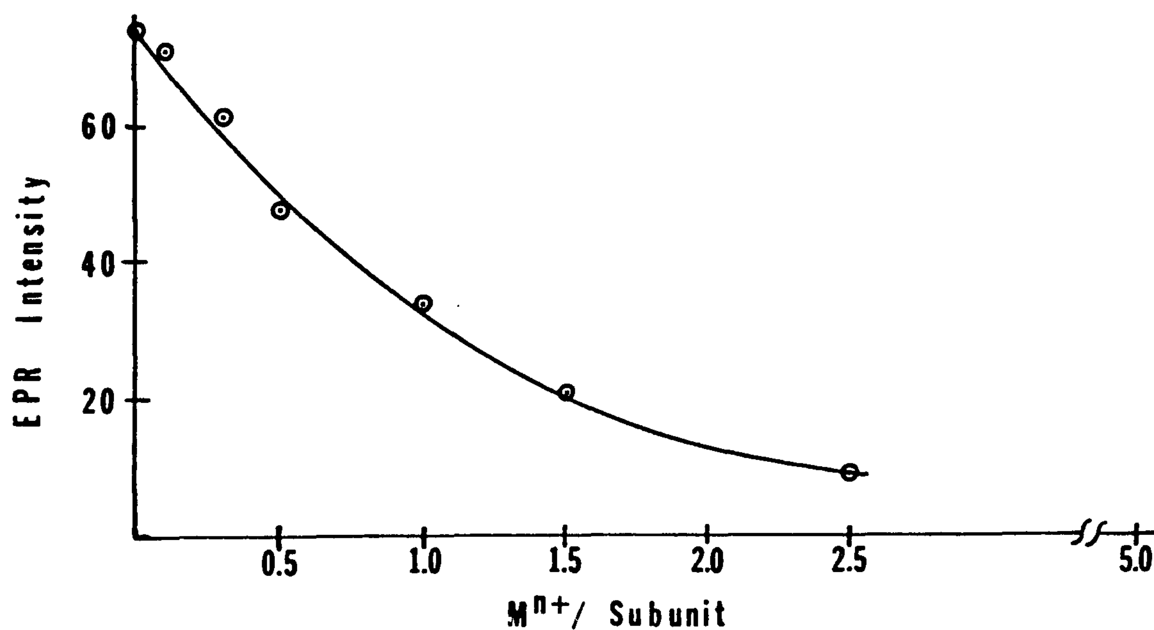


Figure 13. EPR intensity as a function of the Tb^{3+}/subunit ratio present upon addition of 0.5/subunit Fe^{2+} to the protein without prior removal of air. Conditions as in Figure 11. Reference 67.

Tb(III) is binding at the same or a nearby binding site as the Fe(III). Wauthers et al. (64) has shown that Cr(III) binds to apoferritin in a stoichiometry of 0.5 equivalents per subunit. Chasteen and Theil (65) have also shown that vanadium(IV), as vanadyl, forms a specific complex with apoferritin with a stoichiometry of 0.5 equivalents per subunit. They also show that this complex is disrupted by the presence of Fe(II), Fe(III) and Tb(III). The vanadyl EPR signal intensity is reduced by about 25% when 0.5 Fe(III)/subunit is present which is comparable to the amount of EPR active iron that was observed in this work. Stefanine et al. (66) also report that the addition of Fe(II) to Tb(III)-apoferritin quenches Tb(III) fluorescence. These binding sites may be located on the inner surface of the protein shell between subunits as suggested by the results of the above studies and the location of a Tb(III) site in that region by X-ray crystallography reported by Clegg et al. (55).

In summary my results support the Macara model. The data suggest that the crystallite grows at the initial binding site of Fe(III) on the protein. The observed EPR signal appears to be due to mononuclear Fe(III) bound at other such sites on the protein upon which crystallite formation has not yet begun. As iron is added well beyond the 0.5 equivalents/subunit the observed signal is gradually reduced. This suggests that the growing core preferentially takes up the added iron and that occasionally other core

crystallites start to form, reducing the concentration of solitary Fe(III) bound to the protein.

We also observe that Tb(III) inhibits the formation of the EPR active Fe(III) species. This observation suggests that these metals occupy the same or very close sites in the molecule. The stoichiometry also appears to be very near 0.5 equivalents/subunit, in agreement with a binding site between two adjacent subunits, resulting in a total of twelve binding sites in the entire protein.

CHAPTER IV

COMPUTER SIMULATIONS

The use of EPR spectral simulation is a very powerful tool for understanding the chemical systems studied by EPR spectroscopy. A short review of some of the major developments in the simulation of EPR spectra can be found in Poole (73).

Simulation programs have been used extensively in metallobiochemistry research at the University of New Hampshire. In this chapter the modification of our $\underline{S} = 1/2$ EPR simulation program to include $\underline{g}$ and $\underline{A}$ strain in the calculation of spectra is described. The program was first developed by White and Belford at the University of Illinois for the simulation of $\underline{S} = 1/2$ powder pattern spectra and was modified at UNH to include four different hyperfine interactions, more than one paramagnetic center, and $\underline{I} = 7/2$ spins (69). Features of the program include first-order perturbation theory for ligand nuclear hyperfine interactions and second-order perturbation theory for the metal nuclear hyperfine interaction and noncoincident $\underline{g}$ and hyperfine tensors. Equivalent nuclei are placed under one spin value by an intensity weighting feature and all possible orientations were integrated over by using a three-point, Gauss-point method (68).

The program as it stood failed to produce proper line-

widths for outlying peaks in EPR spectra. The modification was undertaken in order to correctly simulate linewidths for the perpendicular and parallel lines for M_I -7/2 and -5/2 in the frozen solutions of aqueous vanadium(IV) ion in acid. Albanese and Chasteen demonstrated that some of the broadening of these lines was due to unresolved proton superhyperfine splitting in the first coordination sphere water molecules (69). Froncisz and Hyde have attempted to explain the observed broadening in wing lines of a copper system in terms of strain resulting from a distribution of parallel hyperfine interactions and parallel g values. They also ascribed some of the line broadening to a correlation of these two effects. Strain arises from distributions of the in-plane pi-bonding parameters and to distributions of the in-plane sigma-bonding parameters and the correlation of these effects. Strain can be attributed to inhomogeneity of the molecular system upon freezing (70).

It has also been shown that g-parallel and A-parallel parameters are correlated for the vanadium system. This was observed by Holyk (34) and has been used to help identify ligation in various systems. For example, Boucher (71) et al. studied ligation of compounds in petroleum and White and Chasteen (72) studied functional groups in protein binding sites for vanadium species.

The program EPRPOW was modified to allow for the introduction of g and A strain parameters. The following variables were added to the program. The variable NS was

added as a flag for the strain calculations. When it was zero the calculations for strain were not executed. The variables DGX, DGY and DGZ in addition to DAAX1, DAAY1 and DAAZ1 were the parameters for the amount of strain to be calculated. The DG group allowed the addition of strain to the g values and the DAA group allowed for the addition of strain in the hyperfine (A) values. The X, Y and Z denote the appropriate axes. The hyperfine parameters are entered in units of 10^{-4} cm^{-1}. Also a parameter NC was added as a flag for correlation of the two types of strain. When this flag was set to 1 the strains were calculated for an uncorrelated system. Parameter NP determines the number of data points that were calculated for each strain packet. When NP is a larger number, more points are simulated over the strain region, giving a smoother simulation at the expense of additional computer time.

The perturbation of the shifts in the principle resonance fields due to g and A strain are calculated as follows. For a correlated system it was assumed that an increase in g resulted in a decrease in A. The resonance peaks (H_{MI}) to first-order are given by equation 9.

$$H_{MI} = \frac{h\nu}{g\beta} + \frac{A\ M_I}{g\beta} \qquad (9)$$

Here h is Planck's constant, ν is the spectrometer operating frequency, β is the Bohr magneton, M_I is the resonance line and g is the EPR g-factor. This factor is

sensitive to molecular environment of the metal. It is the ratio of the magnetic moment to the angular momentum of the electron (26).

With strain the $\underline{g}$ and $\underline{A}$ factors will be perturbed leading to a new magnetic field position for the resonances, $\underline{H}_{MI}$(Strain), equation 10,

$$H_{MI}(\text{strain}) = \frac{h\nu}{(g+\Delta g)\beta} + \frac{(A+\Delta A)M_I}{(g+\Delta g)\beta} \qquad (10)$$

where $\underline{\Delta g}$ and $\underline{\Delta A}$ are the amounts of strain in each parameter. The amount of shift in each resonance field $\underline{\Delta H}_{MI}$ is given in equation 11.

$$\Delta H_{MI} = \frac{\Delta g h\nu + (\Delta g A + g\Delta A)M_I}{g(g+\Delta g)\beta} \qquad (11)$$

Once the shift in resonance field is calculated the EPR lines are then generated and summed into a spectral array. With strain present the line is spread in a Gaussian distribution about the unstrained line. The magnitude of the spread being directly related to the magnitude of $\underline{\Delta H}_{MI}$. This is done to give a smooth strain resonance line.

With the uncorrelated case we do not assume that an increase in $\underline{g}$ due to strain leads to a concomitant decrease in $\underline{A}$. Therefore the changes induced by strain are combined to give a root mean square result in change felt by $\underline{H}_{MI}$ (equation 12).

$$\delta H_{MI} = \sqrt{\delta G(g)^2 + \delta A(A,g)^2} \qquad (12)$$

$\underline{G}(g)$ are the $\underline{g}$ term effects and $\underline{A}(A,g)$ are the hyperfine effects. $\underline{\delta H}_{MI}$ is the perturbation in $\underline{H}_{MI}$ due to strain and $\underline{\delta G}$ and $\underline{\delta A}$ are the perturbations. $\underline{\delta G}$ and $\underline{\delta A}$ are calculated from equations 13 and 14. The value determined for $\underline{\delta H}_{MI}$ is then treated the same as in the correlated case.

$$\delta G = \frac{-h\nu \; \delta g}{g^2 \beta} \qquad (13)$$

$$\delta A = \frac{AM_I}{g\beta} \left(\left(\frac{\delta A}{A}\right)^2 + \left(\frac{\delta g}{g}\right)^2 \right)^{1/2} \qquad (14)$$

Ranges of values used for this work included $\underline{g}$ strains of 0.001 to 0.500 and $\underline{A}$ strains of 0.1 to 25.0 cm^{-1}.

Correlated and uncorrelated simulations were run. The correlated case is expected to give the best simulation of experimental data in that the same physical mechanism leads to both effects. The figures in this chapter show that this is in fact the case. Figure 14 is the EPR spectrum of VO^{2+} in 0.1 M DCl and the next three figures are simulations of this system using the modified program. Figure 15 is an EPR simulation of this system without using the strain calculations. In this simulation we note that the negative perpendicular lines are narrow and do not fit the decreasing peak height trend seen in the experimental system. Figure

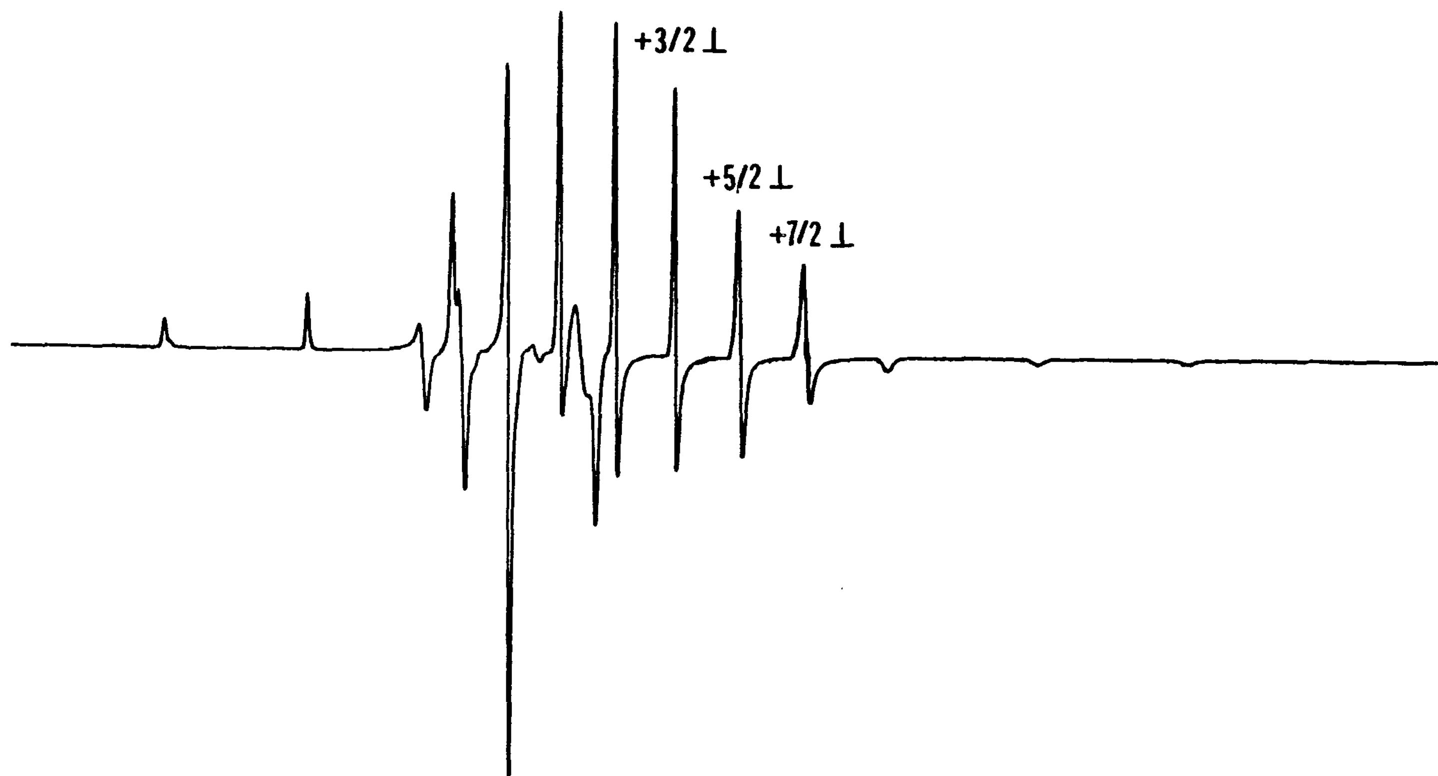


Figure 14. X-band EPR spectrum of VO^{2+} ion in 0.10 M DCl at 77 K. This spectrum was collected with the following conditions: Field set 3400G, scan range 2000 G, 2 G modulation amplitude at 100 kHz, microwave power of 2 mW at 9.088 GHz, receiver gain of 150 and a scan time of 16 minutes with the noise filter time constant set at 0.3 sec. Lines of interest are labeled.

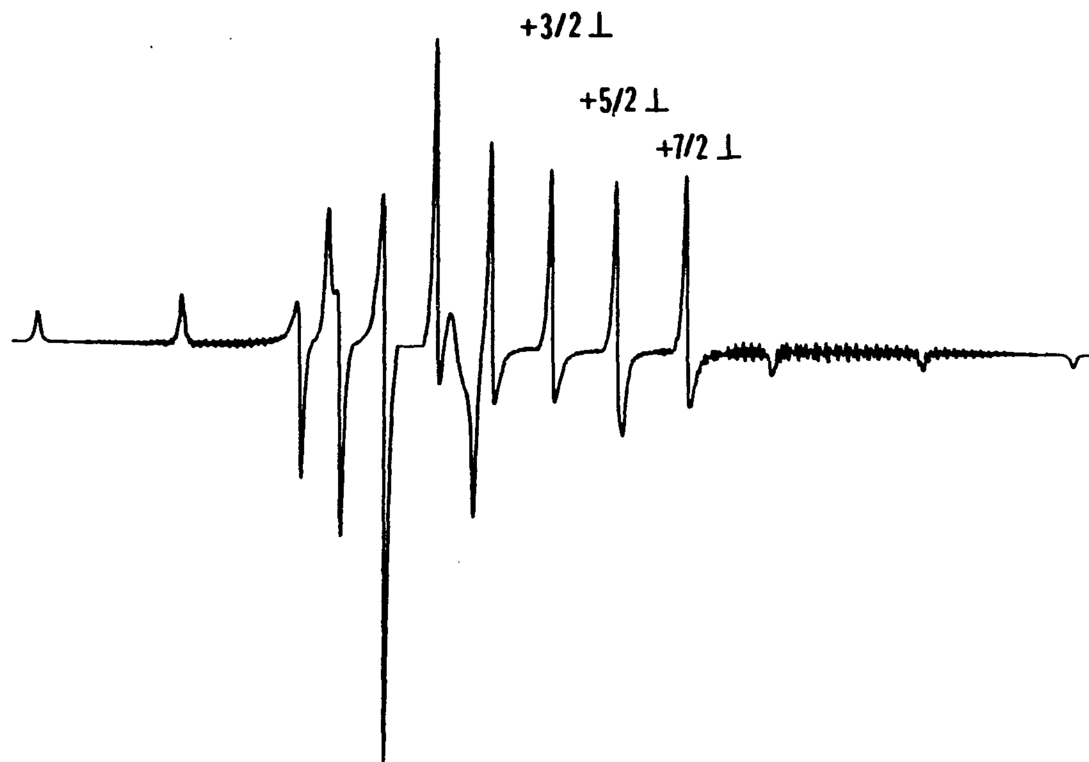


Figure 15. Simulation of the 77K VO^{2+} spectrum from Figure 14. Simulation parameters: *g*-parallel 1.933, *g*-perpendicular 1.979, *A*-parallel 183.6 cm^{-1} and *A*-perpendicular of 69.55 cm^{-1}. Linewidths are 4.0 G. No strain corrections were carried out in this simulation.

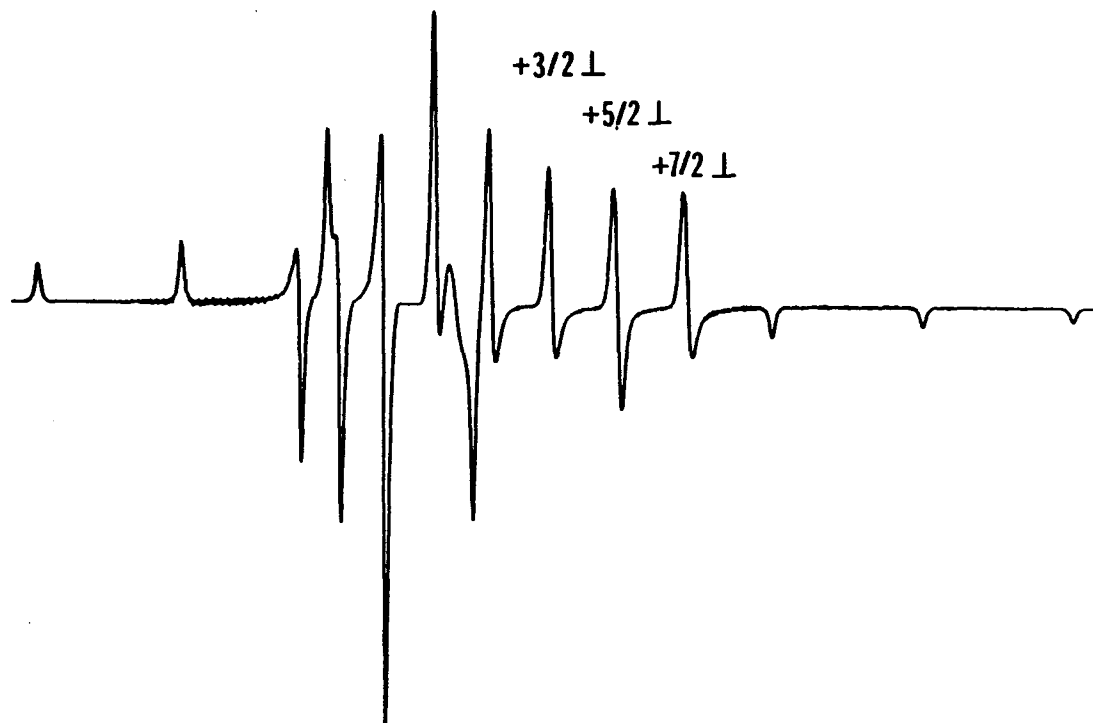


Figure 16. Simulations of the 77 K VO^{2+} spectrum with correlated strains. Same basic simulation parameters used as in Figure 15. Strain parameters applied were: *g* strain parallel, 0.001, *g* strain perpendicular, 0.002, *A* strain parallel, 0.8 cm^{-1}, and *A* strain perpendicular, 0.5 cm^{-1}.

16 is a simulation using correlated strains. In this simulation we see that the negative perpendicular lines are broader than for the no strain case and that they decrease in size in a similar fashion as the experimental spectrum. Finally, Figure 17 presents a simulation of a system using uncorrelated strain. Although some broadening is observed in the negative perpendicular lines the peak heights do not decrease as nicely as in the simulation using correlated strain.

Manipulation of the magnitude of the $\underline{g}$ and $\underline{A}$ strain in the correlated case should allow for a good fit of the experimental and simulated spectra. A listing of the modified program is given in appendix C.

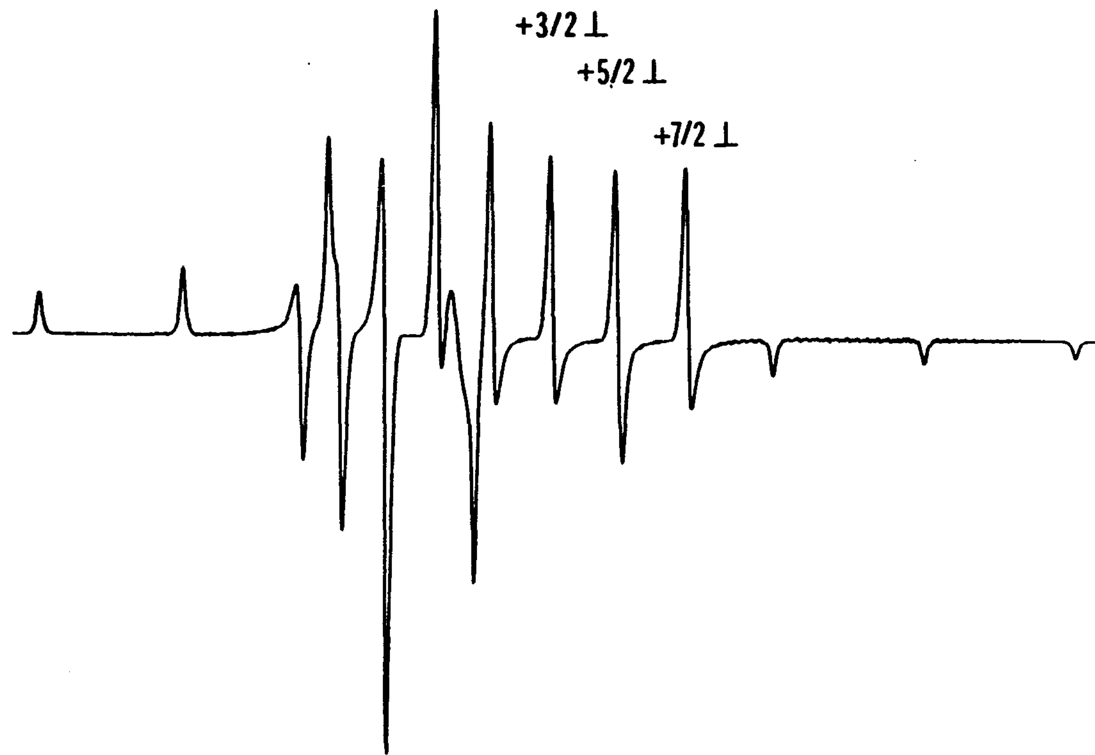


Figure 17. Simulation of the 77 K VO^{2+} spectrum with uncorrelated strain corrections. Parameters as in Figure 16.

APPENDICES

APPENDIX A

A series of programs were developed to assist research utilizing EPR spectroscopy. Most of the programs are collected into the 4K series; however, a few specialized programs are designed to run outside of this series. These programs have specialized functions and do not carry 4K as part of their names. A description of the operation and a listing of each program follows.

Program 4K

Program 4K is the master calling program from which most of the EPR manipulation programs are called. Such a calling format is required due to the limited amount of space available in the computer memory for BASIC language programs. From this program the other EPR programs are accessed by the "CHAIN" command. This command erases the current contents of memory, except for those variables defined in the "COMMON" statement, and reads the new program from disk and executes it. Program execution starts at the first line of the new program. Most programs "CHAIN" back to program "4K" when their tasks have been completed.

This program presents a menu from which the user selects the desired task. The user enters the number from the menu and program execution branches to the appropriate subprogram. The program also checks to see if a disk has been specified from which files will be read and stored, if it

has not the operator is asked to specify it. The file extension that is set in this process is ".4K". This extension denotes EPR spectra that are digitized into 4096 points along the magnetic field axis and that have not been previously manipulated.

The user is reminded that many of the subprograms require prior execution of the "EXTRA_SPACE" command. The large size of some of the subprograms and the very large arrays manipulated require that the MINC be in this extended mode. Typing EXTRA_SPACE when the MINC has prompted the user with a period "." starts this command. Once entered this command prompts the user on completion of it's function. One should be aware that the contents of memory (not disk) are erased by this command. Therefore, any program in memory, with its associated variables, will be lost. Therefore, it is suggested that "EXTRA_SPACE" be specified prior to the start of the EPR program. When the MINC is in EXTRA_SPACE it takes slightly longer to call files from disk; however, this delay is not perceptible under normal conditions.

The variables that are saved in the "COMMON" statement are listed in Table A-1.

Program 4K1

Program 4K1 collects EPR data and then stores this data on MINC disks upon user prompt. The program requires the operator to supply the scan time that has been set on the EPR console. This input is used to generate the time base

Table A-1. Program variables defined by the COMMON command and therefore carried throughout the 4K EPR program.

Variable	Function
Z	Z is set to one (1) when a spectrum is in the spectral array.
A%(4096)	Spectral array file.
D$	The current working disk, either "SY0:" or "SY1:"
R0$	The name of the spectrum in the spectral array.
E$	The extension of the spectrum in the spectral array.

against which the EPR signal is collected. The user should be aware that the actual scanning rate of the field sweep might be slightly different from that time set by the console switch. This difference should be accounted for when entering the scan time. Data collection is initialized when the "Scan Start" pushbutton is depressed on the EPR console. Data is displayed on the MINC VT105 terminal as it is collected. In this strip chart mode 512 data points are presented on a full screen width. As the spectrum is collected the first 512 points are plotted progressively across the VT105. When the screen is full the most recent point is plotted on the right of the screen and the remaining data points are shifted left one position resulting in the loss of the leftmost data point.

When the full spectrum has been collected the operator is given the option of viewing the entire spectrum. This is recommended in most cases so that one is readily able to see if the dynamic range of the analog-to-digital converter has been exceeded, which would be evidenced by truncated peaks.

The operator then has the option to save the spectrum on disk. A negative response will prevent the writing of the spectrum to disk; however, the spectrum can be manipulated by the other EPR subprograms, but there will not be another opportunity to store this spectrum. A positive reply will store the spectrum into an integer array on the MINC disk. The user is then prompted to enter the appropriate EPR spectral parameters and is then directed to enter a

line of text. This text line allows the user a scratch pad to store any information that might later be helpful (such as experimental conditions). The date and time are also stored as part of this scratch pad file. The spectrum is stored on disk under the user supplied name with the extension ".4K". The parameters, stored as a real number array, are stored with the same file name but with the extension ".4KP" and the text is stored as a literal variable under the same file name with the extension ".4KT". This is summarized in table A-2.

If a series of spectra are being collected the program allows the user to enter a zero (0) for any parameter that has not changed from a previous entry. This saves considerable time when collecting a large number of spectra under the same, or similar conditions. This is also true for setting the scan time at the start of each data collection, a zero entered will result in the next spectrum being collected at the same rate as last. This would be very useful if the operator had determined the exact scan time of the EPR and would circumvent the entry of a multidigit number at the start of each scan. Should the user wish to enter a parameter that has been changed to zero it is suggested that a very small number be entered (i.e. 1E-6). However, the only parameter that would be legitimately be zero would be a time constant set to the "OUT" position.

Program 4K2

Program 4K2 provides for the addition or subtraction of

Table A-2. Files in which spectra and associated information are stored.

	Extension	Data type	Disk blocks
Spectral Data	.4K	Integer	17
Parameters	.4KP	Real	1
Text	.4KT	Literal	1

spectra. The results are automatically stored on the specified disk. Although this program is called from the 4K master program none of the COMMON variables are carried forward to this program. This is due to space limitations presented by the MINC hardware. Spectra are manipulated block by block from disk and the result is stored on disk with the extension ".SUB". The result is also saved in computer memory so that it may be plotted on the VT105 terminal.

The user specifies the disk that contains the files and also supplies the spectral file names. Addition or subtraction is selected upon computer prompt. The operation is specified by the sign of variable "S", a negative one (-1) will cause the spectra to be added.

Files to be added of subtracted must carry the extension of either ".4K" or ".SHF". Files that have different extensions would require that they be copied to one of these extensions with the "COPY" command prior to the execution of the 4K program. It is important that files be shifted prior to use of this program as misaligned spectra will produce spurious results. Repetitive additions or subtractions can be carried out on each call to this program.

Program 4K3

Program 4K3 will print stored file texts on both the VT105 terminal and the LA34 printer. These files are created at the time of data collection and allow the user to store information pertinent to the experiment.

The operator my enter up to fifty file names for print-

ing on each call to the program. This program usually requires that the MINC be in the EXTRA_SPACE mode for proper operation.

Once the file names have been entered the program will print the contents of all selected files. No further operator intervention is required provided all entered file names are valid and exist on disk. Invalid names or names of files not on the disk will cause the program to fail. When all files have been printed the program returns to program 4K.

If the printer is not connected or not powered up the program will proceed without error, listing the texts on the VT105 terminal only. If the LA34 printer is in the fault mode this program will hang until such condition is cleared.

Program 4K4

This program will double integrate a file specified by the user. The program's run time is long due to the large number of operations that must be carried out to complete the double integration. This program is very versatile and has several options and is the most complex program in the EPR series. The program allows for single and double integration and it also provides for single integration of files already singly integrated. The baseline is corrected to bring the baseline to zero and to correct for any baseline slope. A selected area of a spectrum may also double integrated allowing for the integration of single peaks. The user could integrate an entire spectrum one peak at a time.

If a spectrum is singly integrated and this integration is then saved on disk, the program labels it with a scaling factor. This insures that if the spectrum is integrated again that the result will be normalized to the same value that would be obtained if a different initial scaling factor were used. Scaling is required so that the resulting integrations yield numerical values that can be plotted and manipulated within the limitations imposed by the MINC software.

When integrating a segment of the spectrum, it is required that the user know the starting and stopping positions prior to entering this program. Program 4K11 is designed to provide these array positions. (Note: a large scaling factor supplied to 4K11 will help the user to accurately determine points where peaks can be separated.)

Upon entering the program the operator is asked to input the scaling factor. Sharp spectra such as isotropic Vanadyl species usually require a factor of about 0.01. If spectra are of a more broad nature a smaller factor will probably be required. The appropriateness of the magnitude of this parameter will be evidenced by the size of the plotted first integral. If this spectrum is too small it would be beneficial to restart the program using a larger scaling factor, a spectrum that is too large (i. e. tops of peaks clipped off in the first integral display) should be restarted using a smaller scaling factor.

The program may be terminated after a single integra-

tion and the results saved for subsequent plotting. The program allows the user to store the results of any integration, either first of second. In addition, a file named "INTER.SCR" is used for a work area in this program. This file is a real number array file and requires 33 blocks of disk storage space; however, under normal operating conditions this file is reused and will be present on "SY0:" after the first use of the program.

After the integration has been completed the results are called back from the temporary storage file and scaled and stored in the file array in MINC memory. The results may now be stored on disk. In this way it is possible, starting from the same EPR spectrum file to integrate a spectrum by parts with repetitive passes through this program.

Notes on program algorithm.

Integration is carried out by summing the EPR spectrum point by point. This sum is stored on the working file on disk. Maximum and minimum values of this summed array are detected and this difference is presented as "peak height" on the displayed integral spectrum. If it is the second integration, this peak height is printed on the hard copy terminal (if connected) as the result of the double integration.

In all cases the baseline is corrected. This is done by determining the mean of the first and the last ten points of the integrated segment. The difference between these

means is divided by the length of the spectrum, this defines the slope. The mean of these end points is taken as the displacement of the spectral baseline from zero. The spectral segment is then corrected for this slope and displacement. This procedure assumes that the baseline drift is linear.

Program 4K5

This program plots a spectrum on the MINC VT105 CRT terminal. This terminal is only able to plot 512 points along its x-axis and spectra are stored in a 4096 point array; therefore, only one in eight points are plotted when a full spectrum is presented. This program allows the user to examine expanded sections of a spectrum. The user may opt to view from one to eight eighths of a spectrum with manual selection of the initial point. When one eighth is selected the MINC will plot 512 sequential points starting from the user selected starting point. When two eighths are selected the MINC plots every other point for a 1024 point span. If a partial spectral plot results in plotting of points beyond the end of the spectral array, the user is warned by a terminal message and asked to supply new plotting limits.

The initial point to plot is selected by using the MINC VT105 terminal indexing cursor. A full description of its use and limitations can be found in volume 4 of the MINC manual series (MINC Graphic Programming). A brief description is given here. After the computer plots the full spec-

trum, a flashing brad is presented on the left most point of the plotted spectrum. This brad is the cursor and can be moved by using the right and left arrow keys on the MINC terminal keyboard. An attempt to move the cursor beyond the extreme right or left of the display will cause the terminal to 'beep'. Holding the right arrow key down will cause the cursor to scan through the spectrum. The key is released when the desired position is reached. If the position is overshot the cursor movement can be reversed with the left arrow key. A single depression of either of these keys will cause the cursor to move one displayed point. Remember that when the entire spectrum is plotted only one point in eight is presented; therefore, if more accurate point selection is desired, program 4K11 should be used. When the cursor is moved to the desired location, the position is stored by depressing the VT105's "S" key. This will cause the brad (cursor) to disappear. If the program allows the indexing of more than one point the cursor for the second point would be presented upon the depression of the "2" key. This point in turn will be stored by depressing the "S" key after the cursor has been positioned. If the number of the point has not been allowed by the programmer, the terminal will 'beep' when that number is depressed. Indexed points are changed by pressing the appropriate number after they have been stored by the "S" key. This program only allows for the selection of a single point. This point can be reindexed after depressing the "S" key by pressing the "1" key. Point

selection sessions are terminated when the "RETURN" key is depressed. Once this has been done the selection of points is finalized and can not be changed.

The program allows for several "looks" at each spectrum and allows the user to examine other spectra prior to returning to the main "4K" program.

Program 4K6

Program 4K6 determines maximum and minimum points of a spectral segment or of the full spectrum held in the program spectral array. Additionally the program determines: 1) the number of times this maximum or minimum is found, 2) the first array position that these extrema is found, and 3) the array position of the midpoint between the first maximum and minimum points in the spectral array. This information is useful in helping the user determine the amount spectra should be shifted prior to addition or subtraction.

Long program runs without operator intervention are possible as this this program allows for the entry of up to fifty file names on each pass; however, if multiple files are to be searched over the same array subset the user must know the search limits prior to entering the file names. If these limits are not known the operator should enter a "1" for the number to be searched and enter the first file name to be searched on computer prompt. When manipulating a single file the program allows the user to select search limits using the display cursor. After completion of this first search the results and the limits of the search will

be presented on the VT105 and if the user wishes may be printed on the hard copy terminal. The program allows for additional passes and in these runs multiple files may be entered using the selected search limits from the first spectrum. If the user wishes to search an entire spectrum the start position to be entered would be 0 and the end position 4095.

Output to the printer is automatic on searching multiple files; however, if only a single file is searched hard copy output is a user option. It is highly recommended that this program be run in "EXTRA_SPACE" mode if multiple files are to be searched. Invalid and nonexistent file names will cause the program to fail.

Program 4K7

This program increases or decreases spectral amplitude and also allows right or left shifting of spectra. The scaling and shifting parameters must be known prior to program entry. Information to assist in the determination of these parameters can be obtained from program 4K6.

The resulting spectrum from this program is automatically saved and will carry the extension .SHF. The scaling factor will be a multiplier that will be applied to each point of the spectrum. For example a scaling factor of 1.20 will result in a spectrum file with peak heights 20% larger than the original spectral file. Shifting factors are input as integers. Negative integers will cause the spectrum to be shifted to the right. Points shifted beyond the limits

of the spectral file will be lost, and points shifted into the file will be filled with zeros.

If the user wishes to examine the shifted spectrum program 4K5 should be utilized.

Program 4K8

Printing of the stored file parameters for spectral files on both the MINC VT105 CRT terminal and the LA34 hardcopy printer is the function of this program. Up to fifty file names can be entered at one time. Printing on the hardcopy terminal is done automatically. If the printer is not connected or is powered down the program execution will not be affected; however, a printer fault condition will cause the program to hang on the attempted output.

Invalid file names or nonexistent file names will cause the program failure when access to that file is attempted.

Program 4K9

Program 4K9 will plot an EPR spectral file onto the recorder on the EPR console. Channel 0 of the MINC D/A converter must be connected to J007 on the back of the EPR console. Controls on Channel 0 of the D/A converter should be set as follows: small knob set to +/- and the large knob set to 2.5. The wires from the connector block to J007 on the rear of the EPR console should be connected in the following manner: one wire should run from the channel 0 signal line to pin z on J007 and the other wire should connect the channel 0 analog ground to pin AA on J007. It should be noted here that it is imperative that the "Record-

er Input Switch" on the Systems Function Selector module be set to external. Due to space limitations in the available computer memory variables defined in the COMMON statement in program 4K are not carried forward to this subprogram.

The user may scale the plotted spectra using this program. The MINC calculates and prints the distance the spectral baseline is offset from the center of the EPR chart paper. This information allows for plots offset from the vertical center of the spectrum allowing for the placement of several spectra on the same paper for ease of comparison.

The scan rate of the EPR console must be supplied to the program. This scan rate is used as a time base for the computer output scan. Due to the variation of scan rate set on the console and the actual scanning rate, a word of of caution is warranted. Attempting to read field positions from such hardcopy spectra could result in errors. The program XYPLOT outputs the spectral array versus a linear array and therefore does not encounter this output problem.

Program 4K10

Program 4K10 is a software signal smoothing routine and is used for noise reduction in an EPR spectrum that has been previously collected. This program also calculates the signal-to-noise ratio prior to and after execution of the smoothing routine.

Noise reduction is accomplished by a floating average algorithm. In this method, a small segment of the spectral data points are averaged and the resulting average value

placed into the midpoint position of this array segment. The first data point of this set is then dropped and the next sequential array position is included into the averaged segment and the resulting new average is placed into the new midpoint position. This moving window is advanced until the end of the spectral array is reached. The number of points (N) in the averaging window is user supplied. The number of points should always be odd so that the midpoint is a valid array position. Seven points has proved to be a reasonable number of points. Additional data points will produce more smoothing, but there are two disadvantages: 1) computer execution time and 2) loss of resolution between spectral peaks. Increasing the number of points to be smoothed drastically increases execution time; however, loss of resolution has not been a problem due to the high density of data points collected for the relatively broad metal EPR spectra collected in this work.

The program may be exited after the initial calculation of the signal-to-noise ratio. The resulting smoothed spectrum may be stored or discarded. If the spectrum is saved it is given the extension ".AVE".

It is important to note that the first and last (N/2-1) data points will not be averaged. These points will be set to the value of the first and last averaged points respectively. This is required because these outer positions are not legal midpoints of the averaged segments.

The signal-to-noise ratio is calculated in the follow-

ing manner. The difference in the maximum and minimum values of the first 100 spectral points are taken as noise. The signal is then assumed to be the difference in the maximum and minimum points of the remainder of the spectral array. The signal-to-noise ratio is taken to be twenty times the log of the ratio of the signal amplitude and the noise amplitude. The results of the signal-to-noise calculation are printed on the VT105 terminal and, if it is ready for output, the LA34 printer. Note: The signal to noise should be calculated by taking the peak height and dividing it by the standard deviation of the noise signal.

Program 4K11

This program allows the user to select x-axis array positions within a stored EPR spectrum. The moving cursor on the VT105 display terminal is an integral part of the selection process. DEC calls this operation indexing. The program outputs the array position (i.e. 0 to 4095) and if the user has supplied the magnetic field strength for any array position and the spectral scan range the program will also provide a calculated magnetic field strength for the selected positions.

In that a full spectrum displays only one in eight data points, this program allows the user to select smaller segments of the spectrum to allow for increased accuracy in selecting peak positions or inflection points. Since all data points are displayed when an eighth of the spectrum is presented, this is the suggested level when high accuracy is

required.

The starting position of the spectral segment and the number of eighths of a spectrum to be plotted are operator supplied. If the input parameters would cause plotting beyond the end of the spectrum then the operator is forced to select a new set of conditions that will avoid this situation.

Up to ten points may be indexed on each spectral segment plot and the number is operator selected. The user then selects any number of points up to the number that was requested. The number of points actually indexed is then displayed and the results are displayed as array positions, and if the option for field position is enabled, the field positions are also displayed. Hard copy output is a user option. The results are printed in the same order as they were entered.

The program then allows for indexing of additional points, but the plot starting position and length of plot must be selected once again. This is not usually an inconvenience as one often wishes to work in a different portion of the spectrum for a new set of points.

When selecting points of inflection around the baseline, the display scaling factor is very helpful. Expansion allows the user greater resolution about the baseline region of the spectrum. Reasonable values for the scaling factor range are from 2 to 20 and, of course, the scaled peaks will be truncated on the display.

Program MULINT

This program is an adaptation of program 4K4 that allows the user to double integrate a series of spectra without frequent operator intervention. The program allows the user to input up to fifty file names on each pass through this program; however, these files must be on the same disk with the same file extension. The program allows for the selection of the limits of the integration should the user wish to integrate less than a full spectrum. Display of the integrated spectra (to save time) may be aborted and the output is automatically printed on the LA34 line printer. The user should insure that the printer is turned on and that there is sufficient paper to print the results of the integrations.

The program was designed to double integrate a series of spectra (i.e. a titration) without forcing the user to supply the periodic inputs that program 4K4 requires. Since the process of double integration takes large blocks of computer time this program could be run overnight.

Care should be taken in entering file names because a improper name, either with incorrect syntax or of a file name not present on disk, will cause program failure. It should also be noted that this program requires that the MINC be in the "EXTRA_SPACE" mode due to the large size of the stored arrays.

The program also allows, if a single spectrum has been selected, integrations of different portions of this spec-

trum; however, this feature is not automatic and the user must supply the limits of integration prior to each pass.

This program will print the time and date of completion on the VT105 terminal at the end of its execution.

Program SIGAVE

This program will collect multiple scans and will at operator command sum these spectra. This is analogous to signal averaging commonly done in Fourier Transform NMR and previously done with the CAT attached to the Varian E-4 and E-9 EPR spectrometers. This is useful technique when working with very weak signals, as it is expected from theory that the signal to noise ratio should increase as a function of the square root of the number of spectral scans collected.

The program collects spectra digitized into 4096 steps along the magnetic field axis. Data is collected as a function of time. The program prompts the user to input the scan time set on the spectrometer console. Since the E-9 EPR spectrometer scan runs slightly faster than the value set on the console, the program adjusts its' data collection time down by 3.33% in compensation.

The file name under which the data will be stored and the the starting file number are operator supplied. If no previous data had been collected for this sample it is important that the user enter a 1 (one) in response to this question. If data had been collected for this sample the user should enter the number of the next sequential file to

be collected. Gaps in the numbering of spectra will cause the program to fail. Spectra are given extensions that are numbered in sequence of their collection. The summed spectrum is stored under he user provided file name with the extension ".SUM".

After the collection of any single spectrum the user is given the option of summing the already collected spectra and examining the results up to that point. If it is decided that more scans are required, the program allows for this. Subsequent collection will only sum the spectra collected since the last summation. This is done to save execution time, which is substantial.

It is possible that all the individual scans not fit on the user disk in drive SY1:. If this happens the user should sum the spectral files already collected, leave the program, and then "UNSAVE" the files already summed. On restarting the program enter the appropriate starting number and continue collecting data. In this way no data will be lost in the summation process.

When collecting spectra it is important that the baseline be kept as close as possible to the center of the field, or that they are offset from the center both equally high and low from scan to scan. If there is a systematic offset of the baseline, this offset will cause the baseline to be summed out of the range of the computers ability to plot the summed spectrum, or worse, to be summed beyond the range of valid computer integers.

The program forces storage of the collected spectrum on "SY1:". This is done because this disk has the most available free space. If it is desired that this be changed and data be collected onto disk "SY0:" program line 40 could be deleted.

The program, as written, requires significant operator intervention. The operator is given the option after each spectral scan to either store the spectrum or to discard it. This 'out' is provided in the case that the operator detects something obviously wrong with the spectrum. After the spectrum is stored the operator must depress the "Scan Start" switch on the EPR console after the computer "beeps" and a horizontal line is presented on the VT105 screen.

If at some later date it is decided that a more automated system is needed this program can be easily adapted for this. The key to this would be to bring initializing the "Scan Start" under computer control. This can be done by placing a ground on pin 2 of P603 in the EPR console. This terminal can be accessed by lifting the EPR recorder assembly. An output from the MINC D/A (digital to analog) converter could be programmed to supply this ground signal. The option to either save or discard the spectrum could be removed from the program and subsequent "Scan Starts" could be initialized by the program. The programs ability to collect data would then only be limited by available disk space. It should be possible to collect about 50 spectral scans on one floppy disk.

To avoid this limitation the program could sum the spectra as they were collected; however, this does not allow the operator to correct for spectra that are in some way bad (i.e. liquid nitrogen evaporated during run, loss of AFC lock on spectrometer and other such problems).

Program XYPLOT

This program allows one to transfer an EPR spectrum from MINC disk storage to a two axis plotter. The program uses the analog output module of the MINC. Instructions on connection of the analog output module are provided as part of the program and are printed on user prompt (statements 3000 - 3060). This program has several additional features. It is not part of the "4K" set of programs but is fully compatible with files created using that program. This program requires that a file named "PLOT.SCR" be on the "SY0:" disk. If this file is not present prior to the start of program execution the user must provide for its generation by responding with a 'Y' to the computer question. Data may be scaled either up or down, but if the magnitude of the resulting data fall outside the digital range of 2047 to -2048 the program will fail. The program also allows the selective plotting of portions of a spectrum. A useful example might be the plotting of a small feature of a spectrum with greater magnification subsequent to the plot of the entire spectrum. However, each plotted segment requires a full execution of the program. The program also provides an option to have the data smoothed with a seven point

floating average routine. (See program 4K11 for details of such a program.)

The program also provides outputs to position the plotter pen in the upper right and lower left corners of the paper thus allowing the plotted spectrum to use a the full output chart. As an aid in setting these margins properly the following procedure might be of assistance. When the program is providing the margin output the control on the HP 7044A X-Y plotter are adjusted to correctly frame the output area. The controls that are required to set up the proper pen positioning are the zero adjust knobs, the check push-buttons and the range 'cal' control knob. Make sure that the outer knob on the range control is set to 1 V. Each axis is independent of the other. With the pen at either extreme press the check knob (either axis, but do one axis at a time). While holding the check knob down use the zero knob to set the pen to the center position for that axis of the paper. Now adjust the 'cal' knob after releasing the zero button to bring the pen to the corner of the paper. When this has been properly set, pressing and releasing the check button will drive the pen from the center of that edge to the paper to the corner of the paper. Repeat the procedure by prompting the computer to supply the other output signal and then adjusting the other corner. The program allows as many margin setting sequences as required.

When margin setting is completed, the MINC prompts the user for a scan time. A scan time of 120 seconds is reason-

able for most applications. Spectra with much fine structure will require a slower scan rates. After the scan time is input, the user is prompted to lower the pen and plotting is started by the pressing of the "RETURN" key on the terminal. The spectrum is output in 256 word blocks and there is a short delay between the plotting of each block. Consequently, it will take approximately two minutes longer to plot the spectrum than the scan time input even though the actual plotting is at the supplied rate.

For proper operation the X-Y recorder should have the power turned on and the servo on. The paper hold down switch controls an electrostatic hold down circuit and should be on for plotting and off for placing paper down or taking paper up. The MINC prompts pen up and down selections; however, the program should be started with the pen up.

The program allows for repeat runs. The paper can be removed after a run, or left in place if one wished to plot another full or partial spectrum on the same sheet of paper.

Program listing of the developed EPR data collection and manipulation.

```
5 REM *************  PROGRAM 4K  ********************

10 COMMON Z,A%(4096),D$,RO$,E$
20 DISPLAY_CLEAR
30 PRINT " THIS IS THE 4K MASTER PROGRAM"
40 PRINT " ALL 4K SUBSYSTEMS CAN BE RUN FROM THIS PROGRAM"
50 PRINT  \ PRINT
60 PRINT "ENTER THE NUMBER OF THE PROGRAM YOU WISH TO RUN"
70 PRINT
80 PRINT "THE PROGRAMS ARE:"
90 PRINT
110 PRINT "     1...Input from the  EPR and store on disk"
120 PRINT "     2...Subtract two spectra"
130 PRINT "     3...Read/Print file text"
140 PRINT "     4...Double integration"
150 PRINT "     5...Display single spectrum from disk"
160 PRINT "     6...Find spectral max/min"
170 PRINT "     7...Shift and scale spectra on disk"
180 PRINT "     8...Read/Print spectrum parameters"
190 PRINT "     9...Output spectrum on disk to the EPR
recorder"
200 PRINT "    10...Signal/Noise calculation and Software
signal smoothing"
210 PRINT "    11...Index array and magnetic field
positions"
230 PRINT "    12...Exit from the program"
235 PRINT
237 IF D$='' THEN  GO TO 400
240 PRINT "PROGRAM NUMBER"; \ INPUT N%
250 IF N%<1 THEN  GO TO 240
260 IF N%>12 THEN  GO TO 240
270 IF N%=12 THEN DISPLAY_CLEAR \ GO TO 9999
280 CHAIN "4K"+STR$(N%)
400 REM SEGMENT TO RESET PARAMETERS
403 PRINT 'Many program require you to run in
EXTRA_SPACE.'
404 PRINT 'You may wish to select this feature now!'
405 PRINT
410 PRINT 'Do you wish your files to be on SY1: ?'
420 D$='SY0:'
430 GOSUB 1300
440 ON R GO TO 445,450
445 D$='SY1:'
450 E$='.4K'
460 Z=0
470 GO TO 237
1300 PRINT ' Answer Y or N.'
1310 INPUT X$
1320 IF X$<>'Y' THEN IF X$<>'N' THEN  GO TO 1300
1330 IF X$='Y' THEN R=1
1340 IF X$='N' THEN R=2
```

```
1350 RETURN
9999 END

10 REM  ***************  PROGRAM 4K1   *******************

20 REM
30 COMMON Z,A%(4096),D$,R0$,E$
40 DIM P1(9)
50 REM THIS IS THE INPUT PROGRAM WITH A TIME BASE INPUT
60 PRINT 'Enter the SCAN TIME in minutes' \ INPUT S9
70 IF S9<>0 THEN S=S9
80 PRINT
90 PRINT 'ST2 Input is required, data into channel 10'
100 PRINT 'Remember to double the gain of optimum chart
output'
110 PRINT
120 PRINT 'Hit Scan Start when ready to go'
130 SET_GAIN(,2,10)
140 T=(S*60)/4096
150 AIN('DISPLAY,ST2',A%(),4096,T,10)
160 PRINT 'Do you wish to see the entire spectrum'
170 GOSUB 1000
180 ON R GO TO 190,210
190 WINDOW('EXACT',0,-2048,4095,2047)
200 GRAPH('-HLINES,EXACT,SHADE,LINES,-TICKS',
512,,A%(0),8)
210 PRINT 'Do you wish to store this spectrum on disk'
220 GOSUB 1000
230 ON R GO TO 240,570
240 PRINT 'Enter the name for your file' \ INPUT R0$
250 OPEN D$+R0$+'.4K' FOR OUTPUT AS FILE #1
260 DIM #1,A1%(4096)
270 FOR I=0 TO 4095 \ A1%(I)=A%(I) \ NEXT I
280 CLOSE #1
290 OPEN D$+R0$+'.4KP' FOR OUTPUT AS FILE #2
300 DIM #2,P(9)
310 C=-1
320 PRINT 'Enter FIELD SET in gauss' \ GOSUB 2000
330 P(0)=P1(C)
340 PRINT 'Enter SCAN RANGE in gauss' \ GOSUB 2000
350 P(1)=P1(C)
360 PRINT 'Enter TIME CONSTANT in seconds, if it is set to
OUT then enter 1E-5'
370 GOSUB 2000
380 P(2)=P1(C)
390 P9=S \ GOSUB 2010
400 P(3)=P1(C)
410 PRINT 'Enter MODULATION AMPLITUDE in gauss' \ GOSUB
2000
420 P(4)=P1(C)
430 PRINT 'Enter RECEIVER GAIN' \ GOSUB 2000
440 P(5)=P1(C)
450 PRINT 'Enter TEMPERATURE in degrees Kelvin' \ GOSUB
2000
```

```
460 P(6)=P1(C)
470 PRINT 'Enter MICROWAVE POWER in milliwatts' \ GOSUB
2000
480 P(7)=P1(C)
490 PRINT 'Enter MICROWAVE FREQUENCY in gigahertz' \ GOSUB
2000
500 P(8)=P1(C)
510 CLOSE #2
520 OPEN D$+R0$+'.4KT' FOR OUTPUT AS FILE #3
530 PRINT 'Enter a line of text if you wish'
540 LINPUT P2$
550 PRINT #3,DAT$,CLK$,P2$
560 CLOSE #3
570 Z=1
580 PRINT 'Do you wish to input another spectrum?'
590 GOSUB 1000
600 ON R GO TO 610,700
610 DISPLAY_CLEAR
620 PRINT 'On the input of the next set of parameters an
entry'
630 PRINT 'of zero (0) will not change that value from the
last'
640 PRINT 'spectrum.'
650 PRINT
660 GO TO 60
700 CHAIN '4K'
1000 PRINT 'Answer Y for yes, N for no' \ INPUT R$
1010 R=0
1020 IF R$='Y' THEN R=1
1030 IF R$='N' THEN R=2
1040 IF R<>1 THEN IF R<>2 THEN  GO TO 1000
1050 RETURN
2000 INPUT P9
2010 C=C+1
2020 IF P9=0 THEN RETURN
2030 P1(C)=P9
2040 RETURN
9999 END

10 REM   ***************   PROGRAM 4K2  *****************

20 REM
30 DISPLAY_CLEAR
40 DIM D%(4096)
50 REM THIS PROGRAM WILL SUBTRACT OR ADD SPECTRA. YOU FIRST
ENTER
60 REM THE FILE THAT YOU WILL HAVE SOME FEATURE SUBTRACTED
OR ADDED
70 S=1
80 PRINT 'Spectra must be scaled and shifted, (if
required), prior to use of '
90 PRINT 'this program segment. '
100 PRINT
110 PRINT 'If you wish to continue type Y, if not type N'
```

```
120 INPUT R2$
130 IF R2$<>'Y' THEN IF R2$<>'N' THEN  GO TO 110
140 IF R2$='N' THEN CHAIN '4K'
150 PRINT
160 PRINT 'Are the spectra on SY1:'
170 PRINT 'Answer Y for yes, N for no.'
180 INPUT R4$
190 IF R4$<>'Y' THEN IF R4$<>'N' THEN  GO TO 170
200 IF R4$='Y' THEN D$='SY1:'
210 PRINT 'Respond with an A if you wish to add these
spectra. '
220 PRINT 'Enter an S and they will be subtracted!'
230 INPUT R3$
240 IF R3$<>'S' THEN IF R3$<>'A' THEN  GO TO 210
250 IF R3$='A' THEN S=(-1) \ PRINT 'SPECTRA WILL BE ADDED'
\ PRINT
260 PRINT 'Enter the name of the first file, this is the
file that'
270 PRINT 'you will be subtracting or adding some feature.'
280 INPUT R$
290 GOSUB 520
300 OPEN D$+R$+R5$ FOR INPUT AS FILE #1
310 DIM #1,A%(4096)
320 PRINT 'Enter name of second file' \ INPUT R1$
330 GOSUB 520
340 OPEN D$+R1$+R5$ FOR INPUT AS FILE #2
350 DIM #2,B%(4096)
360 OPEN D$+R$+'.SUB' FOR OUTPUT AS FILE #3
370 DIM #3,C%(4096)
380 FOR I=0 TO 4095
390 C%(I)=A%(I)-(S*B%(I))
400 D%(I)=C%(I)
410 NEXT I
420 CLOSE
430 WINDOW('EXACT',0,-2048,4095,2047)
440 GRAPH('-HLINES,-TICKS,EXACT,SHADE',511,,D%(0),8)
450 LABEL('R,B,F',R$+'   Resultant')
460 PRINT 'File is automatically saved'
470 PRINT
480 PRINT 'Do you wish to add/subtract more spectra?' \
INPUT R8$
490 IF R8$<>'Y' THEN IF R8$<>'N' THEN  GO TO 480
500 IF R8$='Y' THEN  GO TO 210
510 CHAIN '4K'
520 PRINT
530 PRINT 'Is this a shifted file  i.e. extension .SHF'
540 PRINT
550 PRINT 'Y for yes, N for no'
560 INPUT R9$
570 IF R9$<>'Y' THEN IF R9$<>'N' THEN  GO TO 550
580 IF R9$='Y' THEN R5$='.SHF'
590 IF R9$='N' THEN R5$='.4K'
600 RETURN
610 END
```

```
1 REM  *****************   PROGRAM 4K3  ******************

2 REM
10 COMMON Z,A%(4096),D$,R0$,E$
20 DIM N$(50)
30 REM THIS PROGRAM PRINTS THE DESCRIPTIVE TEXT OF UP TO 50
FILES
40 IF Z=1 THEN N$(1)=R0$ \ F1%=1 \ GO TO 110
50 PRINT 'Enter the number of files you wish to print out'
60 INPUT F1%
70 FOR I1=1 TO F1%
80 PRINT 'Enter file name #'+STR$(I1)
90 INPUT N$(I1)
100 NEXT I1
110 FOR I2=1 TO F1%
120 R0$=N$(I2)
130 OPEN D$+R0$+".4KT" FOR INPUT AS FILE #10
140 LINPUT #10,R2$
150 PRINT 'File name---';R0$
160 PRINT R2$
170 CLOSE #10
180 PRINT
190 OPEN 'LP:' FOR OUTPUT AS FILE #9
200 PRINT #9,'File name ---';
210 PRINT #9,R0$
220 PRINT #9,R2$
230 CLOSE #9
240 NEXT I2
250 CHAIN '4K'
260 END

5 REM ***************   PROGRAM 4K4  ******************

6 REM
10 COMMON Z,A%(4096),D$,R0$,E$
20 REM THIS IS THE DOUBLE INTEGRATION PROGRAM
30 PRINT 'Enter the display scaling factor, 0.01 is
suggested for'
40 PRINT 'sharp featured spectra such as VO(2+). A lower
value for'
50 PRINT 'more broad spectra.'
60 INPUT S
70 IF Z=1 THEN  GO TO 150
80 PRINT 'Enter file name' \ INPUT R0$
90 PRINT 'Enter the file extension' \ INPUT E$
100 Z=1
110 OPEN D$+R0$+E$ FOR INPUT AS FILE #1
120 DIM #1,A1%(4096)
130 FOR I=0 TO 4095 \ A%(I)=A1%(I) \ NEXT I
140 CLOSE #1
150 E=4094
160 PRINT 'Do you wish to integrate the whole spectrum?'
170 PRINT 'Answer Y for yes, N for no.' \ INPUT R7$
```

```
180 IF R7$<>'Y' THEN IF R7$<>'N' THEN  GO TO 160
190 IF R7$='Y' THEN  GO TO 220
200 PRINT 'Enter the array starting position.' \ INPUT B
210 PRINT 'Enter the array stopping position.' \ INPUT E
220 T9=E-B+1
230 PRINT "Working"
235 GOSUB 1000
240 WINDOW('EXACT',0,-2048,4095,2048)
250 GRAPH('-HLINES,SHADE,-TICKS,EXACT',512,,A%(0),8)
260 LABEL('B',R0$+E$,'Baseline Corrected')
270 REM TRAPEZOID BASE WILL EQUAL 1
280 Z2=0
290 OPEN 'INTER.SCR' AS FILE #2
300 DIM #2,B(4096)
305 M4=0 \ M5=0
310 FOR I=B TO E
320 Z2=A%(I)+Z2*1
325 IF Z2<M4 THEN M4=Z2 \ REM M4 IS MIN VALUE
330 IF Z2>M5 THEN M5=Z2 \ REM   M5 IS MAX VALUE
350 B(I)=Z2 \ REM B WILL HAVE SPECTRUM
360 NEXT I
365 M6=M5-M4
370 IF C=0 THEN FOR I=B TO E \ A%(I)=INT(B(I)*S) \ NEXT I
380 IF C=1 THEN FOR I=B TO E \ A%(I)=INT(B(I)*S*.2)-1800
\ NEXT I
390 CLOSE #2
400 WINDOW('EXACT',0,-2048,4095,2048)
410 GRAPH('-HLINES,SHADE,-TICKS,EXACT',512,,A%(0),8)
411 S5=1/S
412 IF E$='.1ST' THEN S5=10000/A%(4095)
420 IF C=0 THEN LABEL('B','Peak height=
'+STR$(M6),'Integral Spectrum')
425 IF C=1 THEN LABEL('B','Second Integral=
'+STR$(M6*S5),'Second Integral')
427 IF E$='.1ST' THEN C=1
430 PRINT 'Do you wish to save this spectrum?'
440 PRINT 'Y for yes, N for no.'
450 INPUT R4$
455 IF R4$<>'Y' THEN IF R4$<>'N' THEN  GO TO 430
460 IF R4$='N' THEN  GO TO 590
470 IF C=1 THEN  GO TO 540
480 PRINT 'Do you wish to have the baseline corrected.'
490 PRINT 'Answer Y for yes, N for no.' \ INPUT R4$
500 IF R4$<>'Y' THEN IF R4$<>'N' THEN  GO TO 480
510 IF R4$='N' THEN  GO TO 530
520 F=1 \ GOSUB 1000
530 IF C=0 THEN E$='.1ST'
540 IF C=1 THEN E$='.2ND'
545 IF C=0 THEN A%(4095)=INT(10000*S)
550 OPEN D$+R0$+E$ FOR OUTPUT AS FILE #3
560 DIM #3,C%(4095)
570 FOR I=B TO E \ C%(I)=A%(I) \ NEXT I
575 IF C=0 THEN C%(4095)=A%(4095)
580 CLOSE #3
```

```
590 IF C=1 THEN  GO TO 670
600 C=1
610 PRINT 'Do you wish to calculate a second integral
curve'
620 PRINT 'Answer Y for yes, N for no' \ INPUT R5$
630 IF R5$<>'Y' THEN IF R5$<>'N' THEN  GO TO 610
640 IF R5$='N' THEN  GO TO 740
650 IF F=1 THEN  GO TO 240
660 GO TO 230
670 OPEN 'LP:' FOR OUTPUT AS FILE #4
680 PRINT #4,'File ----';R0$+E$
690 PRINT #4,'Double integral=';M6*S5
700 PRINT #4
710 CLOSE #4
720 PRINT 'Hit c/r when you wish to return to the main
program'
730 INPUT R$
740 CHAIN '4K'
1000 REM BASELINE CORRECTION SUBROUTINE
1005 T1=0 \ T2=0
1010 FOR I=0 TO 9
1020 T1=A%(B+I)+T1 \ T2=A%(E-I)+T2
1030 NEXT I
1040 L1=T1/10 \ L2=T2/10
1050 M=(L2-L1)/(T9-10) \ REM CALCULATE SLOPE
1060 B1=B
1070 FOR I=-T9/2 TO T9/2-1 \ REM CORRECT SLOPE
1080 A%(B1)=(A%(B1)-I*M)-(L2+L1)/2
1090 B1=B1+1
1100 NEXT I
1150 RETURN
1160 END

1 REM   ****************   PROGRAM 4K5   ******************

2 REM
5 COMMON Z,A%(4096),D$,R0$,E$
20 REM THIS PROGRAM WILL PLOT A SINGLE SPECTRUM
27 REM THE PROGRAM ALSO ALLOWS THE PLOTTING
28 REM OF SMALLER SEGMENTS OF THE SPECTRUM
29 IF Z=1 THEN  GO TO 80
30 PRINT 'Enter the file name ' \ INPUT R0$
35 PRINT 'Enter file extension.' \ INPUT E$
40 OPEN D$+R0$+E$ FOR INPUT AS FILE #3
50 DIM #3,S3%(4096)
60 FOR I=0 TO 4096 \ A%(I)=S3%(I) \ NEXT I
70 CLOSE #3
75 Z=1
80 PRINT 'The spectrum may be expanded after'
90 PRINT 'the initial plot.   Do you want this?'
100 GOSUB 310
110 ON R GO TO 140,120
120 F(0)=0 \ F(1)=0 \ S1=8 \ E=4095
130 GO TO 235
```

```
140 PRINT 'State the number of eights of the spectrum you
wish to plot'
150 PRINT 'and then place cursor at the desired starting
position on the'
152 PRINT 'following full spectrum.'
160 PRINT
170 PRINT 'How many eights?' \ INPUT S1
180 F(0)=1
190 WINDOW('EXACT',0,-2048,4095,2047)
200 GRAPH("EXACT,-TICKS,INDEX,SHADE,-HLINES",512,,
A%(0),8,,,F(0))
205 LABEL('B,R',R0$+E$)
210 S3=S1*512
220 E=S3+F(1)
230 IF E>4096 THEN PRINT 'Attempted plot exceeds spectrum
array' \ GO TO 170
235 WINDOW('EXACT',F(1),-2048,E,2047)
240 GRAPH('EXACT,-TICKS,SHADE,-HLINES',512,,A%(F(1)),S1)
245 LABEL('R,B',R0$+E$)
250 PRINT 'Hit c/r to continue' \ INPUT R3$
260 DISPLAY_CLEAR
270 PRINT 'Do you wish another look at another part of this
spectrum?'
280 GOSUB 310
290 ON R GO TO 140,291
291 PRINT 'Do you wish to look at another spectrum' \
GOSUB 310
292 ON R GO TO 30,300
300 CHAIN '4K'
310 PRINT 'Answer Y for yes, N for no'
320 INPUT R2$
330 IF R2$<>'Y' THEN IF R2$<>'N' THEN  GO TO 310
340 IF R2$='Y' THEN R=1
350 IF R2$='N' THEN R=2
360 RETURN
390 END

10 REM PROGRAM ************   4K6    ********************

20 REM
30 COMMON Z,A%(4096),D$,R0$,E$
40 DIM N$(50)
50 IF Z=1 THEN  GO TO 220
53 PRINT 'Enter the extension of the file(s) to be
searched'
55 INPUT E$
57 PRINT
60 PRINT 'Enter the number of files to be searched (50
max), if you need'
70 PRINT 'to find array positions then do first file alone
and then'
80 PRINT 'the remaining files on the next pass.'
85 PRINT
90 PRINT 'MANY FILES MIGHT REQUIRE EXTRA_SPACE'
```

```
93 PRINT
96 PRINT 'Number of files';'   '; \ INPUT F1%
100 IF F1%=1 THEN  GO TO 130
110 IF F1%>1 THEN IF F1%<51 THEN  GO TO 4000
120 PRINT 'Invalid entry' \ GO TO 60
130 PRINT 'Enter file name.'
140 INPUT RO$
150 OPEN D$+RO$+E$ FOR INPUT AS FILE #1
160 DIM #1,S1%(4096)
170 FOR I=0 TO 4095
180 A%(I)=S1%(I)
190 NEXT I
200 Z=1
210 CLOSE #1
220 PRINT 'Do you wish to search to whole spectrum?'
230 GOSUB 1000
240 ON R GO TO 250,270
250 F(0)=0 \ F(1)=0 \ F(2)=4095
260 GO TO 350
270 F(0)=2
280 PRINT 'Do you know the array positions of start and
stop of search'
290 GOSUB 1000
300 ON R GO TO 310,340
310 PRINT 'Enter start position' \ INPUT B
320 PRINT 'Enter end position' \ INPUT E
330 GO TO 390
340 PRINT 'Use first marker to mark start, second to mark
stop'
350 WINDOW('EXACT',0,-2048,4095,2047)
360 GRAPH('EXACT,-HLINES,-TICKS,SHADE,INDEX',512,,
A%(0),8,,,F(0))
370 B=F(1) \ E=F(2)
380 PRINT 'Begin =';B,'End =';E
390 GOSUB 6000
600 PRINT 'Do you want a hard copy output?'
610 GOSUB 1000
620 ON R GO TO 630,640
630 GOSUB 7000
640 PRINT 'Do you want another pass of this spectrum?'
650 GOSUB 1000
660 ON R GO TO 220,670
670 PRINT 'Do you wish to do another spectrum'
680 GOSUB 1000
690 ON R GO TO 60,700
700 CHAIN '4K'
1000 PRINT 'Enter a Y for yes, an N for no'
1010 INPUT R$
1020 IF R$<>'Y' THEN IF R$<>'N' THEN  GO TO 1000
1030 IF R$='Y' THEN R=1
1040 IF R$='N' THEN R=2
1050 RETURN
2000 H=A%(I)
2010 X1=I
```

```
2020 H1=0
2030 RETURN
3000 L=A%(I)
3010 X2=I
3020 L1=0
3030 RETURN
4000 FOR I1=1 TO F1%
4010 PRINT 'Enter file name #'+STR$(I1)
4020 INPUT N$(I1)
4030 NEXT I1
4040 PRINT 'Enter start position.' \ INPUT B
4050 PRINT 'Enter ending position.' \ INPUT E
4060 OPEN 'LP:' FOR OUTPUT AS FILE #3
4070 PRINT #3,'Searching from ';B;' to ';E
4080 PRINT #3
4090 CLOSE #3
4100 FOR I2=1 TO F1%
4110 R0$=N$(I2)
4120 OPEN D$+R0$+E$ FOR INPUT AS FILE #1
4130 FOR I=0 TO 4095 \ A%(I)=S1%(I) \ NEXT I
4140 GOSUB 6000
4150 CLOSE #1
4160 GOSUB 7000
4170 NEXT I2
4180 CHAIN '4K'
6000 H=A%(B) \ L=A%(B) \ X1=B \ X2=B
6010 FOR I=B TO E
6020 IF A%(I)>H THEN  GOSUB 2000
6030 IF A%(I)<L THEN  GOSUB 3000
6040 IF A%(I)=H THEN H1=H1+1
6050 IF A%(I)=L THEN L1=L1+1
6060 NEXT I
6070 DISPLAY_CLEAR
6080 PRINT 'File name ';
6090 HTEXT('B',,13,R0$)
6100 PRINT
6110 PRINT ,'Value','Times','At X='
6120 PRINT 'Max',H,H1,X1
6130 PRINT 'Min',L,L1,X2
6140 PRINT
6150 PRINT 'Distance between peaks = ';X2-X1
6160 PRINT 'X at midpoint is = ';(X2+X1)/2
6170 PRINT
6180 PRINT 'Full scale = ';H-L
6185 PRINT
6190 RETURN
7000 OPEN 'LP:' FOR OUTPUT AS FILE #2
7002 IF F1%=1 THEN PRINT #2,'Begin =';B,'End =';E
7004 PRINT #2
7010 PRINT #2,'File name  ';R0$
7020 PRINT #2
7030 PRINT #2,,'Value','Times','At X='
7040 PRINT #2,'Max',H,H1,X1
7050 PRINT #2,'Min',L,L1,X2
```

```
7060 PRINT #2
7070 PRINT #2,'Distance between peaks = ';X2-X1
7080 PRINT #2,'X at midpoint is = ';(X2+X1)/2
7090 PRINT #2
7100 PRINT #2,'Full scale = ';H-L
7110 PRINT #2 \ PRINT #2
7120 CLOSE #2
7130 RETURN
9999 END

1 REM  ******************  PROGRAM 4K7  *****************

2 REM
10 COMMON Z,A%(4096),D$,R0$,E$
20 REM THIS PROGRAM WILL SHIFT, SCALE AND STORE A SPECTRUM
30 REM THE NEW SAVED SPECTRUM WILL HAVE THE EXTENSION  .SHF
40 PRINT 'Enter the scaling factor' \ INPUT C
50 PRINT 'Enter the shifting factor as a SIGNED integer'
60 PRINT 'Negative integers will shift the spectrum right.'
\ INPUT S%
70 IF Z=1 THEN  GO TO 140
80 PRINT 'Enter the name of the spectrum file' \ INPUT R0$
90 Z=1
100 OPEN D$+R0$+E$ FOR INPUT AS FILE #1
110 DIM #1,A1%(4096)
120 FOR I=0 TO 4095 \ A%(I)=A1%(I) \ NEXT I
130 CLOSE #1
140 OPEN D$+R0$+'.SHF' AS FILE #2
150 DIM #2,B%(4096)
160 FOR I=0 TO 4096
170 IF I+S%<0 THEN B%(I)=0 \ GO TO 200
180 IF I+S%>4096 THEN B%(I)=0 \ GO TO 200
190 B%(I)=INT(C*A%(I+S%))
200 NEXT I
210 FOR I=0 TO 4095 \ A%(I)=B%(I) \ NEXT I
220 CLOSE #2
230 PRINT 'To look at the shifted spectrum use 4K5'
240 E$='.SHF'
245 PRINT
250 PRINT 'Hit c/r to continue' \ INPUT R1$
260 CHAIN '4K'
270 END

1 REM   ****************  PROGRAM 4K8  ******************

2 REM
5 COMMON Z,A%(4096),D$,R0$,E$
7 DIM N$(50)
10 REM READ FILE PARAMETERS PROGRAM
20 DIM A$(9)
25 IF Z=1 THEN N$(1)=R0$ \ F1%=1 \ GO TO 32
26 PRINT 'Enter the number of files you wish to print out'
27 INPUT F1%
28 FOR I1=1 TO F1%
```

```
29 PRINT 'Enter file name #';STR$(I1)
30 INPUT N$(I1)
31 NEXT I1
32 FOR I2=1 TO F1%
33 R0$=N$(I2)
50 OPEN D$+R0$+'.4KP' FOR INPUT AS FILE #3
60 DIM #3,B(9)
70 A$(0)='Field set  '
80 A$(1)='Scan range  '
90 A$(2)='Time constant  '
100 A$(3)='Scan time  '
110 A$(4)='Modulation amplitude  '
120 A$(5)='Receiver gain  '
130 A$(6)='Temperature  '
140 A$(7)='Microwave power  '
150 A$(8)='Microwave frequency  '
155 PRINT 'File name---';
157 PRINT R0$
160 FOR I=0 TO 8
170 PRINT A$(I);B(I)
180 NEXT I
185 PRINT
220 OPEN 'LP:' FOR OUTPUT AS FILE #4
222 PRINT #4
224 PRINT #4,'File name -----';
226 PRINT #4,R0$
230 FOR I=0 TO 8
240 PRINT #4,A$(I);B(I)
250 NEXT I
252 PRINT #4 \ PRINT #4
260 CLOSE #3,#4
265 NEXT I2
290 CHAIN '4K'
300 END
```

```
1 REM  *****************  PROGRAM 4K9  *******************

2 REM
10 DIM D1%(4096)
20 REM THIS PROGRAM WILL OUTPUT AN EPR SPECTRUM TO THE EPR
RECORDER
30 REM
40 REM REMEMBER THAT CHANNEL 0 OF THE D/A CONVERTER MUST BE
CONNECTED
50 REM TO PINS Z AND AA ON CONNECTOR J007 ON THE BACK OF
THE CONSOLE
60 REM   ON THE D/A THE SMALL KNOB MUST BE SET TO +/- AND
THE LARGE
70 REM   KNOB MUST BE SET TO 2.5
80 REM
90 REM ALSO NOTE THAT THE SYSTEM FUNCTION SELECTOR ON THE
EPR CONSOLE
100 REM MUST HAVE THE RECORDER INPUT SWITCH SET TO EXTERNAL
110 PRINT 'This program will output to the chart'
```

```
120 PRINT 'Enter the file name, with extension and disk
drive' \ INPUT D$
130 OPEN D$ FOR INPUT AS FILE #4
140 DIM #4,D%(4096)
150 PRINT 'Input scale factor' \ INPUT S
160 FOR I=0 TO 14 \ D9=D9+D%(I) \ NEXT I
170 D8=D9/(15*5)
180 PRINT 'The baseline is 'D8/71'blocks away from center'
190 PRINT 'Input the scale offset in blocks, it may be
positive or negative'
200 INPUT S1 \ S1=S1*71
210 D9=0
220 FOR I=0 TO 4096 \ D1%(I)=INT(D%(I)/5*S+S1) \ NEXT I
230 PRINT 'Enter scan time in minutes'
240 INPUT S
250 T=(S*60)/4096
260 PRINT 'Hit scan to start! '
270 AOUT('ST2',D1%(),4096,T,0,1)
280 CLOSE #4
290 PRINT 'Do you wish to output another spectrum?'
300 GOSUB 330
310 ON R GO TO 120,320
320 CHAIN '4K'
330 PRINT 'Answer Y for yes, N for no' \ INPUT R$
340 IF R$='Y' THEN R=1 \ RETURN
350 IF R$='N' THEN R=2 \ RETURN
360 GO TO 330
370 END

1 REM  **************  PROGRAM 4K10  *******************

2 REM
10 COMMON Z,A%(4096),D$,R0$,E$
20 PRINT 'Software signal smoothing Routine'
30 IF Z=1 THEN  GO TO 110
40 PRINT
50 PRINT 'Input File Name' \ INPUT R0$
55 PRINT
57 PRINT 'Enter file extension' \ INPUT E$
60 OPEN D$+R0$+E$ FOR INPUT AS FILE #1
70 DIM #1,A1%(4096)
80 FOR I=0 TO 4096 \ A%(I)=A1%(I) \ NEXT I
90 CLOSE #1
100 Z=1
110 WINDOW('EXACT',0,-2048,4095,2047)
120 GRAPH('-HLINES,-TICKS,EXACT,SHADE',512,,A%(0),8)
130 LABEL('B',R0$+E$)
140 GOSUB 550
150 PRINT 'Do you wish to continue with the smoothing?'
160 PRINT 'Answer Y for yes, N for no.' \ INPUT R1$
170 IF R1$<>'Y' THEN IF R1$<>'N' THEN  GO TO 150
180 IF R1$='N' THEN CHAIN '4K'
190 PRINT 'How many POINTS to be averaged, it must be odd'
200 PRINT 'seven is suggested'
```

```
210 INPUT N
220 IF N/2<>INT(N/2) THEN  GO TO 240
230 PRINT 'Must be odd' \ GO TO 190
240 N9=(N-1)/2
250 REM DO THE AVERAGING
260 FOR I=0 TO 4095
270 S=0
280 FOR J=I TO (N+I-1)
290 S=A%(J)+S
300 NEXT J
310 A=S/N
320 A%(I+N9)=INT(A)
330 IF I=(4096-N) THEN  GO TO 350
340 NEXT I
350 FOR I=0 TO N9
360 A%(I)=A%(N9)
370 A%(4095-I)=A%(4095-N9)
380 NEXT I
390 GRAPH('-HLINES,EXACT,-TICKS,SHADE',512,,A%(0),8)
400 LABEL('B',R0$+E$,'Signal Averaged')
410 PRINT 'Calculating S/N'
420 GOSUB 550
430 PRINT 'Do you wish to save this result'
440 INPUT R1$
450 IF R1$='Y' THEN  GO TO 480
460 IF R1$='N' THEN  GO TO 540
470 GO TO 430
480 PRINT 'File will be stored with the extension  .AVE'
490 OPEN D$+R0$+'.AVE' FOR OUTPUT AS FILE #2
500 DIM #2,A3%(4096)
510 FOR I=0 TO 4096 \ A3%(I)=A%(I) \ NEXT I
520 CLOSE #2
530 E$='.AVE'
540 CHAIN '4K'
550 REM THIS ROUTINE CALCULATES THE SIGNAL TO NOISE RATIO
560 REM NOISE IS THE MAX/MIN OF THE FIRST 100 ARRAY
POSITIONS
570 REM SIGNAL IS THE MAX/MIN OF THE REST OF THE SPECTRUM
580 N0=A%(0) \ N1=A%(0) \ S0=A%(0) \ S1=A%(0)
590 FOR I=0 TO 100
600 IF A%(I)>N1 THEN N1=A%(I)
610 IF A%(I)<N0 THEN N0=A%(I)
620 NEXT I
630 FOR I=101 TO 4095
640 IF A%(I)<S0 THEN S0=A%(I)
650 IF A%(I)>S1 THEN S1=A%(I)
660 NEXT I
665 F1=20*LOG10((S1-S0)/(N1-N0))
670 PRINT 'Signal=';S1-S0;'Noise=';N1-N0;'S/N=';F1
680 OPEN 'LP:' FOR OUTPUT AS FILE #9
690 PRINT #9,'File --- ';D$+R0$+E$
700 PRINT #9
710 PRINT #9,'Signal=';S1-S0;'Noise=';N1-N0;'S/N=';F1
720 CLOSE #9
```

```
730 RETURN
740 END

5 REM  *****************   PROGRAM 4K11   *****************

6 REM
10 REM PROGRAM 4K11
20 COMMON Z,A%(4096),D$,R0$,E$
30 REM THIS IS THE PROGRAM TO INDEX A SPECTRUM
40 IF Z=1 THEN  GO TO 130
50 PRINT 'Enter the file name ' \ INPUT R0$
55 PRINT 'Enter extension' \ INPUT E$
60 Z=1
70 OPEN D$+R0$+E$ FOR INPUT AS FILE #1
80 DIM #1,S1%(4096)
90 FOR I=0 TO 4095
100 A%(I)=S1%(I)
110 NEXT I
120 CLOSE #1
130 PRINT 'This program prints array positions, do you wish
'
140 PRINT 'to know the field positions'
150 GOSUB 2000
160 IF R=1 THEN M=1
170 PRINT 'Place cursor where expanded spectrum is to
start,'
171 S1=8 \ F(0)=1 \ L=0 \ E=0
172 GOSUB 3000
180 PRINT 'How many eighths of a spectrum do you wish to
plot?'
181 PRINT 'One is suggested for accuracy'
182 INPUT S1
220 LABEL('B,R',R0$+E$)
230 E=(512*S1-1)+F(1)
240 IF E>4096 THEN PRINT 'Attempted plot exceeds spectrum
array' \ GO TO 170
250 L=F(1)
260 IF M1=1 THEN  GO TO 280 \ REM SKIP FIELD SET IF
ALREADY DONE
270 IF M=1 THEN  GOSUB 1000
280 PRINT 'How many points do you wish to index'
290 INPUT F(0)
300 GOSUB 3000
310 LABEL('B','Number of points ='+STR$(F(0)))
320 PRINT 'Array positions';
330 FOR I=1 TO F(0)
340 PRINT (F(I)+L);
350 NEXT I
360 IF M<>1 THEN  GO TO 410
370 PRINT 'Field positions';
380 FOR I=1 TO F(0)
390 PRINT ((F(I)+L)-S2)*S6+S8;
400 NEXT I
410 PRINT 'Hard copy output?',
```

```
420 GOSUB 2000
430 ON R GO TO 440,610
440 OPEN 'LP:' FOR OUTPUT AS FILE #5
450 PRINT #5,'File name ---';
460 PRINT #5,R0$+E$
470 PRINT #5,'Array positions',
480 PRINT #5
490 FOR I=1 TO F(0)
500 PRINT #5,(F(I)+L);
510 NEXT I
520 IF M<>1 THEN  GO TO 590
530 PRINT #5
540 PRINT #5,'Field positions',
550 PRINT #5
560 FOR I=1 TO F(0)
570 PRINT #5,((F(I)+L)-S2)*S6+S8;
580 NEXT I
590 PRINT #5
600 CLOSE #5
610 PRINT 'Do you wish to index more points'
620 GOSUB 2000
630 ON R GO TO 170,640
640 CHAIN '4K'
1000 PRINT 'You must index a point and give its Field
value'
1010 PRINT
1020 PRINT 'Then you must supply the scan range'
1030 F(0)=1
1040 GOSUB 3000
1050 LABEL('B,R','Reference peak selection')
1060 S2=F(1)+L
1070 PRINT 'Input reference peak position is Gauss'
1080 INPUT S8
1090 PRINT 'Input Scan Range'
1100 INPUT S5
1110 S6=S5/4095
1120 M1=1
1130 RETURN
2000 PRINT 'Answer Y for yes, N for no.' \ REM YES/NO
ROUTINE
2010 INPUT R2$
2020 IF R2$<>'Y' THEN IF R2$<>'N' THEN  GO TO 2000
2030 IF R2$='Y' THEN R=1
2040 IF R2$='N' THEN R=2
2050 RETURN
3000 W1=-2048 \ W2=2047 \ REM PLOTTING ROUTINE
3010 IF E=0 THEN E=4095 \ GO TO 3050 \ REM ALLOWS INITIAL
PLOTTING
3020 PRINT 'Enter display scaling factor' \ INPUT W4
3030 W1=INT(1/W4*(-2048))
3040 W2=INT(1/W4*(2047))
3050 WINDOW('EXACT',L,W1,E,W2)
3060 GRAPH("EXACT,-TICKS,INDEX,SHADE,-HLINES",512,,A%(L),
S1,,,F(0))
```

```
3070 RETURN
4000 END

1 REM  **************  PROGRAM FIXPAR  ********************

2 REM
10 REM THIS PROGRAM CAN BE USED TO FIX INCORRECT PARAMETERS
20 REM CAUTION !!!!!   USE IT CAREFULLY
30 DIM A$(9)
40 PRINT 'Enter file name---' \ INPUT R$
50 PRINT
60 PRINT 'Is the file on SY1'
70 PRINT 'Answer Y for yes, N for no.' \ INPUT R5$
80 IF R5$<>'Y' THEN IF R5$<>'N' THEN  GO TO 60
90 IF R5$='Y' THEN D$='SY1:'
100 OPEN D$+R$+'.4KP' AS FILE #1
110 DIM #1,B(9)
120 GOSUB 1000
240 PRINT 'Enter the number of the parameter to be changed'
250 INPUT X
260 PRINT 'Now enter the new value'
270 INPUT V
280 B(X-1)=V
290 PRINT 'Do you wish to change another parameter'
300 PRINT 'Answer Y for yes, N for no.'
310 INPUT R3$
320 IF R3$<>'Y' THEN IF R3$<>'N' THEN  GO TO 290
330 IF R3$='Y' THEN  GO TO 120
340 PRINT 'Final parameters'
350 PRINT
360 GOSUB 1000
370 CLOSE
380 STOP
1000 PRINT 'File name---';
1010 HTEXT('B',,17,R$) \ PRINT
1020 PRINT '1....Field set   ';B(0)
1030 PRINT '2....Scan range   ';B(1)
1040 PRINT '3....Time constant   ';B(2)
1050 PRINT '4....Scan time   ';B(3)
1060 PRINT '5....Modulation amplitude   ';B(4)
1070 PRINT '6....Receiver gain   ';B(5)
1080 PRINT '7....Temperature   ';B(6)
1090 PRINT '8....Microwave power   ';B(7)
1100 PRINT '9....Microwave frequency   ';B(8)
1110 PRINT
1120 RETURN
2000 END

1 REM   **************  PROGRAM FIXTXT  ****************

2 REM
10 REM THIS PROGRAM CAN BE USED TO REPLACE A FILE TEXT
20 REM CAUTION!!!!! IS RECOMMENDED IN ITS USE
30 PRINT 'Input the name of the file to be changed' \
```

```
INPUT R$
40 PRINT
50 PRINT 'Is your file on SY1:'
60 PRINT 'Answer Y for yes, N for no.'
70 INPUT R5$
80 IF R5$<>'Y' THEN IF R5$<>'N' THEN  GO TO 50
90 IF R5$='Y' THEN D$='SY1:'
100 OPEN D$+R$+'.4KT' FOR INPUT AS FILE #1
110 LINPUT #1,R1$
114 PRINT
115 PRINT 'File name---';R$
116 PRINT
120 PRINT R1$
125 PRINT
130 PRINT 'Do you still wish to change this text' \ INPUT
R2$
140 IF R2$<>'Y' THEN IF R2$<>'N' THEN  GO TO 130
150 IF R2$='N' THEN  GO TO 300
160 PRINT 'Enter new file text'
170 PRINT
180 LINPUT R4$
182 OPEN D$+R$+'.4KT' FOR OUTPUT AS FILE #2
190 PRINT #2,'Corrected text  ';R4$
300 CLOSE
309 PRINT
310 PRINT 'Do you wish to change another file text?'
320 PRINT 'Answer Y for yes, N for no' \ INPUT R6$
330 IF R6$<>'Y' THEN IF R6$<>'N' THEN  GO TO 310
340 IF R6$='Y' THEN PRINT  \ GO TO 30
350 STOP
9999 END

10 REM  *************  PROGRAM XYPLOT  ****************

20 REM
30 DIM A1%(256)
40 REM THIS THE PLOT ROUTINE
50 REM THIS PROGRAM WILL READ A SPECTRUM FROM DISK AND
60 REM PLOT IT ON AN X/Y RECORDER
70 PRINT 'If you wish instructions on how to use this
program type Y'
80 PRINT 'If not type N' \ INPUT R$
90 GOSUB 1300
100 ON R GO TO 70,3000,110
110 OPEN 'PLOT.SCR' AS FILE #5
120 DIM #5,A%(8191)
130 A=0
140 PRINT "Is 'PLOT.SCR' on your working disk?" \ INPUT R$
150 GOSUB 1300
160 ON R GO TO 140,200,170
170 FOR I=-2048 TO 2047
180 A%(A)=I \ A=A+2
190 NEXT I
200 PRINT 'Enter data scaling factor, 1 for no scaling' \
```

```
INPUT S2
210 PRINT
212 PRINT 'Input the file name, include disk and extension
if needed'
214 INPUT N$
215 PRINT 'Do you wish to plot only a portion of the
spectrum'
216 INPUT R$
217 GOSUB 1300
218 ON R GO TO 215,219,224
219 PRINT 'Enter starting position' \ INPUT B1 \ B1=B1*2
221 PRINT 'Enter stopping position' \ INPUT E1 \ E1=E1*2
223 GO TO 229
224 B1=0 \ E1=8192
229 OPEN N$ FOR INPUT AS FILE #1
230 DIM #1,B1%(4096)
240 FOR B=0 TO 4095
250 B9=2*B+1
260 A%(B9)=INT(S2*B1%(B))
270 NEXT B
280 IF S2<>1 THEN PRINT 'Data Scaled'
290 CLOSE #1
300 PRINT 'Do you wish to have a 7-point smoothing routine
run'
310 INPUT R$
320 GOSUB 1300
330 ON R GO TO 300,340,350
340 GOSUB 4000
350 REM        GO TO SET MARGINS
360 GOSUB 1000
370 PRINT 'Enter the scan time in seconds' \ INPUT S4 \
S5=S4/4096
380 M(0)=A%(B1) \ M(1)=A%(B1+1)
390 AOUT(,M(),2,,2,2)
400 PRINT 'Lower pen and hit RETURN to start' \ INPUT R$
410 N=B1
420 FOR I=0 TO 255
430 A1%(I)=A%(N)
440 N=N+1
445 IF N=E1 THEN  GO TO 480
450 NEXT I
460 AOUT(,A1%(),256,S5,2,2)
470 IF N<>8192 THEN  GO TO 420
480 PRINT 'If you wish you may now lift pen and remove
paper'
485 PRINT
490 PRINT 'If you want another scan type Y' \ INPUT R$
500 GOSUB 1300
510 ON R GO TO 490,200,520
520 CLOSE
530 STOP
1000 PRINT 'Set upper right point on next output'
1010 M(0)=2047 \ M(1)=M(0)
1030 GOSUB 1100
```

```
1040 PRINT 'Now set lower left point'
1050 M(0)=-2048 \ M(1)=M(0)
1051 GOSUB 1100
1052 PRINT 'Do you want another margin setting sequence' \
INPUT R$
1054 GOSUB 1300
1056 ON R GO TO 1052,1000,1090
1090 RETURN
1100 AOUT(,M(),2,,2,2)
1110 PRINT 'When point is set hit RETURN' \ INPUT R$
1120 RETURN
1300 IF R$<>'Y' THEN IF R$<>'N' THEN R=1
1310 IF R$='Y' THEN R=2
1320 IF R$='N' THEN R=3
1330 RETURN
3000 PRINT 'THIS PROGRAM IS WRITTEN TO OUTPUT SPECTRA TO AN
X/Y PLOTTER'
3005 PRINT
3010 PRINT 'CONNECT CHANNEL 0 TO THE X AXIS, CHANNEL 1 TO
THE Y AXIS'
3015 PRINT 'SHORT THE - AND GROUND TERMINAL TOGETHER ON THE
RECORDER'
3020 PRINT 'FOR EACH CHANNEL'
3025 PRINT
3030 PRINT 'SET EACH CHANNEL TO THE 1V SETTING AND SET THE
KNOB TO THE'
3035 PRINT 'RIGHT TO VAR, USE THE VARIABLE AND ZERO
ADJUSTMENT TO '
3040 PRINT 'CENTER YOUR SPECTRA ON THE SHEET (THE PROGRAM
SUPPLIES SIGNALS'
3045 PRINT 'TO AID YOU IN DOING THIS'
3050 PRINT
3055 PRINT 'ON THE D/A CONVERTER SET THE INNER KNOB TO +/-
AND THE OUTER'
3060 PRINT 'KNOB TO 10','GOOD LUCK'
3070 GO TO 110
4000 FOR I=7 TO 8185 STEP 2
4010 FOR I1=-6 TO 6 STEP 2
4020 Z=A%(I+I1)+Z
4030 NEXT I1
4040 M=Z/7
4050 A%(I)=M
4060 Z=0
4070 NEXT I
4080 RETURN
5000 END

10 REM   ****************  PROGRAM SIGAVE  ***************

20 REM
30 DIM A%(4096)
40 D$='SY1:'
50 PRINT 'Spectra will be stored on SY1: unless program
changed'
```

```
60 PRINT
70 PRINT 'Enter the file name you wish to use' \ INPUT R0$
80 PRINT
90 PRINT 'Enter starting file number.' \ INPUT E \ I5=E
100 REM THIS PROGRAM WILL COLLECT SPECTRA AND STORE THEM ON
DISK
110 REM THEY WILL BE LISTED WITH THE EXTENSION
.1,.2,.3,......
120 PRINT 'Enter the scan time in minutes' \ INPUT R
130 DISPLAY_CLEAR
140 R9=R-1*R/30 \ REM CORRECTS FOR FAST SWEEP OF E-9
150 SET_GAIN(,2,10)
160 AIN('ST2,DISPLAY',A%(),4096,R9*60/4096,10)
170 PRINT 'Enter a 1 to save this spectrum,2 to discard' \
INPUT R5
180 ON R5 GO TO 190,130
190 PRINT 'Saving ';D$+R0$+'.'+STR$(E)
200 OPEN D$+R0$+'.'+STR$(E) FOR OUTPUT AS FILE #1
210 DIM #1,A1%(4096)
220 FOR I=0 TO 4095 \ A1%(I)=A%(I) \ NEXT I
230 CLOSE #1
240 E=E+1
250 PRINT 'Do you wish to sum spectra now?'
260 PRINT 'Answer Y for yes, N for no.'
270 INPUT R$
280 IF R$<>'Y' THEN IF R$<>'N' THEN  GO TO 250
290 IF R$='N' THEN  GO TO 310
300 GO TO 340
310 PRINT 'Ready for next spectrum after display is
cleared'
320 PRINT ''
330 GO TO 130
340 OPEN D$+R0$+'.SUM' AS FILE #5
350 DIM #5,S%(4096)
360 FOR I=I5 TO (E-1)
370 OPEN D$+R0$+'.'+STR$(I) FOR INPUT AS FILE #6
380 DIM #6,B%(4096)
390 FOR I1=0 TO 4095
400 S%(I1)=S%(I1)+B%(I1)
410 NEXT I1
420 CLOSE #6
430 NEXT I
440 I5=E
450 FOR I3=0 TO 4095 \ A%(I3)=S%(I3) \ NEXT I3
460 WINDOW('EXACT',0,-2048,4095,2047)
470 GRAPH('-HLINES,-TICKS,EXACT,SHADE',512,,A%(0),8)
480 CLOSE
490 PRINT 'Do you wish to collect more spectra?'
500 PRINT 'Answer Y for yes, n for no' \ INPUT R$
510 IF R$<>'Y' THEN IF R$<>'N' THEN  GO TO 490
520 IF R$='N' THEN STOP
530 GO TO 310
540 END
```

APPENDIX B

CALCULATOR PROGRAM FOR PROTEIN ASSAY

A program was developed for a TI 58 of TI 59 hand held calculator which allowed, in conjunction with the experimental procedure, a rapid determination of protein concentrations. The process consumed minimum protein and allowed replicate analyses.

Typical experimental procedure utilized a spectrophotometer operating at 280 nm. The analysis was carried out using semimicro quartz cuvettes. Both sample and reference cuvettes were filled with 1.000 ml of appropriate buffer. Protein was then added in 2.0 to 5.0 ul increments to the sample cell. Five additions could be made without causing the sample absorbance to exceed 1.0.

The calculator is then supplied with the absorbance of a 1 % protein solution, the initial volume of buffer in the sample cell, the molecular weight of the protein and the volume and absorbances from the experimental procedure.

The program calculates and provides the percentage of the protein in the analyte solution, the molar concentration of the protein and the relative standard deviation of the analysis.

The user defined keys for this program and an annotated calculator program listing are presented below.

Key Assignments

Key	Function
A	Total amount of protein added after this increment.
B	Absorbance observed for this increment of added protein.
C	Calculate the percentage protein in your assay solution.
D	Calculate the Molar concentration in your assay solution.
E	Clear data for additions, leave constant values.
A'	Enters value in display as absorbance of a 1% solution.
B'	Enters initial volume of buffer in the cuvette.
C'	Enters the molecular weight of the protein.
D'	Calculates the relative standard deviation of this analysis.
E'	Master clears calculator.

Calculator Program

This program can be positioned in any memory area in the calculator. The listing gives addresses starting at zero followed by the mnemonic code for the calculator keystroke.

000	76	LBL	Clear calculator and calculator memories.
001	10	E'	
002	47	CMS	
003	57	ENG	
004	25	CLR	
005	91	R/S	
006	76	LBL	The entered absorbance of a 1% protein
007	16	A'	solution is stored in memory location 15.
008	42	STO	
009	15	15	
010	91	R/S	
011	76	LBL	The entered initial volume is stored at
012	17	B'	location 16.
013	42	STO	
014	16	16	
015	91	R/S	
016	76	LBL	The entered molecular weight is stored in
017	18	C'	location 17.
018	42	STO	
019	17	17	
020	91	R/S	
021	76	LBL	For each added increment of protein solution
022	11	A	a dilution factor is calculated and stored.
023	42	STO	
024	11	11	
025	68	NOP	
026	68	NOP	
027	68	NOP	
028	68	NOP	
029	55	÷	
030	53	(	
031	43	RCL	
032	16	16	
033	85	+	
034	43	RCL	
035	11	11	
036	54	)	
037	95	=	
038	35	1/X	
039	42	STO	
040	12	12	
041	91	R/S	
042	76	LBL	From the entered absorbance and the dilution
043	12	B	factor for this addition an absorbance is
044	65	x	calculated for the initial protein solution.
045	43	RCL	
046	12	12	

047	95	=	
048	78	Σ+	
049	91	R/S	
050	76	LBL	The average absorbance is determined and
051	13	C	from this average and the absorbance of a 1%
052	79	$\bar{x}$	solution the percent protein is calculated.
053	55	÷	
054	43	RCL	
055	15	15	
056	95	=	
057	42	STO	
058	07	07	
059	91	R/S	
060	76	LBL	The molar concentration of the protein
061	14	D	solution is calculated. The program will
062	43	RCL	halt if the percent concentration has not
063	07	07	been determined.
064	67	EQ	
065	91	R/S	
066	65	x	
067	01	1	
068	00	0	
069	55	÷	
070	43	RCL	
071	17	17	
072	95	=	
073	91	R/S	
074	76	LBL	The relative standard deviation for this
075	19	D'	assay is determined.
076	22	INV	
077	79	$\bar{x}$	
078	55	÷	
079	79	$\bar{x}$	
080	95	=	
081	91	R/S	The results of the last assay are erased;
099	76	LBL	however, constant values are retained.
100	15	E	
101	00	0	
102	42	STO	
103	05	05	
104	42	STO	
105	06	06	
106	42	STO	
107	07	07	
108	42	STO	
109	01	01	
110	42	STO	
111	02	02	
112	42	STO	
113	00	00	
114	91	R/S	

APPENDIX C

```
C                                   ******EPRPOW******
C
C     EPRPOW WAS DEVELOPED BY DRS. L. K. WHITE AND R. L. BELFORD OF THE SCHOOL OF
C    CHEMICAL SCIENCES OF THE UNIVERSITY OF ILLINOIS AND SUBSEQUENTLY MODIFIED BY
C    L. K. WHITE. N. F. ALBANESE. AND N. D. CHASTEEN AT THE UNIVERSITY OF NEW
C    HAMPSHIRE TO INCLUDE BOTH LORENTZIAN AND GAUSSIAN LINE SHAPE FUNCTIONS. AN
C    I=7/2 NUCLEUS. A FOURTH HYPERFINE INTERACTION. AND MULTIPLE SITES HAVING DIFF
C    ERENT LINE WIDTHS.  PLEASE ACKNOWLEDGE THE ORIGINAL DEVELOPERS OF THE PROGRAM
C    . DRS. WHITE AND BELFORD. WHEN REPORTING RESULTS BASED ON THE USE OF EPRPOW.
C    THANK YOU.
C
C
C    N. D. CHASTEEN
C    CHEMISTRY DEPARTMENT
C    UNIVERSITY OF NEW HAMPSHIRE
C    NOVEMBER 2. 1976
C
C
C     MODIFIED TC INCLUDE STRAIN  IN G AND A TENSOR .  JANUARY 1981
C
C
C      MODIFIED TO INCLUDE LINESHAPE  VARIATION WITH G AND A STRAIN
C*******************************************************************************
C
C
       INTEGER ALPHA.GGX.GGY.GGZ.AX1.AY1.AZ1.AX2.AY2.AZ2.AX3.AY3.AZ3.
      1AX4.AY4.AZ4.WX.WY.WZ.H.STAR.HAM.II.DHX.DHY.DHZ.WWWX.WWWY.WWWZ
       REAL KK. LX. LY. LZ.KH.LH
       REAL*4 X(2502).Y(2502).STORE(2502)
       DIMENSION D(600).CX(600).SX(600).SP(600).CP(600).DPHI(600).FI(600)
       DIMENSION INT1(20).INT2(20).INT3(10).INT4(10)
       DIMENSION PTITLE(12)
       DIMENSION LABEL(24)
         DIMENSION DHX(8).DHY(8).DHZ(8)
       DATA (LABEL(II).II=1.24)/'SITE#'.'WT X'.'GX'.'GY'.'GZ'.'AAX1'.
      1'AAY1'.'AAZ1'.'AAX2'.'AAY2'.'AAZ2'.'AAX3'.'AAY3'.
      2'AAZ3'.'AAX4'.'AAY4'.'AAZ4'.'SPIN1'.'SPIN2'.'SPIN3'.'SPIN4'.'WWX'.
      3'WWY'.'WWZ'/
C   PLOT SETUP. INTEGRATION. FREQUENCY. AND LINEWIDTH DATA READ IN HERE
C   PLOT TITLE READ IN HERE
       READ(5.11) PTITLE
       WRITE(6.11) PTITLE
    11 FORMAT(12A5)
       READ(5 .1)NN.MM.ALPHA.STAR.HAM
       WRITE(6.1) NN.MM.ALPHA.STAR.HAM
     1 FORMAT(5(2X.I3))
       READ(5 .2)L.LTCT.SPAC.PL.PHGT
     2 FORMAT(2(2X.I5).2X.F6.3.2X.F5.2.2X.F5.2)
       READ(5.3)XNU.NSITES
     3 FORMAT(2X.F7.4.I3)
       READ(5.10)WWX.WWY.WWZ.NS
    10 FORMAT(3(2X.F5.1).I3)
                 NP=3
                 DH=1.0
       CUTOFF=4.00
       IF(STAR.EQ.1) CUTOFF=3.0
       IF(HAM.EG.1) CUTOFF=8.0
       L=L*100
       LTOT=LTCT*100
       LINT=SPAC*100.0+0.5
       WX=WWX*100.0+0.5
       WY=WWY*100.0+0.5
       WZ=WWZ*100.0+0.5
       KLOT=(2*(WX+WY+WZ))/LINT
       DEL=KLOT*LINT
       KMAX=LTOT+DEL
       KMIN=L-DEL
       KMOT=((KMAX-KMIN)/LINT)
       KTOT=(LTOT-KMIN)/LINT
       WRITE(6.19) KLOT.KMAX.KMIN.KMOT.KTOT.L.LTOT.DEL.SPAC
    19 FORMAT(7I8.2X.2F7.2)
       WRITE(6.303)XNU
   303 FORMAT(1X.F7.4.24H GHZ MICROWAVE FREQUENCY)
       DO100 KL=1.KMOT
       STORE(KL)=0
       X(KL) = 0.
       Y(KL) = 0.
   100 CONTINUE
       HMIN=L
       HMAX=LTOT
    92 NN3=3*NN
       MM3=3*MM
C   GAUSS-POINT SELECTION OF ANGLES.WEIGHTING FACTORS FOR INTEGATION
C   SELECTION USES LINEWIDTHS OF 1ST SITE ONLY.
C   SUBROUTINE THETA SELECTS FOR XZ OR YZ PLANES
C   SUBROUTINE PHI SELECTS FOR XY PLANE
       IF(HAM.EQ.1) GO TC 89
       CALL THETA(NN.CX.SX.D)
       IF(HAM.EQ.2) GO TO 90
       CALL ORTHRH(MM.CP.SP.DPHI.ALPHA)
       GO TO 91
    89 NN3=1
       SX(1)=0.0000000
       CX(1)=1.0000000
       D(1)=1.0C000000
    90 MM3=1
       CP(1)=1.00000000
       SP(1)=0.00000000
       DPHI(1)=1.00000000
```

```
C   PLOT AND TABLE OF DATA INITIATED HERE
C   HL=LETTER HEIGHT. WLINE= LINE SPACING. BX AND BY= X AND Y COORDINATES TO START TABLE
C
91       HL=0.25
         WLINE=0.35
         BX=2.0
         BY=28.5
         CALL  PLOTS('C')
         CALL  PLOT(1.0.1.0.-3)
         CALL  SYMBOL(BX.BY+3*WLINE.HL.PTITLE.0.0.45)
         CALL  NUMBER(BX.BY+2*WLINE.HL.FLOAT(NN).0.0.-1)
         CALL  NUMBER(BX+2.0.BY+2*WLINE.HL.FLCAT(MM).0.0.-1)
         CALL  NUMEER(BX+4.0.BY+2*WLINE.HL.FLOAT(ALPHA).0.0.-1)
         CALL  NUMEER(BX+6.0.BY+2*WLINE.HL.FLCAT(STAR).0.0.-1)
         CALL  NUMEER(BX+8.0.BY+2*WLINE.HL.FLOAT(HAM).0.0.-1)
         CALL  NUMEER(BX.BY+WLINE.HL.FLCAT(L).0.0.-1)
         CALL  NUMBER(BX+2.0.BY+WLINE.HL.FLCAT(LTCT).0.0.-1)
         CALL  NUMBER(BX+4.0.BY+WLINE.HL.SPAC.0.0.1)
         CALL  NUMEER(BX+6.0.BY+WLINE.HL.PL.0.0.1)
         CALL  NUMEER(BX+8.0.BY+WLINE.HL.PHGT.0.0.1)
         DO 1000 II=1.24
         CALL  SYMBOL(BX.BY-WLINE*FLOAT(II-1).HL.LABEL(II).0.0.5)
 1000    CONTINUE
C
C   EPR PARAMETERS READ IN FOR EACH SITE
C   LINE#IDTH OF A PARTICULAR SITE USED FOR LINESHAPE FUNCTION OF THAT SITE ONLY
C
         DO 108IN=1.NSITES
         IF(IN.EQ.1) GO TO 25
         READ (5.10) WWX.WWY.WWZ.NS
         WX=WWX*100.0+0.5
         WY=WWY*100.0+0.5
         WZ=WWZ*100.0+0.5
   25    CONTINUE
C                       NC=0    STRAIN PARAMETERS CORRELATED
C                       NC=1    STRAIN PARAMETERS  UNCORRELATED
         READ(5.70) DGX.DGY.DGZ.DAAX1.DAAY1.DAAZ1.NC.NP
  70     FORMAT(3(2X.F5.0).3F10.5.2X.I1.2X.I2)
  31     READ(5.4)GGX.GGY.GGZ.INT
         DGX=DGX/10000.
         DGY=DGY/10000.
         DGZ=DGZ/10000.
         GX=GGX/10000.
         GY=GGY/10000.
         GZ=GGZ/10000.
    4 FORMAT(4(2X.I5))
      READ(5  .5)AAX1.AAY1.AAZ1
    5 FORMAT(3F10.5)
      READ(5  .5)AAX2.AAY2.AAZ2
      READ(5  .5)AAX3.AAY3.AAZ3
      READ(5.5)AAX4.AAY4.AAZ4
      READ(5.8)SPIN1.SPIN2.SPIN3.SPIN4
    8 FORMAT(4(2X.F3.1))
      NSP1=2.*SPIN1+1.5
      NSP2=2.*SPIN2+1.5
      NSP3=2.*SPIN3+1.5
      NSP4=2.*SPIN4+1.5
      READ(5.9)   ((INT1(I).I=1.NSP1).(INT2(J).J=1.NSP2).
     1(INT3(K).K=1.NSP3).(INT4(LM).LM=1.NSP4))
    9 FORMAT(20I4/20I4)
C                   INSERTED PROGRAM
         IF(NS.EQ.0) GO TO 26
                              IF(NC.EQ.1) GO TO 72
                 FMI=-SPIN1
         DO 71 J1=1.NSP1
         DHX(J1)=((GX*DAAX1+AAX1*DGX)*FMI-333.564*XNU*DGX)
     1   /(0.466877*GX*(GX+DGX))*100.0+0.5
         DHX(J1)=ABS(DHX(J1))
         DHY(J1)=((GY*DAAY1+AAY1*DGY)*FMI-333.564*XNU*DGY)
     1    /(0.466877*GY*(GY+DGY))*100.0+0.5
         DHY(J1)=ABS(DHY(J1))
         DHZ(J1)=((GZ*DAAZ1+AAZ1*DGZ)*FMI-333.564*XNU*DGZ)
     1    /(0.466877*GZ*(GZ+DGZ))*100.0+0.5
         DHZ(J1)=ABS(DHZ(J1))
         FMI=FMI+1.0
                          WRITE(6.900)DHX(J1).DHY(J1).DHZ(J1)
900              FORMAT(1X.4HDHX=.I6.1X4HDHY=.I6.1X4HDHZ=.I6)
71               CONTINUE
C        DHX.DHY.AND DHZ ARE THE SHIFTS IN THE PRINCIPLE
C        RESONANCE FIELDS DUE TC THE G AND A STRAIN
                           GO TO 73
72                         FGX=DGX/GX
                           FGY=DGY/GY
                           FGZ=DGZ/GZ
                           FAX=DAAX1/AAX1
                           FAY=DAAY1/AAY1
                           FAZ=DAAZ1/AAZ1
                           HGX=333.564*XNU*FGX/(0.466877*GX)
                           HGY=333.564*XNU*FGY/(0.466877*GY)
                           HGZ=333.564*XNU*FGZ/(0.466877*GZ)
                           FMI=-SPIN1
                           DO 73 J1=1.NSP1
                 HAX=SQRT(FGX**2+FAX**2)*AAX1*FMI/(0.466877*GX)
                 HAY=SQRT(FGY**2+FAY**2)*AAY1*FMI/(0.466877*GY)
                 HAZ=SQRT(FGZ**2+FAZ**2)*AAZ1*FMI/(0.466877*GZ)
                 DHX(J1)=SQRT(HGX**2+HAX**2)*100.0+0.5
                 DHY(J1)=SQRT(HGY**2+HAY**2)*100.0+0.5
                 DHZ(J1)=SQRT(HGZ**2+HAZ**2)*100.0+0.5
                 FMI=FMI+1.0
                 WRITE(6.900)DHX(J1).DHY(J1).DHZ(J1)
73               CONTINUE
                 IF(NS.EQ.1) GO TO 28
   26    DO 27 J1=1.NSP1
         DHX(J1)=0.0
         DHY(J1)=0.0
         DHZ(J1)=0.0
   27    CONTINUE
```

```
C
C  DATA FOR EACH SITE PLOTTED HERE
C
28        BX=BX+2.5
 BX=BX+2.5
CALL  NUMBER(BX.BY.HL.FLCAT(IN).0.0.-1)                       ).-1)
CALL  NUMBER(BX.BY-WLINE.HL.FLOAT(IWT).0.0.-1)                ).0.-1)
CALL  NUMBER(BX.BY-2*WLINE.HL.FLOAT(GGX).0.0.-1).0.-1)
CALL  NUMBER(BX.BY-3*WLINE.HL.FLOAT(GGY).0.0.-1).0.-1)
CALL  NUMEER(BX.BY-4*WLINE.HL.FLOAT(GGZ).0.0.-1)
CALL  NUMEER(BX.BY-5*WLINE.HL.AAX1.0.0.2)
CALL  NUMBER(BX.BY-6*WLINE.HL.AAY1.0.0.2)
CALL  NUMBER(BX.BY-7*WLINE.HL.AAZ1.0.0.2)
CALL  NUMBER(BX.BY-8*WLINE.HL.AAX2.0.0.2)
CALL  NUMEER(BX.BY-9*WLINE.HL.AAY2.0.0.2)             )
CALL  NUMEER(BX.BY-10*WLINE.HL.AAZ2.0.0.2)            )
CALL  NUMBER(BX.BY-11*WLINE.HL.AAX3.0.0.2)            )
CALL  NUMEER(BX.BY-12*WLINE.HL.AAY3.0.0.2)            )
CALL  NUMEER(BX.BY-13*WLINE.HL.AAZ3.0.0.2)            )
CALL  NUMEER(BX.BY-14*WLINE.HL.AAX4.0.0.2)            )
CALL  NUMEER(BX.BY-15*WLINE.HL.AAY4.0.0.2)            )
CALL  NUMBER(BX.BY-16*WLINE.HL.AAZ4.0.0.2)            1)
CALL  NUMBER(BX.BY-17*WLINE.HL.SPIN1.0.0.1)           1)
CALL  NUMEER(BX.BY-18*WLINE.HL.SPIN2.0.0.1)           1)
CALL  NUMEER(BX.BY-19*WLINE.HL.SPIN3.0.0.1)           1)
CALL  NUMEER(BX.BY-20*WLINE.HL.SPIN4.0.0.1)
CALL  NUMBER(BX.BY-21*WLINE.HL.WWX.0.0.2)
CALL  NUMEER(BX.BY-22*WLINE.HL.WWY.0.0.2)
CALL  NUMEER(BX.BY-23*WLINE.HL.WWZ.0.0.2)
        RALPH=ALPHA
        ALP=ALPHA*0.0174533
        SALP=SIN(ALP)
        CALP=COS(ALP)
        WRITE(6.298)
   298 FORMAT(12A5//)
        WRITE(6.299) IN.IWT
   299 FORMAT(14H***SITE NUMBER.I4.14H***WEIGHT X    .I3.3H***)
        WRITE(6.300)SPIN1.SPIN2.SPIN3.SPIN4
   300 FORMAT(2X.6HSPIN1=.F4.2.2X.6HSPIN2=.F4.2.2X.6HSPIN3=.F4.2.
      12X.6HSPIN4=.F4.2)
        WRITE(6.301)GX.GY.GZ.AAX1.AAY1.AAZ1.AAX2.AAY2.AAZ2.AAX3.AAY3.AAZ3.
      1AAX4.AAY4.AAZ4.DGX.DGY.DGZ.DAAX1.DAAY1.DAAZ1

   301 FORMAT(1X.3HGX=.F6.4.1X3HGY=.F6.4.1X3HGZ=.F6.4/
      11X.5HAAX1=.F10.5.1X.5HAAY1=.F10.5.1X.5HAAZ1=.F10.5/
      21X.5HAAX2=.F10.5.1X.5HAAY2=.F10.5.1X.5HAAZ2=.F10.5/
      31X.5HAAX3=.F10.5.1X.5HAAY3=.F10.5.1X.5HAAZ3=.F10.5/
      41X.5HAAX4=.F10.5.1X.5HAAY4=.F10.5.1X.5HAAZ4=.F10.5/
      51X.4HDGX=.F10.5.1X.4HDGY=.F10.5.1X.4HDGZ=.F10.5/
      61X.6HDAAX1=.F10.5.1X.6HDAAY1=.F10.5.1X.6HDAAZ1=.F10.5)
        WRITE(6.302)WWX.WWY.WWZ.ALPHA
   302 FORMAT(1X.4HWWX=.F4.1.1X.4HWWY=.F4.1.1X.4HWWZ=.F4.1.1X.5HALPH=.I3)
                    WRITE(6.333)NS.NC.NP
 333                FORMAT(1X3HNS=.I3.1X3HNC=.I3.1X3HNP=.I3)

        WRITE(6.304)((INT1(I).I=1.NSP1).(INT2(J).J=1.NSP2).
      1(INT3(K).K=1.NSP3).(INT4(LM).LM=1.NSP4))
   304 FORMAT(20I4/20I4)
        GXX=GX*GX
        GYY=GY*GY
        GZZ=GZ*GZ
        WISO=1.0000
        AX1=AAX1*100.0+0.5
        AY1=AAY1*100.0+0.5
        AZ1=AAZ1*100.0+0.5
        AX2=AAX2*100.0+0.5
        AY2=AAY2*100.0+0.5
        AZ2=AAZ2*100.0+0.5
        AX3=AAX3*100.0+0.5
        AY3=AAY3*100.0+0.5
        AZ3=AAZ3*100.0+0.5
        AX4=AAX4*100.0+0.5
        AY4=AAY4*100.0+0.5
        AZ4=AAZ4*100.0+0.5
        AXX=AX1*AX1
        AYY=AY1*AY1
        AZZ=AZ1*AZ1
C   CX(I) AND CS EQUALS COSINE(THETA)
C   SX(I) AND SN EQUALS SINE(THETA)
C   CP(I) AND CC EQUALS COSINE(PHI)
C   SP(I) AND SS EQUALS SINE(PHI)
C   D(I) INTERGRATION FACTORS FOR THETA
C   DPHI(I) INTERGRATION FACTORS FOR PHI
        DO 108 I=1.NN3
        CS=CX(I)
        SN=SX(I)
        DO 108 M=1.MM3
        CC=CP(M)
        SS=SP(M)
        SN2=SN*SN
        CS2=CS*CS
        SS2=SS*SS
        CC2=CC*CC
   402 LX=SN*SN*CC*CC
        LY=SN*SN*SS*SS
        LZ=CS*CS
        GLX=GXX*LX
        GLY=GYY*LY
        GLZ=GZZ*LZ
        G=SQRT(GLX+GLY+GLZ)
        GG=0.466877*G
C   'U' IS THE ANISOTROPIC G WEIGHTING FACTOR
        U=(GXX*GYY*SN2+GYY*GZZ*(SS2+CS2*CC2)+ GZZ*GXX*(CC2+CS2*SS2))/(G*
      1*3.)
C   'W' INCLUDES INTEGRATION WEIGHTING FACTOR AND SITE WEIGHTING FACTOR
        W=U*D(I)*DPHI(M)*IWT
C   'HO' EQUALS 'G' CENTER OF A SPECTRUM
        HO=71448.7*XNU/G
```

```
C   SECUND-ORDER PERTURBATIGN FCRMULA BEGIN HERE
        ELX=SN*CC
        ELY=SN*SS
        ELZ=CS
        GELX=GX*ELX
        GELY=GY*ELY
        GELZ=GZ*ELZ
        ACZZ=AZ1
         GPERP=SQRT(GXX*CC2+GYY*SS2)
C   TXY EQUALS OFF-DIAGONAL,TENSOR ELEMENT IN NON-COINCIDENT CASE
  701   TXX=AXX*CALP*CALP+AYY*SALP*SALP
        TYY=AXX*SALP*SALP+AYY*CALP*CALP
        TXY=(AXX-AYY)*SALP*CALP
        AZG=TXX*GELX*GELX+TYY*GELY*GELY+2*GELX*GELY*TXY+AZZ*GELZ*GELZ
        Z=SQRT(AZG)/G
        ZG=Z/GB
        APERP=SQRT(GXX*CC2*TXX+GYY*SS2*TYY+2*GX*GY*SS*CC*TXY)/GPERP
        ABCDE=(SS*CC*(GX*GY*(TYY-TXX)+(GXX-GYY)*TXY))/(Z*G*GPERP)
        AA=(APERP*ACZZ/Z)**2
        AB=(TXX*TYY-TXY**2)/APERP**2
        AC=(ABCDE*ACZZ*GZ*CS/(APERP*GPERP))**2
        AD=(GZ*GPERP*(APERP**2-ACZZ**2)*SN*CS/(Z*G*G))**2
        AE=(ABCDE*SN)**2
        GBBH=GB*GB*HO
        CA=(AA+AB+AC)/(4*GBBH)
        CB=(AD+AE)/(2*GBBH)
        ZN=(SQRT(AZ2*AZ2*CS2*GZZ+SN2*(AX2*AX2*CC2*GXX+AY2*AY2*SS2*GYY)))/G
        ZNG=ZN/GB
        YN=(SQRT(AZ3*AZ3*CS2*GZZ+SN2*(AX3*AX3*CC2*GXX+AY3*AY3*SS2*GYY)))/G
        YNG=YN/GB
        QN=(SQRT(AZ4*AZ4*CS2*GZZ+SN2*(AX4*AX4*CC2*GXX+AY4*AY4*SS2*GYY)))/G
        QNG=QN/GE
C   HH EQUALS PRINCIPAL RESGNANCE FIELD FOR A1 HYPERFINE INTERACTION
  406   DU98 J1=1,NSP1
C   ANISOTROPIC LINEWIDTH INCLUDED HERE
                    IF(NS.EQ.0) GCTC 905
           DH=SQRT(DHZ(J1)**2*CS2+SN2*(DHX(J1)**2*CC2*GXX
     1              +DHY(J1)**2*SS2*GYY))/G
  905      S=(SQRT(WZ*WZ*CS2*GZZ+SN2*(WX*WX*CC2*GXX+WY
     1*WY*SS2*GYY)))/G
  405   NSS=(CUTOFF*S)/LINT
        SS=S*S
        WS=W*S
        SPINM=J1-1-SPIN1
        HH=HO-ZG*SPINM-CA*(SPN-SPINM**2)-CB*(SPINM**2)
        DO98 J2=1,NSP2
        KH=HH+ZNG*(SPIN2-J2+1)
        DO98 J3=1,NSP3
        MH=KH+YNG*(SPIN3-J3+1)
        DO98 J4=1,NSP4
        LH=MH+QNG*(SPIN4-J4+1)
  408   IF(LH.LT.KMIN.OR.LH.GT.KMAX)GO TO 98
                    IF (NS.EQ.0) GOTC 55
                    LH=LH-2.0*DH
                    WTPT=-2.0
                    WTINCR=4.0/(NP-1.0)
  55        NH=(LH-KMIN)/LINT
                    ZNH=0.0
        NI=NH-NSS
        NJ=NH+NSS
        IF (NI.LE.0) NI=1
        IF(NJ.GT.KMOT) NJ=KMCT
C   LINESHAPE OPTION EXERCISED HERE'STAR' EQUALS'1' GAUSSIAN USED
        IF(STAR.EG.1) GO TO 202
C   LORENTIZIAN LINESHAPE 1ST DERIVATIVE
C   'STORE(KL) IS ARRAY POWDER SPECTRUM  IS STORED
  201   DO88KL=NI,NJ
        KK=LH-KMIN-(KL*LINT)
        SK=SS+KK*KK
        STORE(KL)= STORE(KL)+((WS*KK/(SK*SK))*INT1(J1)*INT2(J2)*INT3(J3)*
      1 INT4(J4))*EXP(-(WTPT**2))
   88   CONTINUE
        GO TO 87
C   GAUSSIAN LINESHAPE 1ST DERIVATIVE
  202   DO87KL=NI,NJ
        KK=LH-KMIN-(KL*LINT)
        SK=((KK*KK)*0.693147)/(SS)
        STORE(KL)=STORE(KL)+((KK)/(SS*S))*EXP(-SK)*INT1(J1)*INT2(J2)*
      1 INT3(J3)*INT4(J4)*W*EXP(-(WTPT**2))
   87   CONTINUE
                    IF (NS.EQ.0) GOTO 99
                    LH=LH+WTINCR*DH
                    WTPT=WTPT+WTINCR
                    IF (WTPT.NE.(2.0+WTINCR)) GOTO 55
   99               CONTINUE
   98   CONTINUE
  108   CONTINUE
        WRITE(6,18)I,M,J1,J2,J3,J4
   18   FORMAT(6(2X,I3))
C   WEIGHTING CCMPUTED SPECTRUM TO MOST INTENSE PEAK
  335   PEAK=0
        DO 51N=KLOT,KTOT
        PAK=ABS(STORE(N))
        IF(PEAK.LT.PAK)  GOTO 50
        GO TO 51
   50   PEAK=PAK
   51   CONTINUE
        FACTOR=1.00
C
C   SIMULATED SPECTRUM PLOTTED HERE
C
        DO 52 N= KLOT,KTOT
        X(N-KLOT+1)=  ((N-KLOT)*LINT)
```

```
      Y(N-KLOT+1)= STORE(N)*FACTOR/PEAK
 .52  CONTINUE
      KCOM=KTCT-KLOT
      NTOT =KCCM+1
      TX1=L
      TX2=(LTOT-L)/PL
      TY1=-FACTOR
      TY2=2*FACTOR/PHGT
      X(KCOM+2)=0.0
      X(KCOM+3)=TX2
      Y(KCOM+2)=TY1
      Y(KCOM+3)=TY2
      CALL  SETSIZ(0.30,0.12,0.15,1.00)
      CALL  AXIS(0.0,0.0,'INTENSITY',9,PHGT,90.0,TY1,TY2)
      CALL  PLOT(0.0,0.5*PHGT,-3)

      CALL  LINE(X,Y,NTOT,1,0,0)
 121  STOP
      END
      SUBROUTINETHETA(NN,CX,SX,D)
      DIMENSION CX(600),SX(600),TETA(600),D(600)
      NINT=NN
      SINT=1.570796/NINT
      GSINT=SINT*0.77459667/2.
      J=1
      TETA(J)=0.
      D(J)=0.22222222
      J=J+1
      TETA(J)=SINT/2.-SINT*0.28989794/2.
      D(J)=1.02497166
      J=J+1
      TETA(J)=SINT/2.+SINT*0.68989794/2.
      D(J)=.75280612
      J=J+1
      NNEX=NINT-2
      DO 100  I=1,NNEX
      TETA(J)=(I+1)*SINT-(SINT/2.+GSINT)
      D(J)=.55555555
      J=J+1
      TETA(J)=I*SINT+SINT/2.
      D(J)=.88888888
      J=J+1
      TETA(J)=I*SINT+(SINT/2.+GSINT)
      D(J)=.55555555
      J=J+1
 100  CONTINUE
      TETA(J)=1.5707963-(SINT/2.+SINT*.68989794/2.)
      D(J)=.75280612
      J=J+1
      TETA(J)=1.5707963-(SINT/2.-SINT*0.28989794/2.)
      D(J)=1.02497166
      J=J+1
      TETA(J)=1.5707963
      D(J)=.22222222
      DO 101  NT=1,J
      CX(NT)=CCS(TETA(NT))
      SX(NT)=SIN(TETA(NT))
      D(NT)=D(NT)*SX(NT)
 101  CONTINUE
      RETURN
      END
      SUBROUTINE ORTHRH (MM,CF,SP,DPHI,ALPHA)
      DIMENSICN CP(600),SP(600),DPHI(600),FI(600)
      MINT=MM
      RANGE=3.1415923
      IF(ALPHA.EQ.0.0) RANGE=RANGE/2.
      SINTP=RANGE/MINT
      GSINP=SINTP*0.77459667/2.
      J=1
      FI(J)=0
      DPHI(J)=.22222222
      J=J+1
      FI(J)=SINTP/2.-SINTP*0.28989794/2.
      DPHI(J)=1.02497166
      J=J+1
      FI(J)=SINTP/2.+SINTP*0.68989794/2.
      DPHI(J)=.75280612
      J=J+1
      MINT2=MINT-2
      DO 200  I=1,MINT2
      FI(J)=(I+1)*SINTP-(SINTP/2.+GSINP)
      DPHI(J)=.55555555
      J=J+1
      FI(J)=I*SINTP+SINTP/2.
      DPHI(J)=.88888888
      J=J+1
      FI(J)=I*SINTP+(SINTP/2.+GSINP)
      DPHI(J)=.55555555
      J=J+1
 200  CONTINUE
      FI(J)=RANGE-(SINTP/2.+SINTP*0.68989794/2.)
      DPHI(J)=.75280612
      J=J+1
      FI(J)=RANGE-(SINTP/2.-SINTP*0.28989794/2.)
      DPHI(J)=1.02497166
      J=J+1
      FI(J)=RANGE
      DPHI(J)=.22222222
      DO 201  MP=1,J
      CP(MP)=CCS(FI(MP))
      SP(MP)=SIN(FI(MP))
 201  CONTINUE
      RETURN
      END
```

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